Investigating the Mechanistic Roles of Conserved Residues in Cysteine Dioxygenase: How a Hydrogen-bonding Network and a Rogue Cysteine Effect L-cysteine Oxidation and Crosslink Formation

by

Claire Julia Graham

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy in Chemistry

Auburn, Alabama
May 4, 2019

Approved By

Holly R. Ellis, Chair, Professor of Chemistry and Biochemistry
Douglas C. Goodwin, Associate Professor of Chemistry and Biochemistry
Evert C. Goodwin, Associate Professor of Chemistry and Biochemistry
Steven Mansoorabadi, Assistant Professor of Chemistry and Biochemistry

Abstract

Cysteine Dioxygenase (CDO) catalyzes the oxidation of L-cysteine to L-cysteine sulfinic acid utilizing iron as a cofactor. Adjacent to the iron center is a protein-derived thioether crosslink formed by a cysteine (Cys93) and a tyrosine (Tyr157) residue. The crosslinked isoform of CDO increases catalytic activity ~5-fold compared to non-crosslinked CDO.

Tyr157 forms a hydrogen-bonding network with His155 and Ser153 which has been proposed to participate in cysteine oxidation. Substitutions of His155 were made in CDO to evaluate the importance of the hydrogen-bonding network in both cysteine oxidation and crosslink formation. All substitutions resulted in minimal catalytic activity compared to wild-type CDO. Even though H155Q CDO showed minimal catalytic activity, the variant was able to generate the crosslink as effectively as wild-type CDO, while crosslink formation was limited with the H155A and H155E CDO variants. All substitutions of His155 altered the microenvironment of the metal coordination center of CDO. The different effects of the His155 CDO variants on crosslink formation and cysteine oxidation suggest the hydrogen-bonding network plays dual roles due to the distinct chemical steps utilized in these processes.

Located ~8 Å away from the iron center is a conserved Cys (Cys164) residue at the opening to the active site. Cys164 does not participate in any intramolecular disulfide bonds and exists as a free thiol. Several bacterial CDO homologs contain either an Arg or a Met residue at a comparable position as Cys164 in mammals. Therefore, it has been speculated that Cys164 and amino acids at comparable positions could contribute to the substrate specificity of mammalian

and bacterial CDO. Other studies have shown that Cys164 is involved in a disulfide bond with a free Cys in three-dimensional structures which suggested that Cys164 may serve as a redox switch in CDO. However, this hypothesis has not been adequately evaluated.

Multiple variants of Cys164 in CDO were constructed to evaluate the role of Cys164 in catalysis, crosslink formation, and oxidative regulation. All Cys164 CDO variants displayed diminished activity compared to wild-type CDO. Crosslink formation studies showed that C164A CDO was the only Cys164 variant that could generate the fully crosslinked species at increased L-cysteine substrate concentrations similar to wild-type CDO. In addition, wild-type, C164A, non-crosslinked wild-type, and non-crosslinked C164A CDO did not appear to be modified by hydrogen peroxide or L-cysteine. Although Cys164 is not in the active site, these studies suggest that Cys164 likely plays a key role in L-cysteine substrate oxidation. Since Cys164 is located at the opening of the active site, it may regulate accessibility of the L-cysteine substrate to the active site.

Both of these studies evaluated the mechanistic roles of conserved residues in CDO. The hydrogen-bonding network appears to stabilize iron-oxo intermediates through hydrogen-bonding interactions during catalysis. The Cys164 residue may act as a gate-keeper by regulating accessibility of the L-cysteine substrate during catalysis and crosslink formation. These studies also provided a foundation for future studies regarding the elucidation of the L-cysteine oxidation and crosslink formation mechanisms.

Acknowledgments

First, I would like to thank the Department of Chemistry and Biochemistry, Auburn University for providing me this opportunity. I'd like to thank Dr. Holly Ellis for her guidance and support throughout my research here at Auburn University. I am forever grateful for the skills she has equipped me with for a successful graduate career as well as my future endeavors as a scientist. I'm very appreciative of her patience and listening to my ideas even when they weren't sensical. I'd also like to thank my committee members: Dr. Doug Goodwin, Dr. Steve Mansoorabadi, and Dr. Evert Duin. They have all played an important role in my graduate studies whether it be through the use of instrumentation or research input. I also appreciate Dr. Paul Cobine serving as the University Reader. I'd like to acknowledge former and current lab members: Paritosh Dayal, Dr. Jonathan Musila, Dr. Dianna Forbes, Katie Tombrello, and Richard Hagen. I truly appreciate all of your help with protocols, preparing for talks, discussions regarding research, and just science in general. I'd like to thank David Beuttel for always supporting and believing in me even when I didn't believe in myself. Also, a special thanks to Tabitha Williford for always being there throughout my life and academic career. Last but certainly not least, I would like to thank Chris, Rhonda, and Joan Graham. Without them, none of this would've been possible. Thank you for always pushing me to be my best and supporting me in everything that I do.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

CDO	cysteine dioxygenase
CSA	cysteine sulfinic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
EPR	electron paramagnetic resonance
PCET	proton coupled electron transfer
LC-MS	liquid chromatography-mass spectrometry
HPLC-SEC	high performance liquid chromatography-size exclusion chromatography
MCD	magnetic circular dichroism

Chapter One

Literature Review

1.1 Introduction to Cysteine Metabolism

Cysteine is a multi-functional amino acid known to be a strong nucleophile, with a high reduction potential that is readily oxidized. Additionally, cysteine has been shown to play a role in protein synthesis, protein stability, regulation through redox signaling, and a multitude of metabolic reactions.[1,2] In mammals, cysteine can be acquired through the diet or obtained through the transsulfuration pathway by utilizing methionine and serine. Cysteine can then be used as a precursor to produce important metabolites involved in multiple metabolic processes.[2]

Cysteine can be acquired through the diet or obtained through the transsulfuration pathway utilizing methionine and serine (**Fig. 1.1**). Methionine is converted to S-adenosylmethionine (SAM) by methionine adenosyltransferase which is driven by ATP. SAM is then converted to S-adenosylhomocysteine by methyltransferase. S-adenosylhomocysteine is transformed into homocysteine and adenosine by adenosylhomocysteine hydrolase. Homocysteine is a branchpoint in this pathway since it can be condensed with serine to form cystathionine catalyzed by the pyridoxal 5'-phosphate (PLP) dependent enzyme, cystathionine β-synthase. Alternatively, homocysteine can be converted back to methionine by vitamin B-12 which is catalyzed by methionine synthase. Hydrolysis of cystathionine by cystathionine γ-lyase then yields cysteine, α-ketobutyrate, and ammonia. Cysteine can then be used for several metabolic fates such as glutathione synthesis, oxidation to cysteine sulfinic acid, protein synthesis, and coenzyme A synthesis. [2–4]

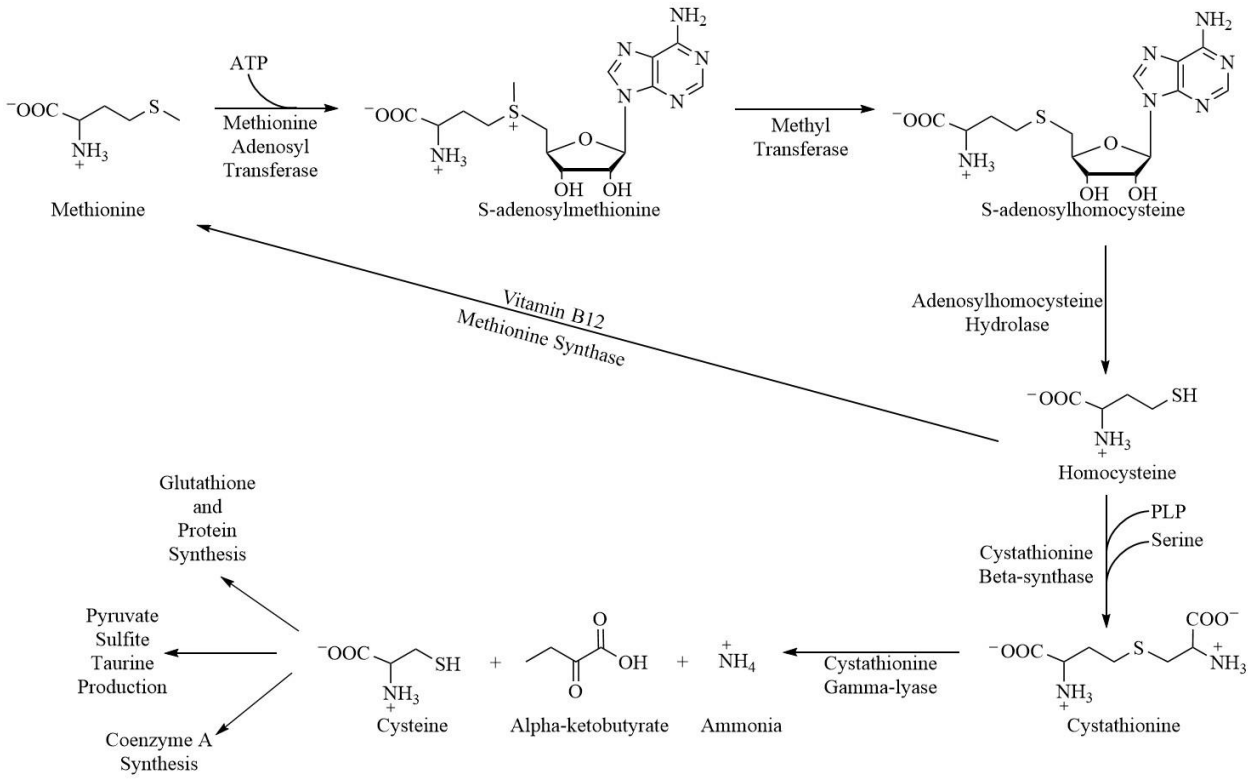


Fig. 1.1 The transsulfuration pathway for the production of cysteine. [4]

Cysteine is a precursor for protein, glutathione, and coenzyme A biosynthesis.[2] Due to their high nucleophilicity, cysteine residues incorporated into proteins often play a role in redox signaling and stability. A large portion of available cysteine is utilized in protein and glutathione biosynthesis. In addition to serving as a cysteine reservoir, glutathione has been shown to serve as an antioxidant for harmful biproducts such as superoxide produced by various metabolic processes.[2,5] Additionally, cysteine is utilized in the synthesis of coenzyme A, a key cofactor in oxidative metabolism and biosynthetic reactions.[6] Cysteine is also susceptible to oxidation and can be oxidized to cysteine sulfinic acid which is catalyzed by cysteine dioxygenase (CDO). Cysteine sulfinic acid is an important branchpoint in sulfur metabolism since it can then form

pyruvate and sulfite, or taurine. [7–9] This branchpoint is an alternate route for pyruvate formation for other metabolic reactions. Additionally, taurine has shown to be important in oxidative stress regulation by regulating mitochondrial protein synthesis which protects against mitochondrial oxidation by enhancing electron transport chain activity. [10]

1.2 Metabolic Functions of Cysteine in Proteins

Increased levels of cysteine can be toxic in biological systems. This is commonly due to cysteine being associated with reactive oxygen species (ROS) especially in the presence of iron (**Fig. 1.2**). ROS are often the byproducts of several biological reactions including mitochondrial electron transport and oxidation reactions involving metals. Types of ROS produced involve peroxides, superoxide anions, hydrogen peroxides, hydroxyl radicals, and hydroxyl ions. [11] Production of ROS results in oxidative stress conditions, which can lead to the modification of macromolecules. The modifications can affect the overall function of macromolecules. ROS also play a role in cell signaling for apoptosis and gene expression. [12]

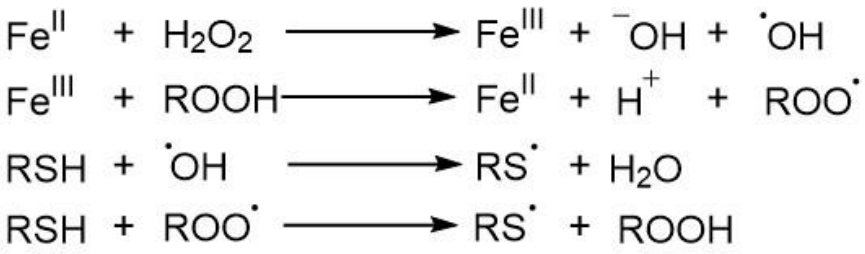


Fig. 1.2 ROS formed in the presence of cysteine and iron. [11]

While increased cysteine concentrations can be hazardous to the cellular environment, the use of free cysteine has been shown to be important in numerous metabolic processes. [1,2] When cysteine is at or slightly above the physiological level, one of the main purposes it serves is for the synthesis of proteins.[13,14] Cysteine is incorporated in proteins due to its chemical versatility. Cysteine is one of the least abundant amino acids; however, it is one of the most commonly found

amino acid at functional sites in proteins. Cysteine residues can undergo multiple oxidative modifications forming cysteine sulfenic acid, cysteine sulfinic acid, cysteine sulfonic acid, S-nitrosylation, S-glutathionylation, and disulfide formation (**Fig. 1.3**). [15] Cysteine residues can be categorized based on their overall function. These categories include: regulatory, catalytic, and structural. Since cysteine residues are one of the most reactive amino acids, they often play multiple roles in proteins and can be classified in more than one category. [94] However, some cysteines found in proteins do not serve a particular function. Typically, these cysteine residues have little to no amino acid conservation. These cysteines also have very little exposure and hinder the accessibility of the sulfur atom. In addition, the cysteines are not in proximity to other cysteines preventing intermolecular disulfide bond formation. High pK_a values (>9) that result in low reactivity are also characteristic of non-functional cysteine residues. [16,17]

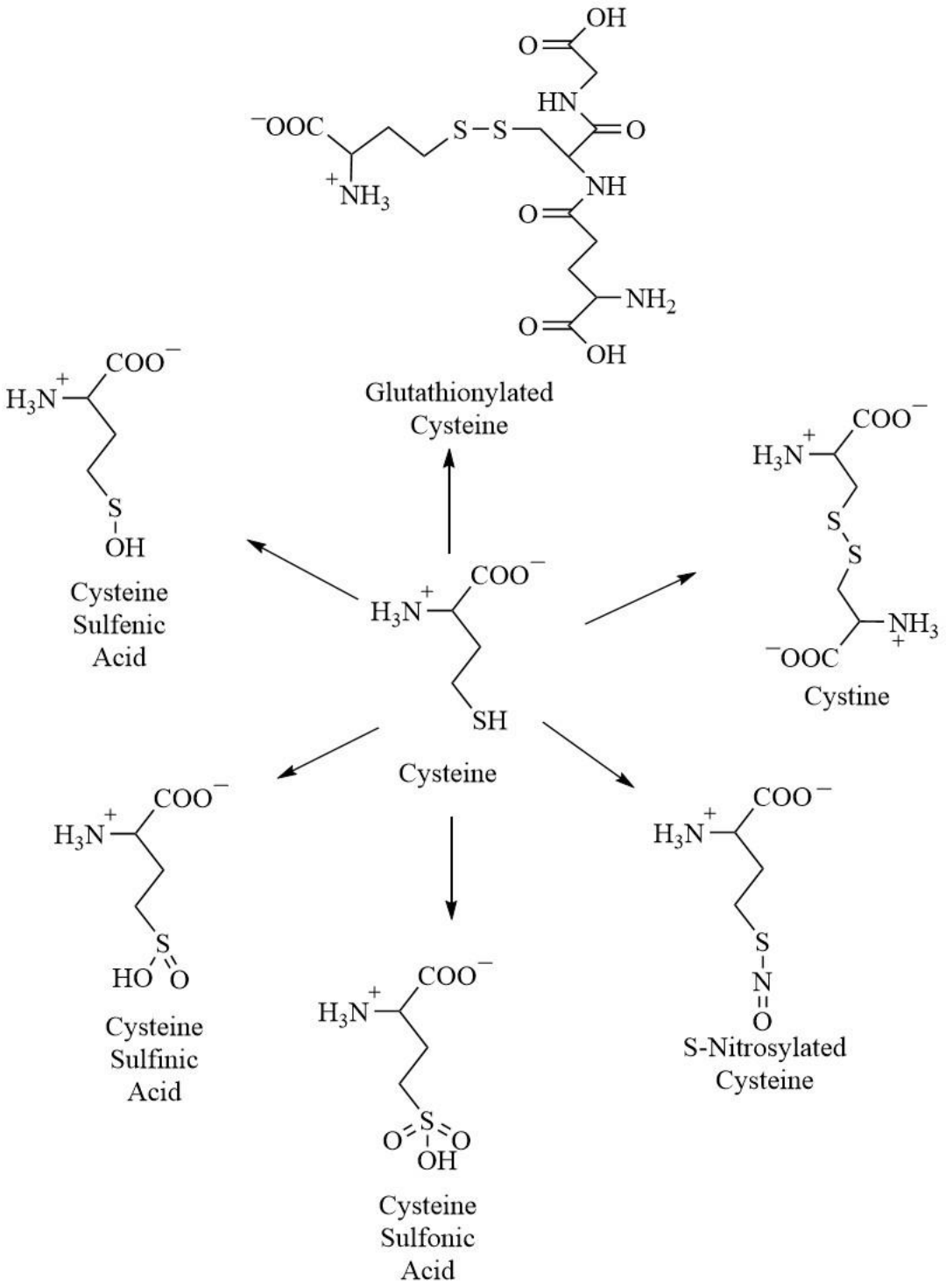


Fig. 1.3 Some of the possible oxidative modifications of cysteine. [15]

1.3 Cysteine-dependent Enzymes Involved in Reactive Oxygen Species Protection

There are several ways biological systems combat ROS to prevent negative effects on metabolic function. Small molecules such as glutathione, ascorbic acid, and vitamin E are known for their antioxidative properties. Several enzymes are also known to remove ROS from the cellular environment such as catalases, peroxidases, and superoxide dismutase.[18] Catalases are responsible for converting hydrogen peroxide to yield water and oxygen while peroxidases utilize hydrogen peroxide as a substrate to oxidize numerous organic or inorganic substrates to yield water and the oxidized form of the organic or inorganic substrate. In contrast, superoxide dismutase (SOD) is responsible for the detoxification of superoxide by catalyzing the disproportion of the superoxide anion to yield dioxygen and hydrogen peroxide.[19] Thioredoxins, glutaredoxins, peroxiredoxins are also responsible for removing ROS and sometimes reactive nitrogen species (RNS) by using a catalytic cysteine residue to reduce or oxidize their substrate.[20] Thioredoxins, peroxiredoxins, and SOD will be further discussed since they contain multiple cysteine residues with various functions important in maintaining the redox properties of the cellular environment.

1.3.1 Thioredoxin

Thioredoxin is a ubiquitous class of redox proteins present in all organisms that helps maintain the reducing environment of the cell utilizing redox-active cysteine residues.[21] Thioredoxin is important in protecting enzymes from oxidative stress. Under oxidative stress conditions, cysteines are susceptible to oxidation which often results in disulfides between intramolecular cysteines.[22] While disulfides are known to provide structural stability in proteins, some cysteines need to remain in the reduced form for protein function.[23] Mammalian thioredoxin contains five cysteine residues, and two of the cysteine residues make up a C-X-X-C motif. This C-X-X-C motif is characteristic of proteins containing catalytic redox-active cysteine residues

where the cysteines are highly conserved, and X is usually a hydrophobic residue. The first cysteine in the motif is typically referred to as the attacking cysteine while the second cysteine is known as the resolving cysteine. The attacking cysteine will attack the oxidized substrate, and the resolving cysteine will stabilize the attacking cysteine.[24] Thioredoxin is capable of reducing oxidized cysteines in proteins by thiol-disulfide exchange utilizing the C-X-X-C motif and thioredoxin reductase, a FAD-bound enzyme that is NADPH-dependent. [23]

Cys32 in thioredoxin is the attacking cysteine in the motif and will attack the oxidized substrate. The resolving cysteine, Cys35, will stabilize Cys32 forming an intramolecular disulfide. Utilizing NADPH, FAD-bound thioredoxin reductase is oxidized as the disulfide between Cys32 and Cys35 in thioredoxin is reduced returning thioredoxin to the catalytically active form (**Fig. 1.4**). [23,25]

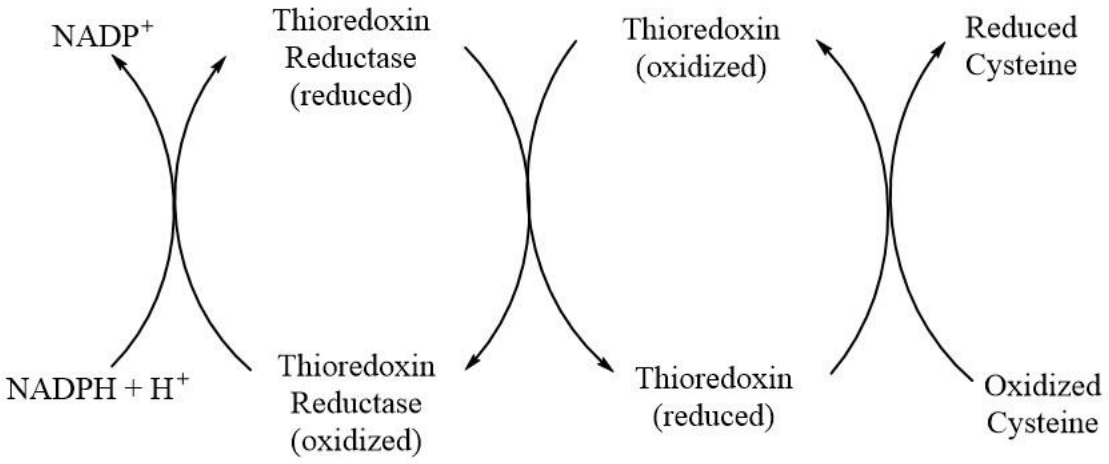


Fig. 1.4. Reactions catalyzed by thioredoxin reductase and thioredoxin in order to reduce undesirable disulfides among cysteine residues. [23,25]

In addition to thiolation, thioredoxin has also been shown to be modified during nitrosative stress.[26] Nitric oxide is an important mediator in vascular function and is produced by nitrous oxide synthase.[27] In the presence of superoxide, peroxynitrite, a reactive nitrogen species (RNS), can be formed which can react with lipids, thiols, amino acids, and other components found in the cell.

In addition, peroxynitrite can also form other types of RNS including nitrogen dioxide and other free radicals.[28] Under nitrosative stress conditions, thioredoxin was shown to be S-nitrosylated at one of the cysteine residues at positions 62, 69, or 73 which does not involve the catalytically active C-X-X-C motif.[26] The cysteine that is modified appears to depend on the oxidative state of thioredoxin.[29] In any case, the S-nitrosylated cysteine will then form a disulfide with one of the other cysteine residues releasing nitric oxide resulting in the denitrosylation of various proteins.[29] In contrast, reactions with other nitrosylated compounds such as nitrosylated glutathione rendered thioredoxin inactive which may suggest a regulatory mechanism since it has been shown to be directly corelated with activation of apoptosis signal-regulating kinase1 (ASK1). [30]

Under oxidative stress conditions, thioredoxin has also been shown to be modified by glutathione or hydrogen peroxide.[29,31] Glutathionylation of thioredoxin abolished catalytic activity. However, thioredoxin can de-glutathionylate itself regaining activity which was shown to be possible through disulfide-thiol exchange. De-glutathionylation of thioredoxin is dependent on the GSH/GSSG ratio in the cell which can be an indicator of the redox state of the cellular environment suggesting that glutathionylation preserves thioredoxin during oxidative stress.[31] Thioredoxin regulates hydrogen peroxide concentrations indirectly by reducing the disulfide between cysteine residues of peroxiredoxin in mammalian systems. However, increased levels of hydrogen peroxide have been shown to inhibit thioredoxin activity through modification of cysteine residues resulting in a disulfide. Although the resulting disulfide can be reduced, this could affect peroxiredoxin activity and may induce higher levels of hydrogen peroxide in the cell.[32]

1.3.2 Peroxiredoxin

In addition to catalases and peroxidases, peroxiredoxins catalyze the reduction of hydrogen peroxide. Peroxiredoxins all contain a cysteine residue that mediates hydrogen peroxide reduction generating a cysteine sulfenic acid. [33] Like thioredoxin, peroxiredoxins have an attacking cysteine residue and a resolving residue. However, these cysteine residues may not be located within the same protein, subunit, or polypeptide chain. There are three difference types of peroxiredoxins including 1-cys, typical 2-cys, and atypical 2-cys. All of these categories involve the cysteine sulfenic intermediate. However, the positions of the cysteine residues and the mechanisms in which the cysteine residues are converted back to their reduced form are different. [34]

The 1-cys peroxiredoxin contains one cysteine residue which is oxidized to cysteine sulfenic acid. The cysteine sulfenic acid intermediate is reduced by a thiol-containing compound which is speculated to be glutathione (**Fig. 1.5A**).[23,35] The typical 2-cys peroxiredoxin exists as a homodimer containing the catalytically active cysteine residues in each monomer. One of the cysteine residues is oxidized to the cysteine sulfenic intermediate which then forms a disulfide bond with the other monomeric cysteine. In mammals, thioredoxin is responsible for the reduction of the disulfide releasing both cysteine residues to their reduced form. The atypical 2-cys peroxiredoxin behaves in the same manner as the typical 2-cys with the exception of both cysteines being located on the same polypeptide (**Fig. 1.5B**). [35]

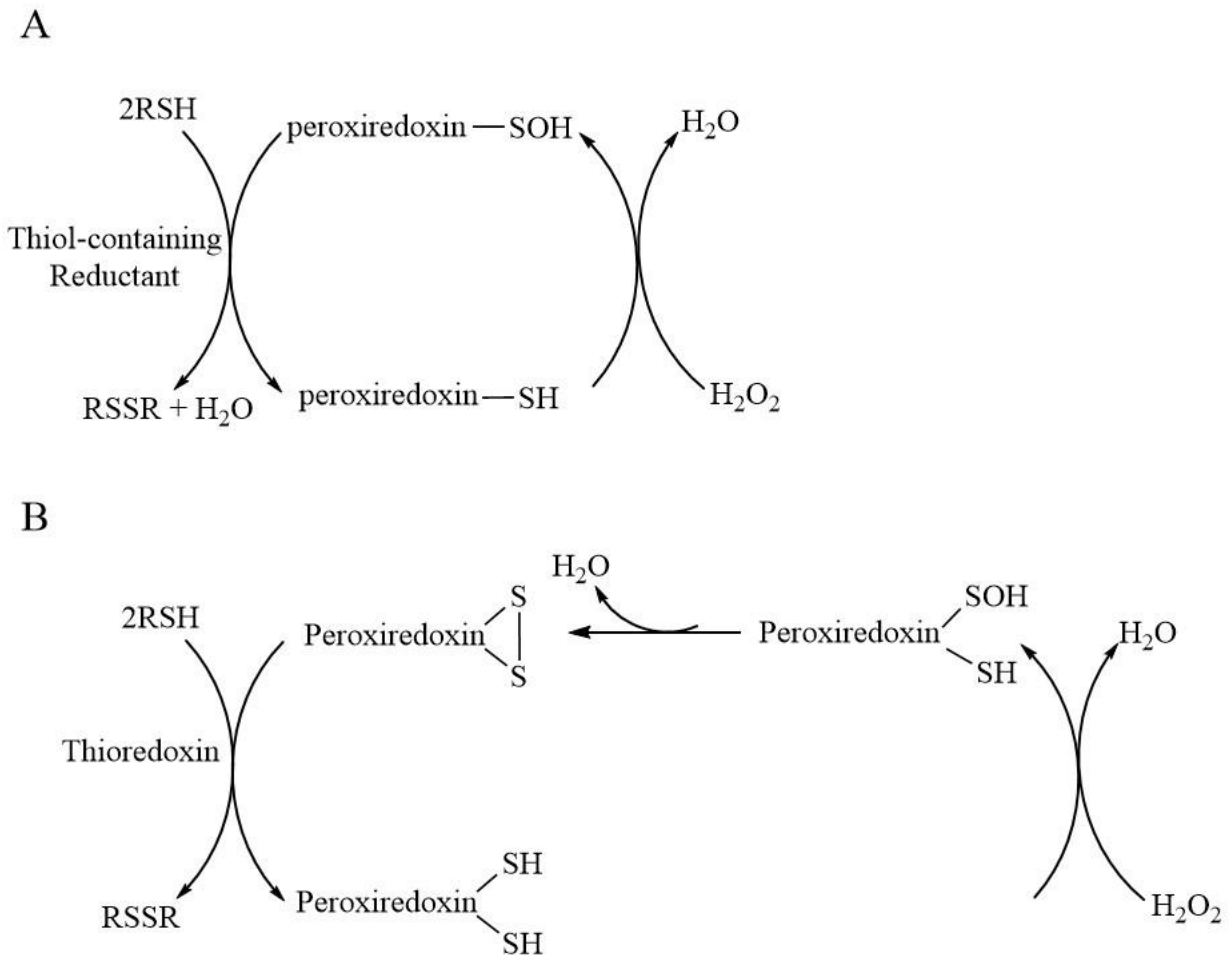


Fig. 1.5. Schematic representation of the peroxiredoxin reaction. A. 1-cys type peroxiredoxin reaction. B. Typical and atypical 2-cys type peroxiredoxin reaction. [35]

Since peroxiredoxin is present during oxidative stress, the cysteine residues can be overoxidized. [33] Cysteines can undergo modifications of one, two, and three oxygen atoms resulting in sulfenic, sulfinic, and sulfonic acids, respectively. [15] Overoxidation of cysteine has been unique to typical 2-cys peroxiredoxins where one of the cysteines accepts one or two additional hydrogen peroxide molecules leading to a cysteine sulfinic or sulfonic acid modification. [33,36]

Modification of cysteine residues to sulfonic acids is irreversible causing most enzymes to lose catalytic activity. [36,37] Sulfonated peroxiredoxins lose the reductase activity; however, studies have shown that they switch to a molecular chaperone by achieving higher oligomeric states. [63-65] The chaperone activity of peroxiredoxin appears to be similar to holdases by non-covalently binding to proteins to prevent their aggregation during oxidative stress.[38] Peroxiredoxin are able to achieve oligomeric states consisting of multiple dodecamers which interact through stacking resulting in a conical shape. Proteins interact with the peroxiredoxins and are partially unfolded. Once the redox environment is no longer under stress, the proteins are sent to chaperones for refolding. [64, 65]

Cysteine residues modified to sulfinic acids were thought to be irreversible; however, sulfiredoxin has been shown to enzymatically reverse this modification (**Fig 1.6**). [33,40]

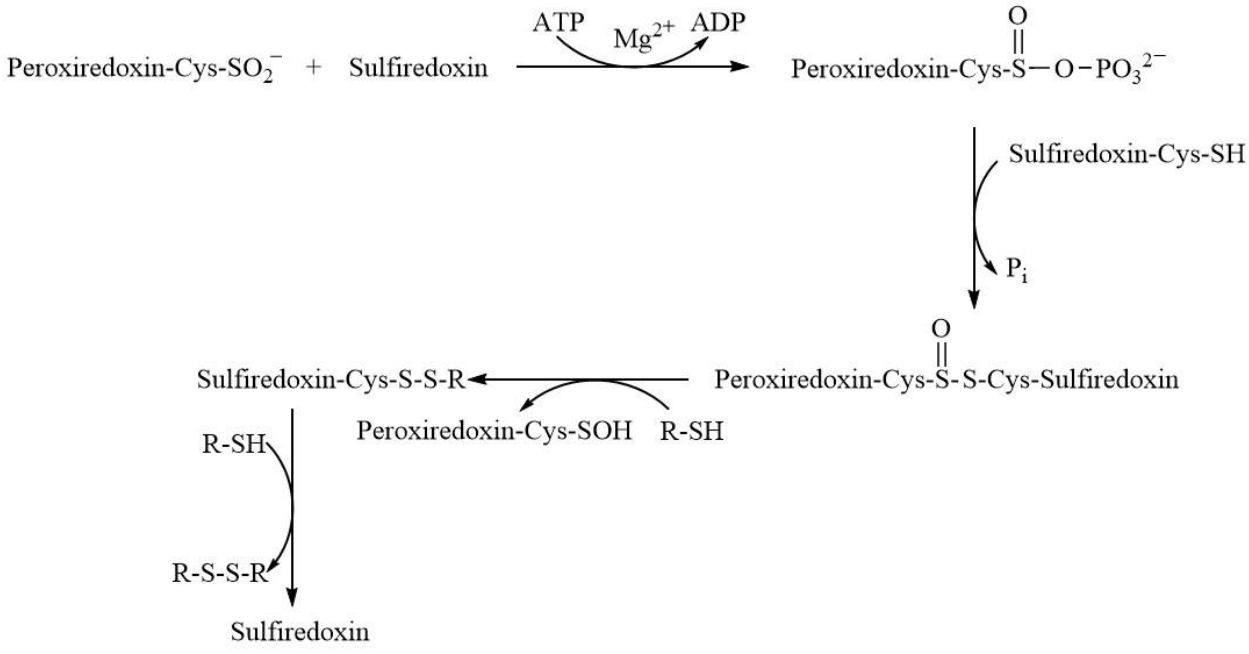


Fig 1.6 Reversal of cysteine sulfinic acid modification found in 2-cys typical peroxidases under extreme oxidative stress conditions. [33,40]

Sulfiredoxin-mediated reversal of the sulfinylated peroxiredoxin requires the presence of ATP and Mn^{2+} or Mg^{2+} (**Fig. 1.6**). The cysteine sulfinic acid attacks ATP resulting in phosphorylation of the modified peroxiredoxin. The free cysteine in sulfiredoxin can then attack phosphorylated peroxiredoxin resulting in a disulfide between sulfiredoxin and peroxiredoxin. A reductant such as thioredoxin may then release sulfenylated peroxiredoxin to its catalytic cycle and sulfiredoxin to the reduced form. [33,40]

1.3.3 Superoxide Dismutase

Superoxide dismutase (SOD) is an enzyme known for detoxification of the superoxide anion to yield dioxygen and hydrogen peroxide during oxidative stress in biological systems.[41] There are three different types of SOD in mammalian systems including SOD1 (cytosolic) and SOD3 (extracellular) which are Cu- and Zn-dependent and SOD2 (mitochondrial) which is Mn-dependent.[42] Cu/Zn SOD1 will be further discussed since it contains cysteine residues that serve multiple purposes.

Cu/Zn SOD1 is a cytosolic enzyme that exists as a homodimer in its catalytically active form and is both Cu- and Zn-dependent with one binding site for each metal per subunit. In the oxidized form, Cu(II) has a 4-His coordination with one His residue also coordinating Zn. In addition to the bridging His residue, Zn is coordinated by two His residues and an Asp residue.[43] Reduced Cu(I) contains a 3-His coordination with the bridging His residue belonging entirely to Zn. The Cu atom is essential for catalytic activity, and Zn is primarily responsible in maintaining the structural integrity of the enzyme (**Fig.1.7**). SOD1 also contains four cysteine residues (Cys 6, 57, 111, and 146) that play important roles in SOD1 function. [43]

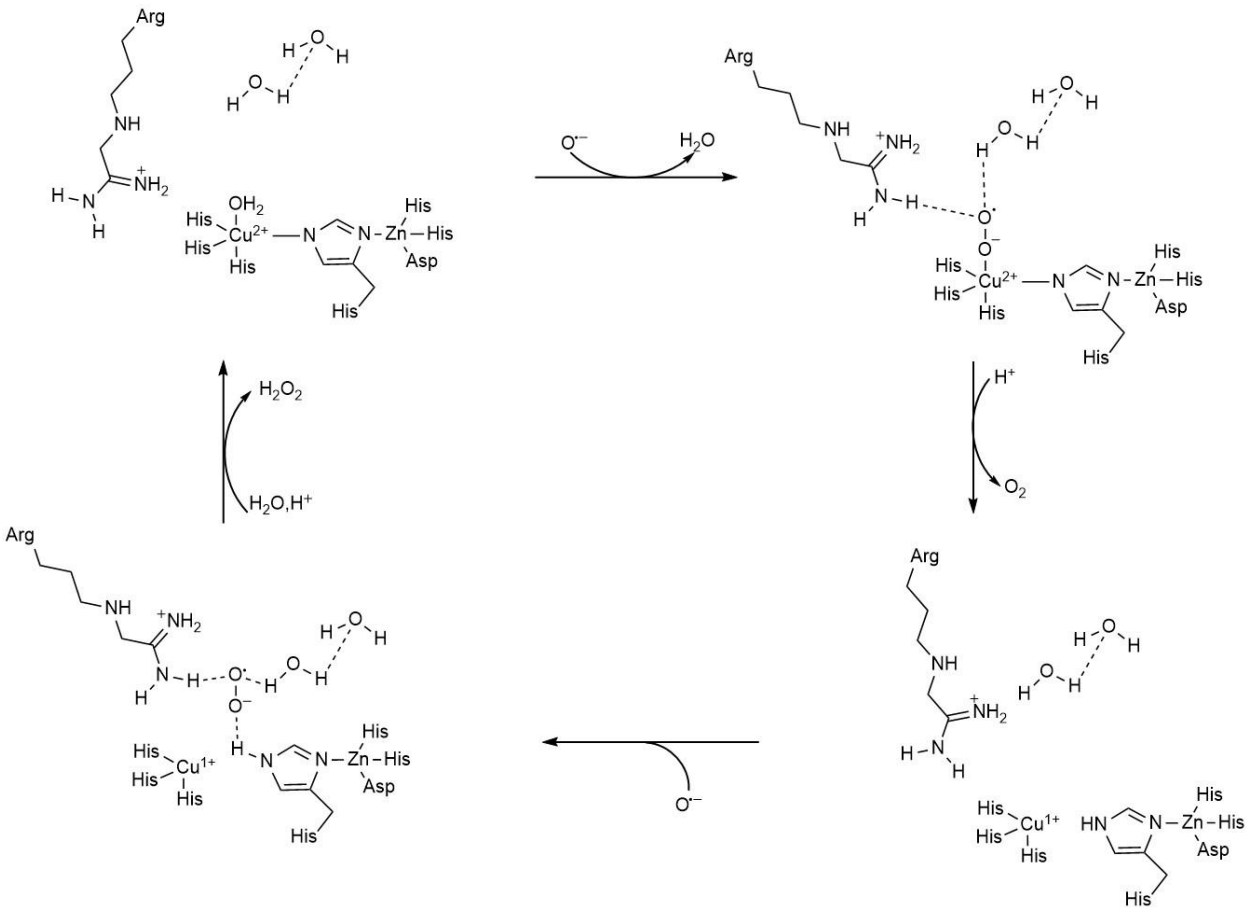


Fig. 1.7 The disproportion of the superoxide anion to yield dioxygen and hydrogen peroxide catalyzed by SOD mechanism.[43]

SOD1 obtains its copper through the metallochaperone, Copper Chaperone for SOD1 (CCS1).[44] The metallochaperone contains three domains all of which play a part in activating SOD1. D1 which is the N-terminal domain contains the copper-binding motif, MXCXXC. The second domain (D2) is utilized to bind SOD1 at the homodimeric interface which is made possible due to the structural similarity that D2 and SOD1 share. D3, the final domain, contains a CXC motif, which is responsible for disulfide formation between C57 and C146 in SOD1 securing Cu delivery.[45,46]

Activation of SOD1 *via* Cu delivery requires a specific thiolate conformation, cysteine sulfenylation, migration of the Cu ion, and disulfide formation. It has been shown that the Cu atom is delivered to SOD1 by CCS1 through a sulfenylation intermediate. CCS1 binds to an electropositive site in SOD1 near C146 with the Cu(I) ion exposed. A superoxide or hydrogen peroxide may be utilized for the sulfenylation of cysteine. In one case, the superoxide is attracted to the electropositive site near C146. The superoxide is then reduced to hydrogen peroxide by Cu(I) which is in turn oxidized to Cu(II). Hydrogen peroxide then oxidizes C146 to a cysteine sulfenic acid in SOD1; however, a cysteine in the D3 domain of CCS1 could form the sulfenylated intermediate initially. Interestingly, sulfenylation is not seen at any other cysteine residue in SOD1 indicating that sulfenylation of SOD1 is C146 specific. As an alternate, hydrogen peroxide could sulfenylate C146 without changing Cu oxidation states. Disulfide exchange reactions with the CCS1 D3 cysteines and sulfenylated C146 in SOD1 then drives the Cu ion to the SOD1 active site. Formation of a disulfide bond between C146 and C57 then terminates the CCS1 binding event locking Cu into the SOD1 active site resulting in the catalytically active SOD1 enzyme. [47]

Inactivation of SOD1 can lead to an influx of superoxide anions resulting in detrimental effects which has been linked to Amyotrophic Lateral Sclerosis (ALS).[48] ALS is a neurodegenerative disease that results in the death of motor neurons leading to muscle weakness and eventually death. [43] One way in which SOD1 is thought to be inactivated is through oxidation of the enzyme itself by its own product, hydrogen peroxide.[49] The Cys residue (Cys111) in SOD1 has been proposed to play a role in regulating SOD1 through cysteinylation. Cys111 is a free thiol located at the dimer interface in SOD1 and has been shown to be highly reactive, and is modified by oxygen, copper, glutathione, and cysteine. *In vitro* and *in vivo* experiments were conducted where the hydrogen peroxide product was incubated in the presence of free cysteine with the SOD1

enzyme. In both *in vitro* and *in vivo* experiments, mass spectrometry revealed an adduct of 119 Da at Cys111 indicative of cysteinylation of Cys111. Interestingly, this modification was specific to Cys111 only. Additionally, mass spectrometry revealed that cysteinylated SOD1 was protected from oxidative damage by hydrogen peroxide compared to wild-type SOD1 which had mostly been modified by both sulfinic and sulfonic acids. Cysteinylation at Cys111 in one monomer is proposed to protect the other monomer from oxidative damage during oxidative stress by changing the protein conformation and maintaining Cu in the correct position (**Fig. 1.8**). [48]

1.4 Maintenance of Cysteine Levels in Mammalian Systems

The physiological range of cysteine concentrations in mammals is typically 90-100 μM. [50] Cysteine must be kept under tight regulation since increased levels of cysteine has been linked to neurodegenerative, autoimmune, and heart disease.[51,52] Mammalian systems utilize several

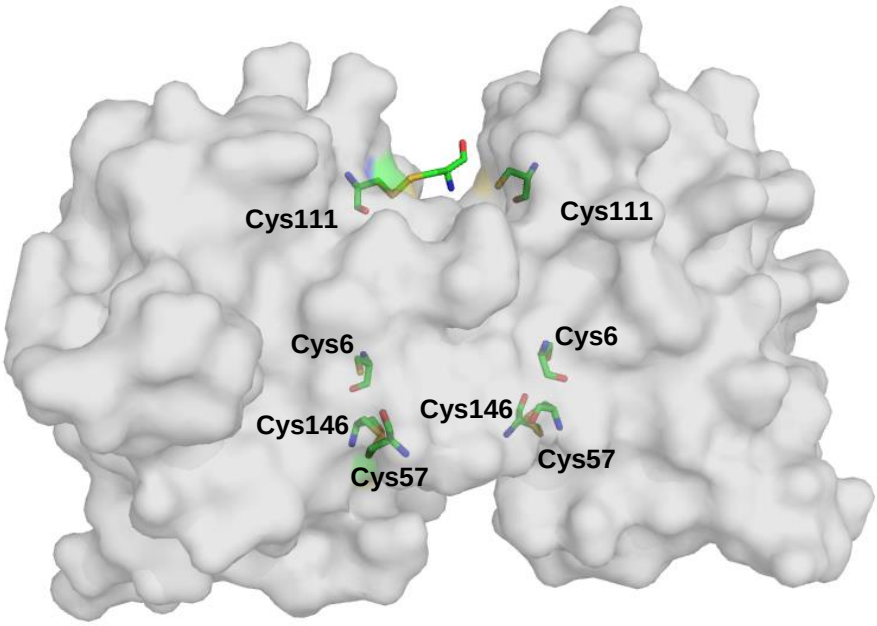


Fig. 1.8. Space-filling model depicting the disulfide bond formed at the dimer interface of SOD between an intramolecular cysteine and free cysteine in order to protect the catalytically active monomer during oxidative stress. [48]

metabolic pathways to maintain appropriate cysteine levels. Cysteine levels can be regulated by protein synthesis, glutathione synthesis, and catabolism of cysteine. [2]

One method in which cysteine levels are managed in the cell is through protein synthesis. While this does not necessarily occur when cysteine concentrations are elevated, a large portion of available cysteine goes towards the synthesis of proteins.[53,54] Cysteine residues in proteins can be involved in structural stability and are post-translationally modified.[1]

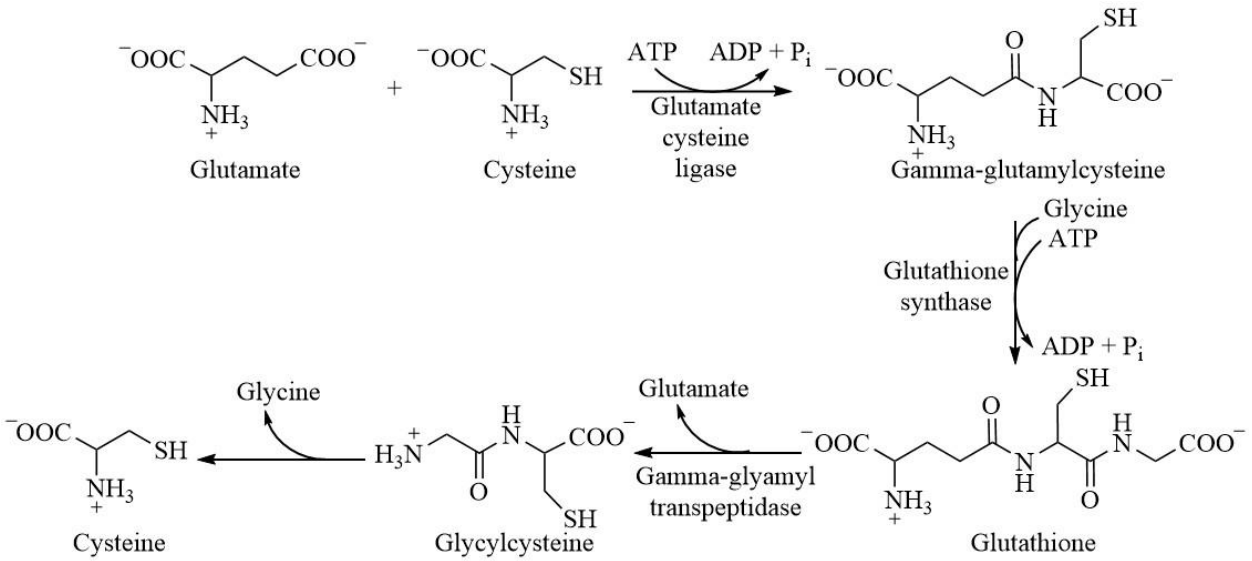


Fig. 1.9. Glutathione synthesis and degradation. [2,15,17-20]

Like protein synthesis, a large portion of available cysteine is incorporated into glutathione synthesis.[54,55] Glutathione is a tripeptide made up of glutamate, cysteine, and glycine. Glutathione is primarily known for its antioxidative properties; however, it can also serve as a reservoir for free cysteine. One of the ways mammalian systems regulate cysteine levels is through glutathione synthesis which is catalyzed by glutamate-cysteine ligase and glutathione synthase (**Fig. 1.9**). Ligation of glutamate and cysteine is catalyzed by the ATP-dependent enzyme glutamate-cysteine ligase. Glutathione synthase catalyzes the ATP-dependent synthesis of glutathione with the

addition of glycine to the glutamate-cysteine intermediate. Glutathione is considered a reservoir for cysteine since cysteine can be released from glutathione when cysteine concentrations are limiting.[56–58] Hydrolysis of the glutamate residue from the glutamate-cysteine-glycine tripeptide is catalyzed by γ-glutamyl transpeptidase, and several dipeptidases can cleave the cysteine-glycine pair resulting in free cysteine (**Fig. 1.9**). Cysteine is utilized for glutathione synthesis, protein synthesis, and other metabolic processes when cysteine is at or marginally above physiological levels. However, protein synthesis and other metabolic processes utilizing cysteine appear to have priority for cysteine over glutathione synthesis due to the observed depletion of glutathione levels at or marginally above physiological cysteine levels.[2,54,59]

Another way cysteine levels are maintained is through the synthesis of coenzyme A. Cysteine and pantothenate are both used as substrates for coenzyme A (**Fig. 1.10**). Phosphorylation of pantothenate utilizing ATP yields 4'-phosphopantothenate and ADP catalyzed by pantothenate kinase. Cysteine is then coupled with 4'-phosphopantothenate and ATP to yield 4'phosphopantothenoylcysteine, AMP, and inorganic diphosphate. Decarboxylation of 4'-phosphopantothenoylcysteine by 4'-phosphopantothenoylcysteine decarboxylase produces 4'-phosphopantetheine. Utilizing ATP, 4'-phosphopantetheine is then converted to dephospho-CoA and inorganic diphosphate by phosphopantetheine adenyltransferase. Dephospho-CoA is then phosphorylated by ATP yielding coenzyme A and ADP which is catalyzed by dephospho-CoA kinases. In this pathway, coenzyme A can also undergo degradation back to pantothenate which can be converted to cysteamine. Cysteamine is oxidized to hypotaurine by cysteamine dioxygenase, and hypotaurine can then be further oxidized to taurine [2,60,61]

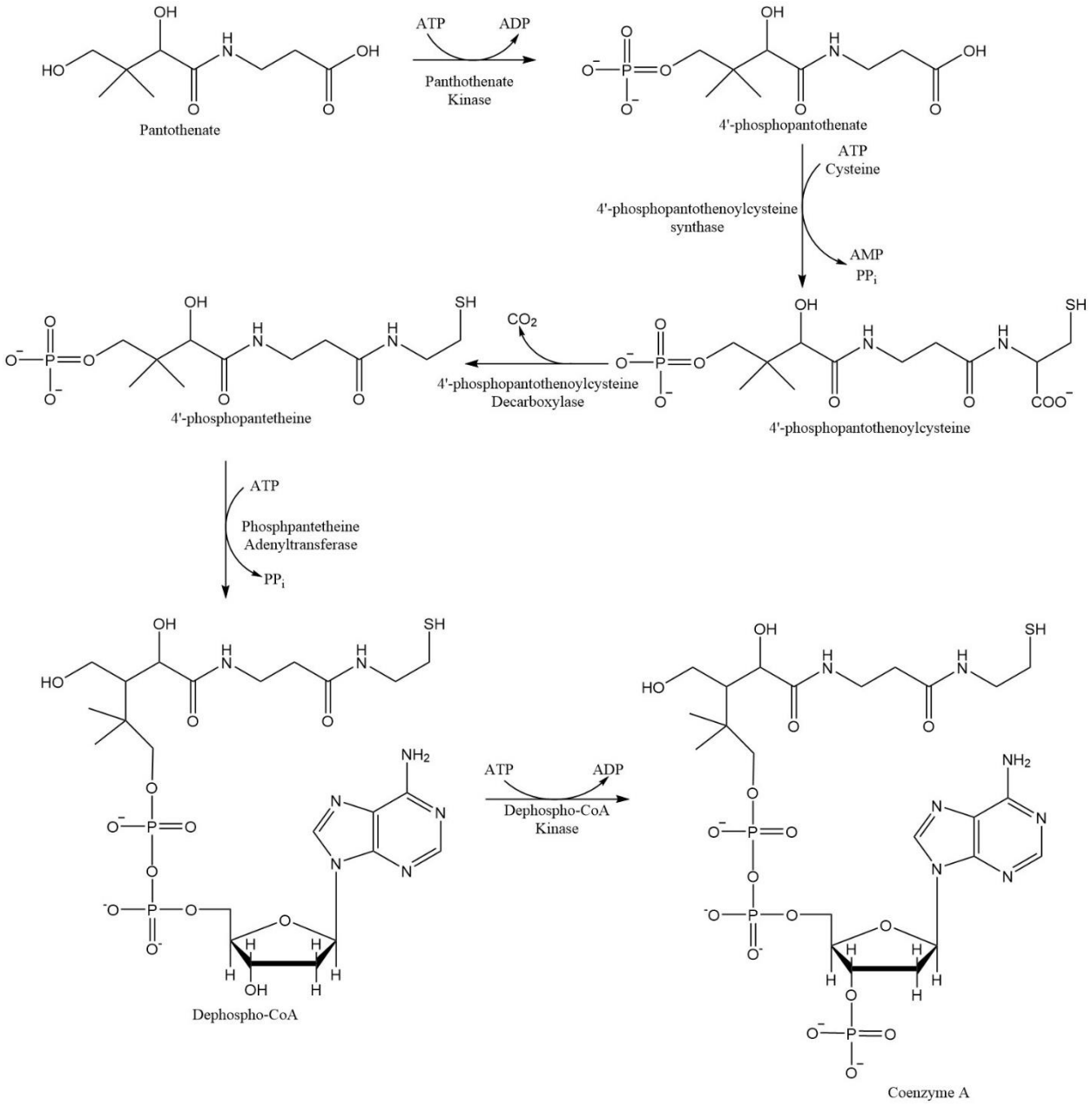


Fig. 1.10 Biosynthesis of coenzyme A. [2,60]

Another way cysteine levels are maintained in mammals is through desulfuration reactions (**Fig. 1.11**). These reactions yield pyruvate and reduced sulfur usually as hydrogen sulfide. There are two pathways that carry out the desulfuration of cysteine which contain H_2S as a possible product. 1) Cystathionine β-synthase catalyzes the release of H_2S from cysteine in the presence of

other thiol-containing compounds.[2,62] 2) Oxidized cysteine (cystine) can be cleaved by cystathionine γ-lyase which produces pyruvate, ammonia, and thiocysteine. Thiocysteine can then react with cysteine nonenzymatically releasing sulfide and cystine. The reduced sulfur from these reactions can then be used for further synthesis of compounds which require reduced sulfur. In contrast, the reduced sulfur may be oxidized to thiosulfate, sulfite, and sulfate.[2,63,64]

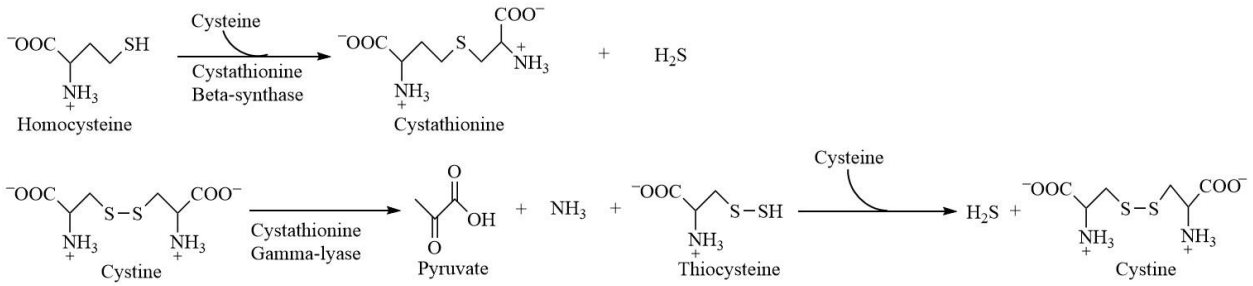


Fig. 1.11 The fate of cysteine through desulfuration reactions. [2,62]

Cysteine can also be converted to taurine or pyruvate and sulfite through oxidation to cysteine sulfinic acid (**Fig. 1.12**). Cysteine sulfinic acid is an important branchpoint in sulfur metabolism since it is able to form either pyruvate and sulfite, or taurine. CDO catalyzes the oxidation of cysteine to cysteine sulfinic acid. Cysteine sulfinic acid and 2-oxoglutarate are then converted to sulfinyl pyruvate and L-glutamate which is catalyzed by aspartate aminotransferase. Spontaneous hydrolysis of sulfinyl pyruvate generates pyruvate and sulfite. Alternatively, cysteine sulfinic acid can also undergo decarboxylation by cysteine sulfinic acid decarboxylase yielding hypotaurine which is then oxidized to taurine. It is not known if the conversion of hypotaurine to taurine occurs enzymatically or nonenzymatically. [65–68] CDO is appears to be transcriptionally regulated by dietary intake. Diets containing high levels of protein or sulfur- containing amino

acids lead to an increase in cysteine concentrations. [19, 30-33] CDO is then expressed giving rise to pyruvate and sulfite, or taurine production. In contrast, CDO is turned over through polyubiquitination when cysteine levels are low. When cysteine levels are below the physiological range, CDO is ubiquitinated and becomes the substrate for proteolysis by the 26S proteasome. When cysteine levels are elevated, ubiquitination is blocked which prevents the degradation of CDO. [69,70]

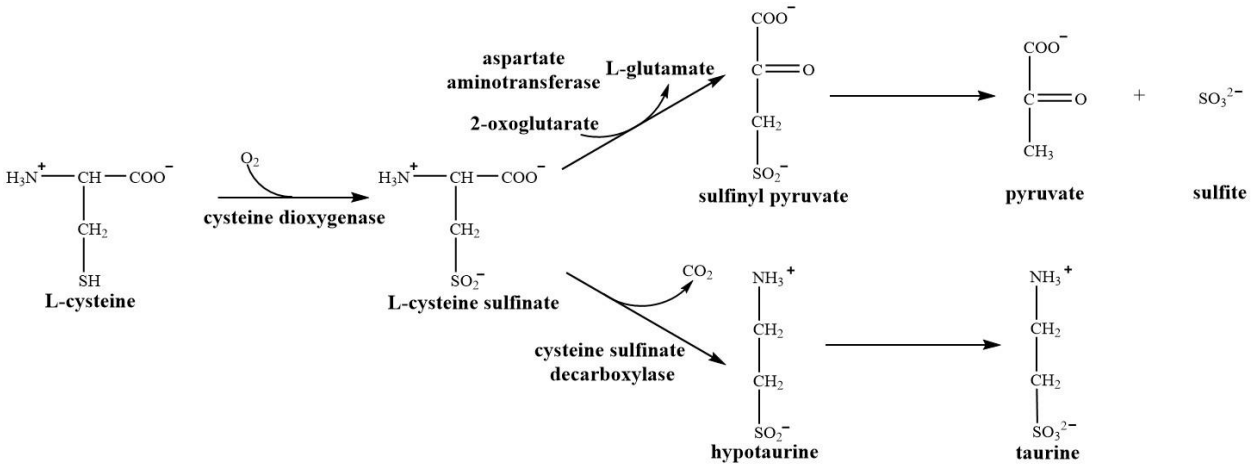


Fig. 1.12 CDO metabolic pathway where L-cysteine is oxidized to L-cysteine sulfinate which can then form pyruvate and sulfite, or taurine. [65–68]

1.5 Structural Properties of Cysteine Dioxygenase

The cupin superfamily is functionally diverse, and enzymes belonging to the superfamily are found in all domains of life.[71] The cupin superfamily originated from the identification of the wheat protein germin. Germin has oxalate oxidase activity which is manganese-dependent and catalyzes the oxidation of oxalate to yield two carbon dioxide molecules and hydrogen peroxide. [72,73] Several other proteins found in plants shared a conserved amino acid sequence

(HI/THPRATEI) with germin and were therefore classified as germin-like proteins.[74] In addition, globulin storage proteins found in plant seeds and spores shared a similar amino acid sequence with the conserved sequence in germin and germin-like proteins. Three-dimensional structures were solved for the seed proteins, and it was noted that the proteins shared common secondary structures which included four β-sheets where two of the β-sheets corresponded to two partially conserved motifs and two other β-sheets which were separated by a less conserved region.[74] This was identified as a "cupin fold" resulting in the cupin superfamily classification (**Fig. 1.13**). The word, "cupin," is derived from the latin word, "cupa," meaning "small barrel."[75] Proteins belonging to the cupin superfamily often have an overall low amino acid sequence similarity and are functionally diverse; however, the commonality among members of the cupin superfamily is the characteristic β-barrel fold. In addition, many cupins contain partially conserved motifs (Motif 1: $G(X)_5\underline{H}X\underline{H}(X)_{3\text{-}6}\underline{E}(X)_6G$ and Motif 2: $G(X)_{5\text{-}7}PXG(X)_2\underline{H}(X)_3N$). However, not all proteins in the cupin superfamily contain these motifs. [76,77]

Typical cupins are metalloproteins where the metal and active site are located in the middle of the β-barrel fold. Most of the metal centers contain iron; however, other metals such as zinc, cobalt, copper, nickel, and manganese are common. The most common type of metal coordination is the 2-His, 1-Glu from motif 1 and 1-His from motif 2, which is characteristic of the germin protein (**Fig. 1.13**). In some cases, Gln or Glu/Asp residues are substituted for one of the His residues from Motif 1.[76,78]

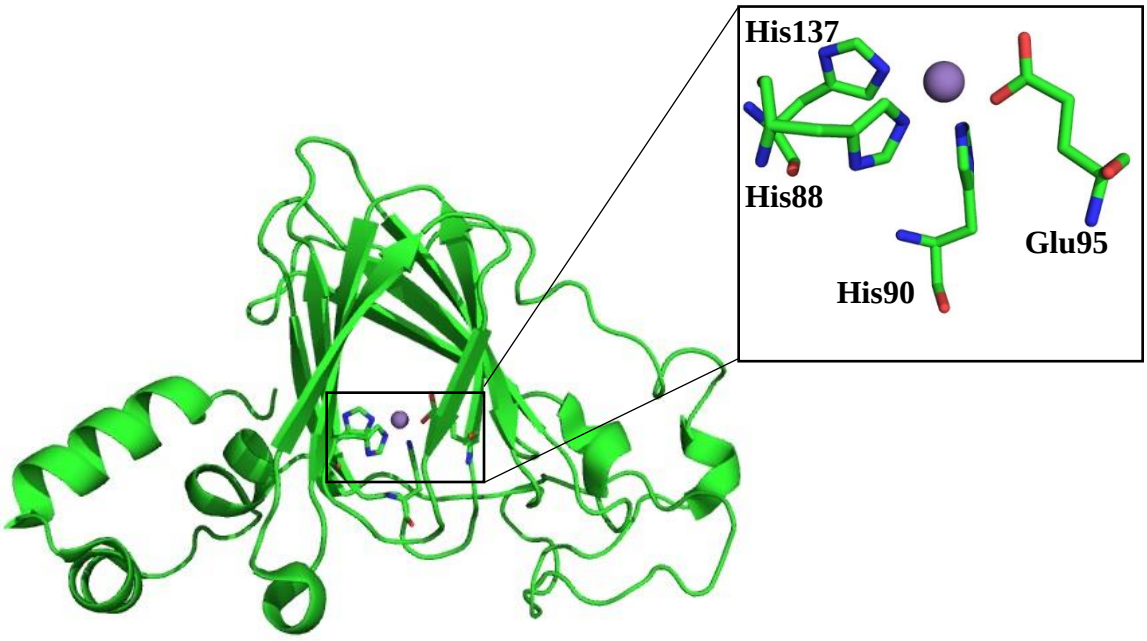


Fig. 1.13. Three-dimensional structure of germin oxalate oxidase depicting the β-barrel or cupin fold and the 3-His/1-Glu manganese (purple sphere) coordination which is characteristic of cupin proteins. PDB:1FI2[76]

The cupin superfamily contains several protein families which include non-enzymatic functions such as auxin binding, nuclear transcription factors, and seed storage. Protein families that have enzymatic functions include dioxygenases, decarboxylases, hydrolases, isomerases, and epimerases.[79] Cupin proteins can also be further classified as monocupins, bicupins, or multicupins. Monocupins are proteins that possess a single cupin domain. Bicupin proteins contain two cupin domains, and multicupin proteins contain multiple (>2) cupin domains. [80,81]

CDO is classified as a monocupin protein; however, it contains variations from the typical cupin uniform (**Fig. 1.14**). Instead of the conserved Glu residue found in Motif 1, CDO contains a Cys residue. Because of this, the iron center in CDO is octahedrally coordinated with three coordinating water molecules and by two His residues found in motif 1 and a single His residue in Motif 2 resulting in a 3-His coordination (**Fig. 1.15**). [82–84] Additionally, the Cys (Cys93) residue that replaces the metal coordinating Glu in most cupin proteins is a component of a thioether crosslink with Tyr157 which does not coordinate the iron center.[85] The crosslink is a post-translational modification proposed to better aid the substrates in coordinating to the iron center resulting in an increase in catalytic activity by ~5-fold compared to non-crosslinked CDO. [86]

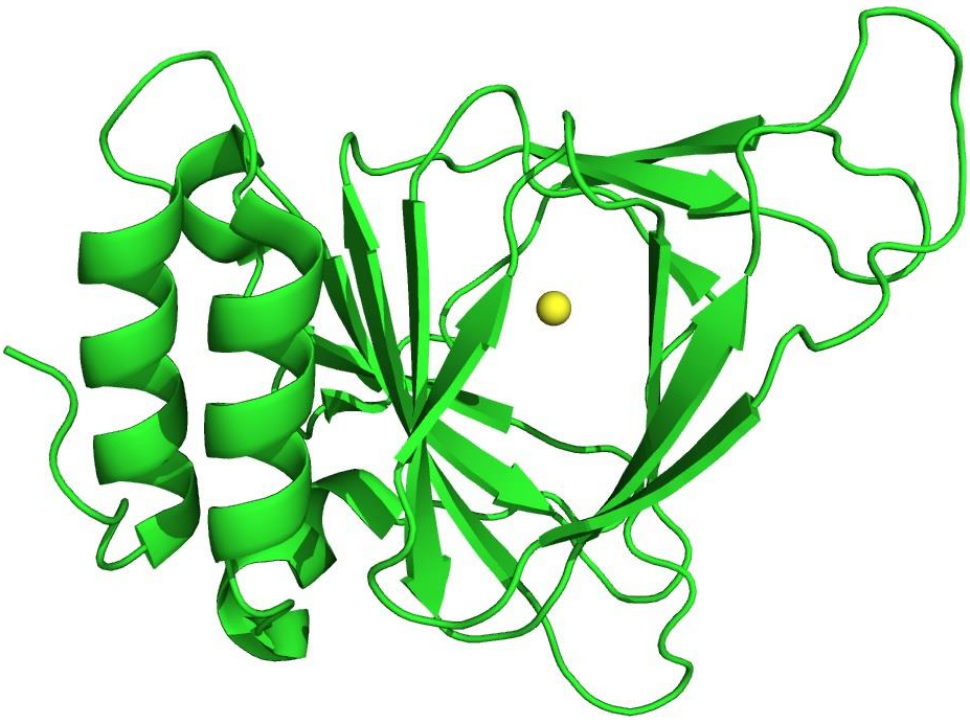


Fig. 1.14. Three-dimensional structure of CDO showing the β-barrel fold around the iron (yellow sphere) indicating that CDO is part of the cupin superfamily. PDB: 2B5H. [87]

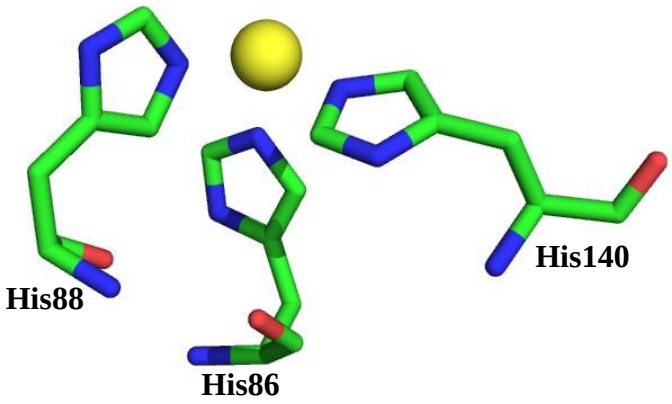


Fig. 1.15. The 3-His iron coordination in CDO. PDB: 2B5H. [87]

In addition, a set of CDO homologs were also identified by the characteristic cupin fold in various bacterial organisms. [88] Bacterial CDO also contains the conserved Tyr157 residue as well as the 3-His iron coordination, but it does contain some structural features that are different from CDO in mammalian systems. Bacterial CDO does not contain the Cys93 residue which is conserved among mammalian CDO. The Cys93 residue in mammalian CDO is replaced with a Gly residue in bacterial CDO and is unable to form the thioether crosslink which is conserved among CDO in mammalian systems. [85]

1.6 Mechanisms of Cysteine Dioxygenase

As mentioned, CDO catalyzes the oxidation of L-cysteine to L-cysteine sulfinic acid utilizing iron as a cofactor (**Fig. 1.16**). L-cysteine, ferrous iron, and dioxygen must be present in order for catalysis to proceed.[82,83,89–91] Based on multiple electron paramagnetic resonance (EPR) studies, the resting iron can exist in the ferrous or the ferric state. The ferrous state is catalytically active whereas the ferric state requires an external reductant for catalysis to occur.[82,86,87,92–97] CDO

catalyzes an ordered sequential mechanism where L-cysteine binds the iron center bidentate by the thiolate and amine groups. Binding of the cysteine substrate displaces the three water molecules allowing an open coordination site for dioxygen to bind (**Fig. 1.16, I**).[82,92] L-cysteine reduces iron to the ferrous state forming a thiyl radical (**Fig. 1.16, II**). Dioxygen oxidizes the iron back to the ferric state resulting in a superoxo iron species (**Fig. 1.16, III**). The resulting radicals from L-cysteine and dioxygen couple to form a cyclic peroxo iron complex (**Fig. 1.16, IV**). Homolytic cleavage of the cyclic complex results in an iron sulfoxy complex (**Fig. 1.16, V**). Three water molecules then displace the cysteine sulfinic acid product (**Fig.1.16, VI**). [86]

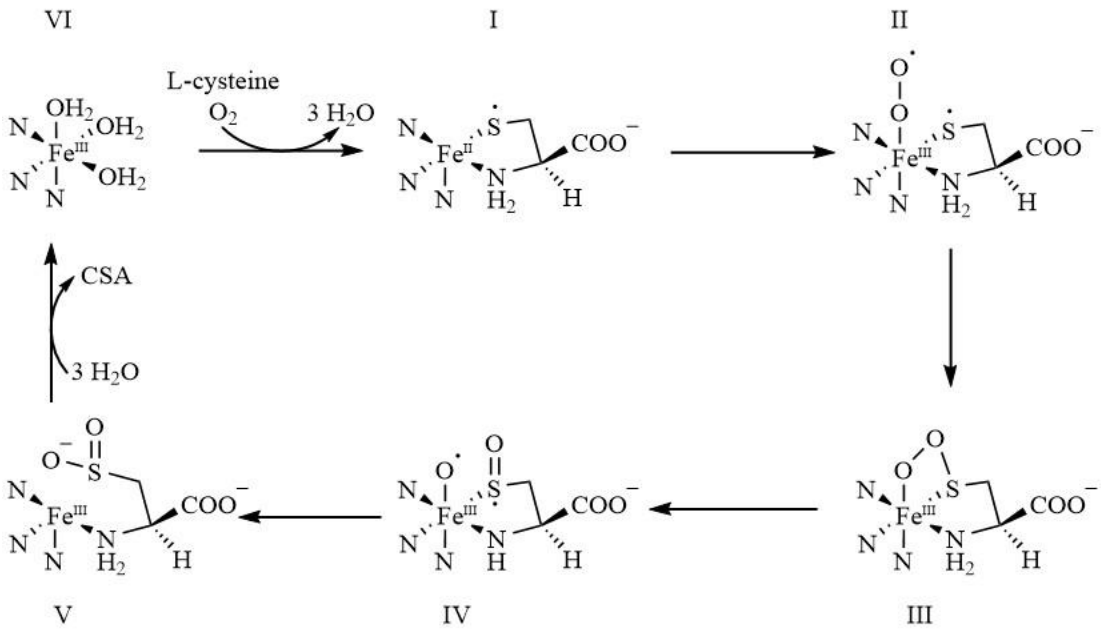


Fig. 1.16. Proposed mechanism for L-cysteine oxidation catalyzed by cysteine dioxygenase. [86]

Catalysis of the thioether crosslink in CDO has been proposed to occur through a separate mechanism from L-cysteine oxidation. [86] *In vivo* studies including the use of rat liver lysates and HepG2/C3A cells showed a heterogenous mixture of non-crosslinked and crosslinked isoforms.[89] The amount of crosslinked and non-crosslinked isoforms can be evaluated by SDS-PAGE. [98] The isoforms have a specific double band migration pattern where the top band represents the non-crosslinked isoform and the bottom band represents the crosslinked isoform (**Fig 1.17**). Because the thioether bond is not reduced, the crosslinked isoform navigates further through the gel than the non-crosslinked species. The double band migration pattern for CDO has also been confirmed utilizing mass spectrometry and mutational analysis. [70,89,99,100]

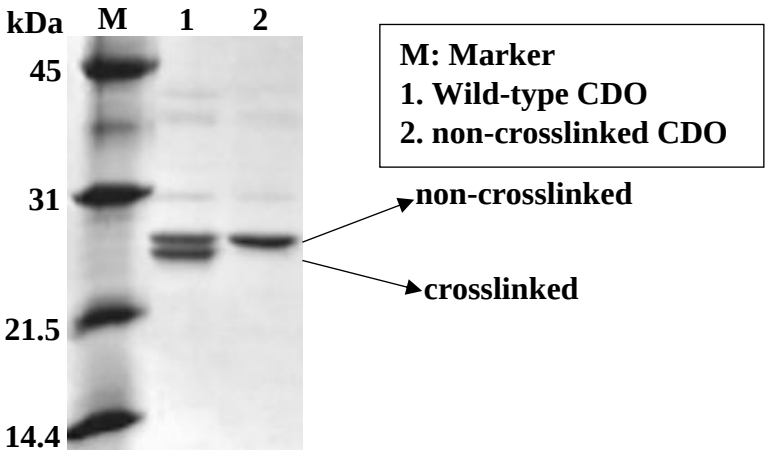


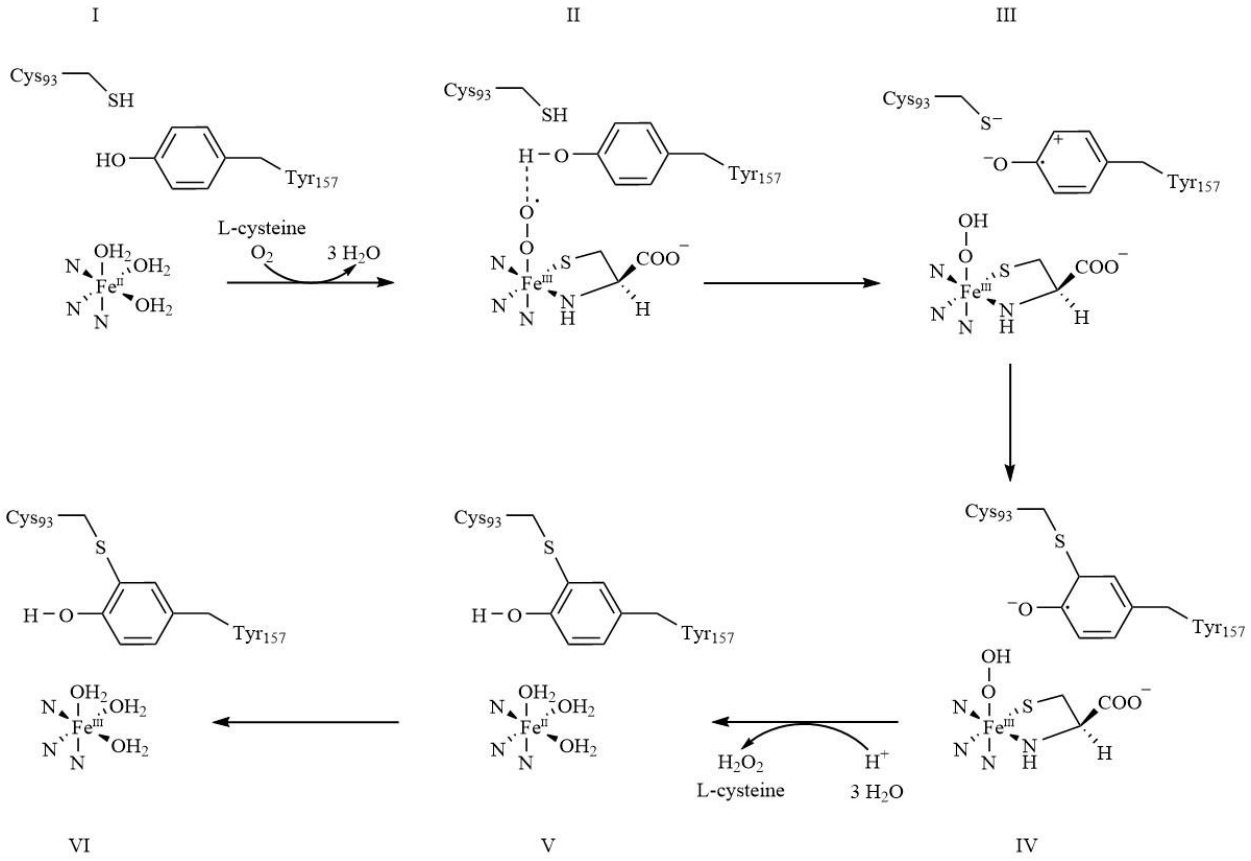
Fig. 1.17. Migration pattern of wild-type CDO where the top band represents the non-crosslinked isoform and the bottom band represents the crosslinked isoform. The non-crosslinked isoform was able to be isolated due to the addition of an iron chelator 1,10-phanthroline prior to induction during cell growth containing the CDO gene.[86]

As mentioned, the crosslink has been shown to increase catalytic activity ~5-fold compared to non-crosslinked CDO.[86] There have been several proposed roles for the crosslink in CDO. One particular study suggested that the crosslink enhanced activity indirectly by eliminating any potential side reactions that could occur with the otherwise free thiol of Cys93.[101] Several studies have suggested that the crosslink better positions the hydroxyl group of Tyr157 for acid/base catalysis during catalysis to stabilize the iron superoxo species generated during catalysis. [82–84,87] Other studies propose that the crosslink is important for substrate specificity since Tyr157 is within hydrogen-bonding distance of the carboxylate of the L-cysteine substrate.[102,103] Additionally, it has been proposed that the crosslink could act as a regulatory mechanism *in vivo*. This is because the crosslink is generated at elevated cysteine concentrations and increases catalytic activity compared to non-crosslinked CDO. However, absence of the crosslink does not completely abolish activity suggesting that catalytic activity can occur but not as efficiently. [98]

Both L-cysteine oxidation and crosslink formation utilize L-cysteine, dioxygen, and ferrous iron. [82,83,89–91] Though L-cysteine oxidation and crosslink formation both utilize ferrous iron, dioxygen, and L-cysteine, it remains unclear if these mechanisms are ordered where crosslink formation . However, L-cysteine oxidation and crosslink formation likely follow separate chemical steps. The resting iron exists in the ferrous iron state in the non-crosslinked isoform.[86] Analagous to the cysteine oxidation mechanism, L-cysteine binds bidentate to the iron center displacing the water molecules so dioxygen can bind (**Fig 1.18, I**). Once dioxgen binds the iron center, it is oxidized to the ferric state which was confirmed by EPR studies, and the resulting ferric superoxo species is stabilized by Tyr157 (**Fig.1.18, II**). Proton coupled electron transfer (PCET) from Tyr157 results in a tyrosyl radical intermediate (**Fig.1.18, III**). A nucleophilic attack from Cys93 to Tyr157 generates a crosslinked radical which undergoes the loss of a proton and electron transfer

reducing ferric iron back to ferrous iron generating the fully crosslinked species (**Fig. 1.18, IV**). Protonation of the hydroperoxy intermediate releases hydrogen peroxide, and L-cysteine is released and the water molecules maintain the open coordination sites (**Fig 1.18, V**). The iron center is oxidized to the ferric state as seen with crosslinked CDO in EPR studies (**Fig. 1.18, VI**).[86]

Fig. 1.18. Proposed crosslink formation mechanism in cysteine dioxygenase. [86]



1.7 Conserved Residues in Cysteine Dioxygenase

CDO contains several conserved residues located in or near the active site and have been shown to play roles in catalysis and/or crosslink formation. Some of these residues include Arg60, Cys93, Ser153, His155, Tyr157, Cys164, and Met179. Most of these residues are conserved among

mammalian CDO. However, bacterial systems maintain the alternative conserved residues at comparable positions. [85,88]

Based on three-dimensional structures and sequence alignments, Arg60 is conserved among mammalian CDO and is proposed to play a role in substrate specificity. [82,85,87] However, Arg60 is only conserved in CDO in some bacterial organisms. In other bacterial organisms, Arg60 is replaced with a Gln residue in CDO. It has been proposed that the bacterial CDO that contains the Arg60 residue utilizes L-cysteine analogous to mammalian CDO, while bacterial CDO containing the Gln residue utilizes other thiol-containing substrates indicating that Arg and Gln residues dictate the substrate specificity in bacterial CDO. [88,104,105]

In three-dimensional structures, the guanidinium group of Arg60 is located within ~3 Å of the carboxylate group of the iron-bound L-cysteine substrate (**Fig. 1.19**).[106] In previous studies, Arg60 was substituted with alanine and glutamine, and both variants showed a ~50% decrease in iron content and a ~70% decrease in catalytic activity compared to wild-type CDO.[82,89] It was suggested that the diminished activity was due to the lack of iron coordination; however, the decrease in iron content did not fully account for the decrease in activity.[82] The proximity of Arg60 to the carboxylate group of the L-cysteine substrate combined with diminished activity observed upon substitution of the Arg60 residue suggests that Arg60 plays a role in substrate specificity by coordinating the carboxylate group of the L-cysteine substrate.[82,106]

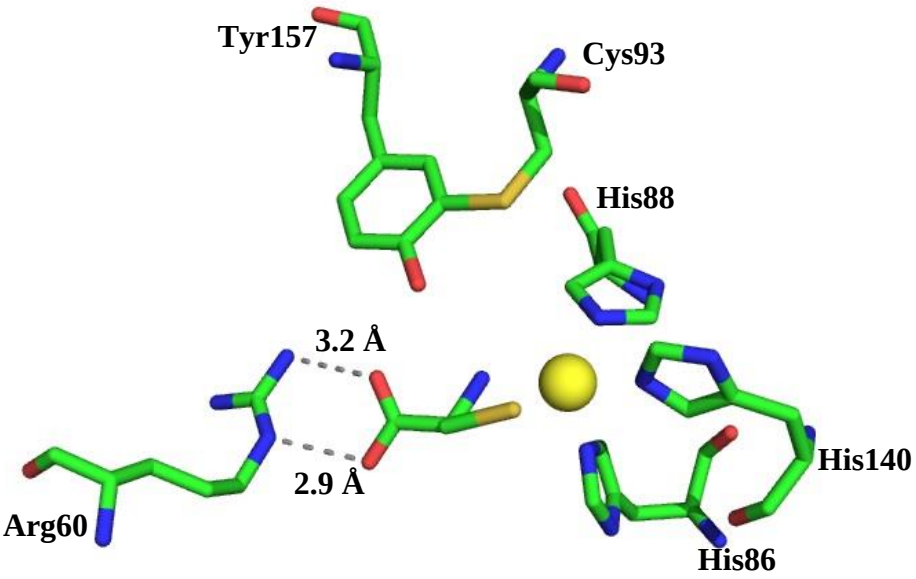


Fig. 1.19. The guanidinium group of Arg60 is ~3 Å away from the carboxylate group of the cysteine substrate in the cysteine-bound wild-type CDO three-dimensional structure indicative of stabilization through hydrogen-bonding interactions. PBB: 4IEV. [106]

Located ~8 Å away from the iron center in the opening of the active site is Cys164 which exists as a free thiol and does not participate in any intramolecular disulfide bonds.[82,87,107–109] This residue is conserved among mammalian CDO; however, in bacterial CDO this residue is replaced with an Arg or a Met residue. If the bacterial CDO maintains an Arg residue comparable to Arg60 observed in mammalian systems, then it will contain a Met residue. Conversely, if bacterial CDO contains a Gln residue in place of Arg60, it will have an Arg residue substituted for Cys164 (**Fig. 1.20**). [87,104,110] Since the Arg and Met residues in bacterial CDO are conserved and in comparable

positions to Cys164 in mammalian systems, these residues are proposed to serve a similar role in substrate specificity. [104]

Cys164 does not appear to be involved in any type of interactions with any other residues. However, the residue in closest proximity to Cys164 is Met179 which is conserved in CDO in most eukaryotic organisms. In three-dimensional structures, Cys164 is 7 Å away from Met179 which is ~5 Å away from Arg60. When L-cysteine was co-crystallized in wild-type CDO, there was a shift among the Met179 and Arg60 residues which resulted in an equidistance of ~4 Å between Cys164, Met179, and Arg60. In several three-dimensional structures, electron densities were detected at Cys164 in wild-type CDO [82,87,106,107,109] In this particular study, a mixed disulfide detected at Cys164 was determined to be the cause of the shift seen among these residues preventing the cysteine-bound CDO three-dimensional structure to be obtained.[87,107] It is unclear whether modification of Cys164 is physiologically relevant *in vivo*; however, it has been proposed that the mixed disulfide could be attributed to substrate inhibition observed *in vitro* although substitution of Cys164 with an Ala or Ser residue resulted in a nominal decrease in catalytic activity compared to wild-type CDO.[82,87]

It is currently proposed that Cys164 could play a role in substrate specificity or regulation.[82,104,107,109] In these studies, excess electron density at the Cys164 position was detected in three-dimensional structures of CDO co-crystallized with L-cysteine. [87,106,109] It was initially proposed that Cys164 had been oxidized to sulfenic, sulfinic, or sulfonic acids. [82,109] However, mass spectrometry revealed that Cys164 was involved in a disulfide bond with free L-cysteine resulting in the formation of a cystine.[87,106,109] The role of this modification is currently unknown; however, it has been proposed to be involved in regulation. [82,109] Since Cys164 is within 4 Å of a water molecule which coordinates the amine group of the L-cysteine substrate in other three-

dimensional structures, it was suggested that Cys164 could play a role in substrate specificity.[107] However, these proposed roles for Cys164 have not been adequately evaluated.

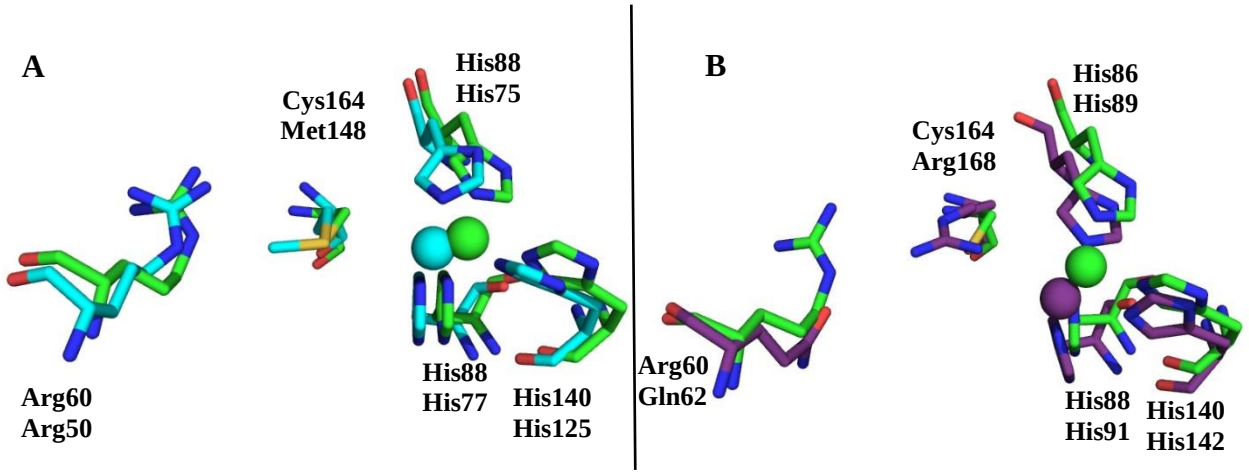


Fig. 1.20. Three-dimensional structure comparing conserved residues in mammalian and bacterial CDO. A. Like CDO in *R. norvegicus* (green), CDO in *B. subtilis* (blue) contains a 3-His coordinated iron and an Arg residue in a comparable position as Arg60. However, *B. subtilis* CDO contains a Met residue in a comparable position is Cys164 in *R. norvegicus* CDO. PDB: 2B5H and 4QM9. B. Both CDO in *R. norvegicus* (green) and *P. aeruginosa* (purple) contain a 3-His coordinated iron. However, CO in *P. aeruginosa* contains a Gln residue in place of Arg60 and an Arg residue is substituted for Cys164. PDB: 2B5H and 4TLF. [104,106,111]

Another conserved residue in CDO is Tyr157 which has been shown to play key roles in catalysis. Tyr157 forms a thioether crosslink with Cys93 which is located adjacent to the iron center in CDO. The crosslink is conserved among mammalian CDO and has been proposed to better position substrates to coordinate the iron center due to the observed 5-fold increase in catalytic activity compared to non-crosslinked CDO.[86] The thioether crosslink has been observed

in other enzymes such as galactose oxidase.[112] Galactose oxidase is a copper-dependent enzyme that catalyzes the oxidation of primary alcohols to the corresponding aldehyde and hydrogen peroxide.[113,114] The crosslink in galactose oxidase coordinates the metal center in galactose oxidase serving as a redox center during catalysis. [112,113] Interestingly, based on sequence alignments, only Tyr157 is conserved across CDO in bacterial systems while Cys93 is replaced with a Gly residue.[84,89,103] Therefore, the crosslink is not essential for bacterial CDO. [84,104,115] Despite the increase in catalytic activity when the fully crosslinked species is present, the crosslink does not appear to be essential for catalysis to occur in mammalian systems. Substitutions of Cys93 and Tyr157 in CDO resulted in reduced catalytic activity compared to wild-type CDO. Because appreciable activity was still observed, the crosslink was not considered essential for catalytic function.[82,86,89,103,116] Crosslink formation studies were also performed with rats fed a low protein diet containing low concentrations of cysteine or cysteine precursors followed by a high protein diet consisting of high concentrations of cysteine or cysteine precursors. The rats were then sacrificed and CDO levels in the hepatic tissues of the rats were analyzed by SDS-PAGE. Once the rats were switched to the high-protein diet, the non-crosslinked isoform of CDO was rapidly formed and was then converted to the crosslinked isoform gradually over several days indicating that the crosslink was generated due to an excess of cysteine present.[98] Since the crosslink is generated at increasing L-cysteine concentrations, the crosslink could act as a regulatory mechanism which would explain the increased activity observed compared to non-crosslinked CDO. [82,89,92]

Adjacent to Tyr157 is a His (His155) residue and a Ser (Ser153) residue. In three-dimensional structures, Tyr157-His155-Ser153 are all equidistantly located ~2.7 Å away from each other resulting in a hydrogen-bonding network (**Fig. 1.21**). Based on sequence alignments,

Tyr157-His155-Ser153 are conserved among most mammalian and bacterial CDO. [84,87,90,101,103,117] This hydrogen-bonding network has been of interest since it involves a component of the crosslink and due to the proximity of the network to the active site.

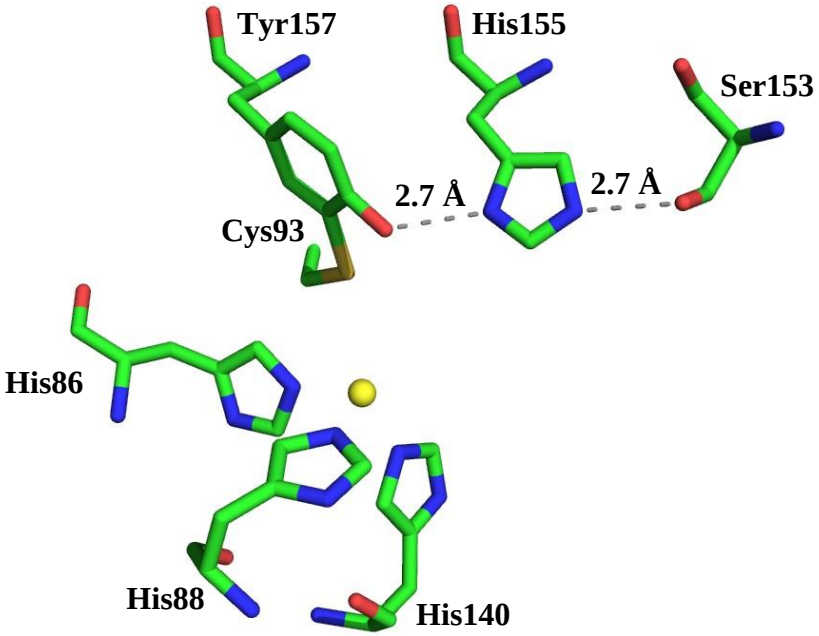


Fig.1.21. Three-dimensional structure depicting the hydrogen-bonding network involving Tyr157-His155-Ser153 and the proximity to the crosslink and iron center in the active site of CDO. PDB: 2B5H. [87]

Because Tyr157 is a component of the crosslink and hydrogen-bonding network, it has been implicated in cysteine oxidation.[82,83,87,89,101,103,117] The hydroxyl group of Tyr157 aids in positioning L-cysteine and dioxygen to bind the iron center. In previous studies, substitution of Tyr157 with Phe and Cys93 with Ala resulted in diminished activity with L-cysteine compared to wild-type CDO. However, the C93A CDO variant resulted in higher product formation with D-

cysteine compared to wild-type CDO with D-cysteine. In wild-type CDO, generation of the crosslink maintains the rigidity of the position of Tyr157 during catalysis. In the C93A CDO variant, Tyr157 may be able to move more freely which may lead to the lowered substrate discrimination. Computational studies suggested that Tyr157 could play roles in substrate recognition and stabilization of Fe-oxo intermediates by acid/base catalysis which could explain the changes in catalytic activities. This suggests that the crosslink is responsible for positioning Tyr157 for these interactions to occur. [103]

Adjacent to Tyr157 is His155 which has been proposed to play a key role in the stabilization of Tyr157 through hydrogen-bonding interactions. Previous studies have suggested that substitution of His155 to Ala resulted in abolished activity compared to wild-type CDO.[103,117] Unlike wild-type CDO, the L-cysteine-bound iron(III) center in H155A CDO was unable to bind cyanide. Computational studies have suggested that His155 hydrogen bonds with Tyr157 to stabilize the Fe-oxo intermediates which could explain the diminished catalytic activity and the inability to bind cyanide.[103] In a separate study, magnetic circular dichroism (MCD) experiments were performed and wild-type CDO had a 5-coordinate iron(II) with L-cysteine bound whereas H155A CDO displayed a 6-coordinate iron(II) with L-cysteine bound. The sixth ligand observed for H155A CDO is a water molecule occupying the otherwise free coordinate site for dioxygen binding. The lack of catalytic activity observed with H155A CDO could be due to the water molecule preventing dioxygen to bind the iron center suggesting that His155 may play a role in discouraging water molecules to remain bound to the iron center during catalysis[117].

Ser153 is the final component of the hydrogen-bonding network. While Ser153 is conserved among CDO in most mammalian and bacterial systems, it is not as highly conserved as Tyr157 or His155. Therefore, the functional relevance of Ser153 in the hydrogen bonding network

is uncertain [102]. Due to the proximity of Ser153 to His155 and Tyr157, Ser153 is thought to play a role in stabilizing the hydrogen-bonding network during catalysis, although it does not appear to play a direct role in L-cysteine oxidation or crosslink formation[118].

1.8 Hydrogen-bonding Network in Cysteine Dioxygenase

Hydrogen-bonding interactions are crucial for protein function. Hydrogen bonds aid in the structure and stability of proteins as well as proton transfer during catalysis. Catalytic triads are often referred to three amino acids within a protein that interact through hydrogen bonds. [119,120] These networks commonly involve three types of amino acids: a nucleophile (Ser/Cys), a base (His), and an acid (Glu/Asp). Hydrogen-bonding networks are often referred to as catalytic triads since they have been shown to play a prevalent role in catalysis in some enzymes.[119,121]

Catalytic triads were first identified in trypsin and chymotrypsin. A Ser residue in both trypsin and chymotrypsin was recognized as a catalytic nucleophile. Later, three-dimensional structures revealed the catalytic triad in several other proteases.[122–126] Catalytic triads can have two different functions in catalysis. They can hydrolyze a substrate (hydrolases), or they can transfer part of a substrate to a second substrate (transferases). [127] Regardless of the function the catalytic triads serve, the overall goal of the triad is to increase nucleophilicity of the nucleophilic amino acid inducing an attack on the substrate to initiate catalysis. [119,120]

The nucleophilic residue, responsible for initiating the attack on the substrate, is usually a Ser or Cys residue. [120] Ser and Cys are known for their nucleophilic capabilities; however, their minor chemical differences can have an impact on catalysis. Cysteine can form longer bonds and has a lower pK_a than Ser making it easier for Cys to be deprotonated for a nucleophilic attack.[128,129] However, Ser has a higher pK_a making it crucial for Ser to be positioned in the correct orientation

with the basic and acidic residues so that the pK_a is lowered and can be effectively deprotonated. Conversely, the lower pK_a for Cys causes it to be vulnerable to the reversal of the nucleophilic attack rather than the resolution of the tetrahedral intermediate. [120] Occasionally, Thr may be utilized as the nucleophile although the methyl group of the side chain often causes steric hindrance resulting in the use of the N-terminal amide as the basic residue. [119,130] While rare, selenocysteine is sometimes recognized as the nucleophilic residue. [87]

The basic amino acid is responsible for donating and abstracting protons throughout catalysis. It is especially known for abstracting the proton from the nucleophile initiating a nucleophilic attack on the substrate. [131] Due to its pK_a, a His residue often serves as the basic residue since it is able to maintain hydrogen-bonding with the acidic residue and readily undergo acid or base catalysis. [87] Occasionally, a lysine residue is observed as the basic residue in catalytic triads. However, due to its high pK_a, it is important for the acidic residue to effectively lower the pK_a of Lys so that it can abstract the proton from the nucleophilic residue. [132] Due to the low pK_a, the acidic residue in catalytic triads maintains the position of the basic residue and increases the electronegativity of the basic residue so that it will abstract a proton from the nucleophile initiating catalysis. The acidic residue is often a Glu or Asp residue. [131] In some cases, His residues are utilized as both the basic and acidic residues while other enzymes utilize a catalytic dyad that lacks the acidic residue. [119]

Serine proteases contain the classic catalytic triad which consists of Ser-His-Asp (**Fig.1.22**). [91] The Asp residue lowers the pK_a of the His residue resulting in proton abstraction from the Ser residue. Ser then makes a nucleophilic attack on the carbonyl of the peptide substrate resulting in a covalent, tetrahedral intermediate. The dense negative charge resulting from the intermediate is known as the oxyanion hole which is stabilized by the amide peptide backbone of

Ser195 and Gly193. His57 abstracts a proton from the tetrahedral intermediate which forms the first product, the N-terminal region of the peptide, and the acylenzyme. His57 abstracts a proton from a water molecule which then performs a nucleophilic attack on the carbonyl of the acylenzyme to generate the second intermediate. The collapse of the intermediate results in the second product which is the C-terminal region of the peptide (**Fig. 1.23**). [134]

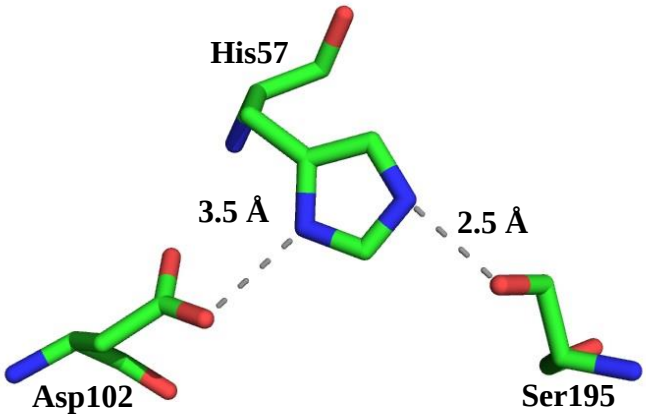


Fig. 1.22. The Ser-His-Asp triad found in serine proteases which is a classic example of a catalytic triad. PDB: 1CHO. [135]

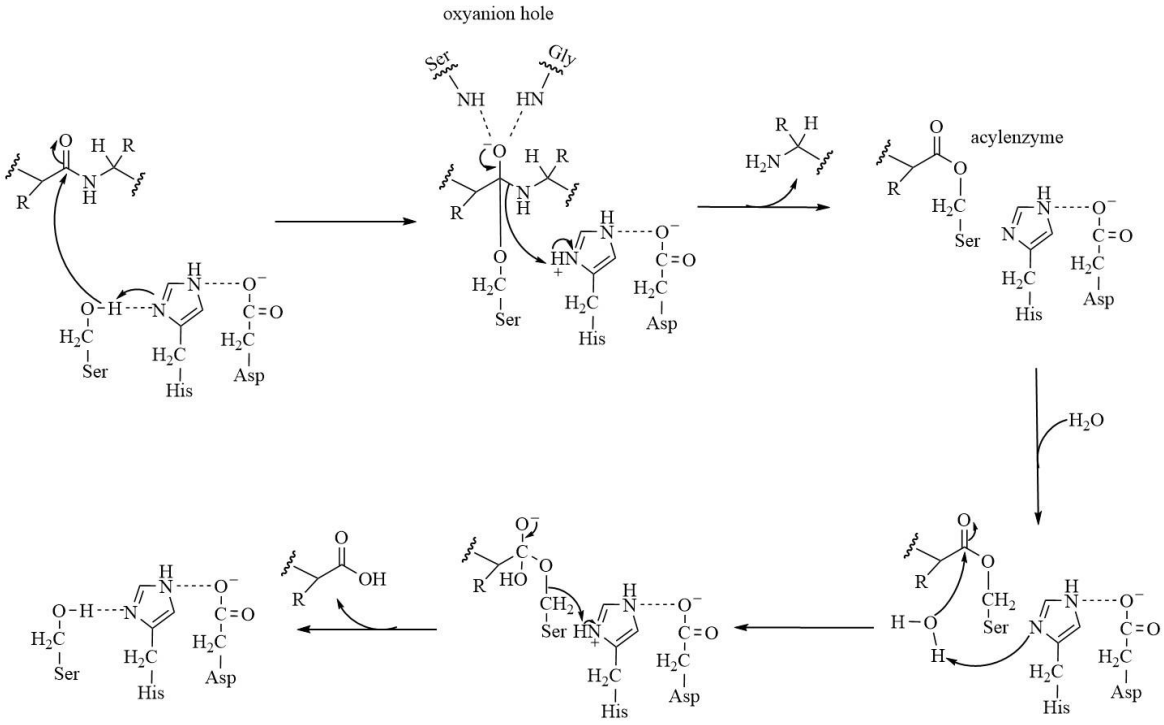


Fig. 1.23. The general catalytic mechanism for serine proteases. [134]

CDO has a hydrogen-bonding network made up of Tyr157-His155-Ser153 (**Fig. 1.24**). [51] This network was identified as a catalytic triad since it resembled defined catalytic triads found in proteases. This network is mostly conserved in CDO among mammalian systems and many strains of bacteria. [87,102,103,117] Additionally, it does contain the nucleophilic (Ser153) and basic (His) residues commonly observed in catalytic triads. His155 is proposed to serve a similar role as the basic residue in catalytic triads by stabilizing Tyr157 during catalysis by donating and accepting protons during catalytic events.[102,117] However, Tyr residues have not been identified as a nucleophile in catalytic triads which is normally the residue that His155 assists in defined catalytic triads. The Ser153 residue which would normally be identified as the nucleophilic residue is not as widely conserved among organisms like Tyr157 and His155. [103] In addition, Ser153 is not known to make a nucleophilic attack on the cysteine substrate which is a key step among catalytic triads. In fact, substitution of Ser153 with Ala has shown negligible structural and catalytic

changes in CDO suggesting that Ser153 may provide stability for Tyr157 and His155 but is not essential for catalysis. [118] Occasionally, an amino acid with a high pK_a has been observed as the acidic residue in catalytic triads; therefore, Tyr157 could be identified as the acidic residue for the catalytic triad. [135,136] However, based on mutational and kinetic studies on Tyr157, it is proposed to be directly involved in catalysis through hydrogen-bonding interactions with the substrates and would therefore not serve the same function as the acidic residue in defined catalytic triads. [82,87,93,98,101,103,117]

While this Tyr157-His155-Ser153 hydrogen-bonding network does possess some of the characteristics of defined catalytic triads, this network does not possess the typical types of amino acids that catalyze these defined reactions. This network also does not appear to follow the typical mechanism characteristic of catalytic triads.[60,65] The Tyr157-His155-Ser153 hydrogen-bonding network does appear to play important roles in both L-cysteine oxidation and crosslink formation in CDO through hydrogen-bonding interactions. Therefore, it does not appear to play the same role as catalytic triads seen in proteases. [103,117]

1.9. Summary

Cysteine is utilized in multiple metabolic processes including protein synthesis, redox signaling, catabolic reactions, and the biosynthesis of thiol-containing compounds. Cysteine is involved in several metabolic pathways. One pathway of interest includes the oxidation of cysteine to cysteine sulfinic acid which is catalyzed by CDO. The product, cysteine sulfinic acid, serves as an important branchpoint in sulfur metabolism since it can then be utilized to form pyruvate and sulfite, or taurine. These metabolites formed from the branchpoint can be used for metabolic processes. Consequently, the absence of CDO can lead to an increase in cysteine concentrations.

The influx of cysteine in the presence of metals often generates ROS which has been linked to numerous disease states.

CDO contains an octahedrally coordinated iron center including three His residues and three water molecules. Adjacent to the iron center is a thioether crosslink which consists of Tyr157 and Cys93. This crosslink has been identified as a protein-derived cofactor due to the increased activity observed compared to non-crosslinked CDO. Tyr157 is also involved in a hydrogen-bonding network consisting of the Tyr157-His155-Ser153 triad. This network is structurally common among CDO in various organisms and was first identified due to the resemblance of defined catalytic triads in other enzymes.

Located almost 8 Å away from the iron center in CDO is a cysteine residue (Cys164). It is in the opening of the active site and exists as a free thiol. Cys164 is conserved in CDO among eukaryotic organisms and one of the proposed functions is substrate specificity. Met or Arg residues have been identified at comparable positions in CDO homologs of bacteria and have been proposed to serve the same function as Cys164 in mammalian CDO. In three-dimensional structures, Cys164 exists as a free thiol in the absence of free cysteine. However, crystals that have been soaked or co-crystallized with free cysteine exhibited an excess electron density at the 164 position in some resolved three-dimensional structures. The electron density was later identified as free cysteine involved in a disulfide linkage with Cys164. It was then proposed that Cys164 could serve as a regulatory switch potentially under oxidative stress. Cumulatively, this work evaluates the functional and mechanistic roles of a hydrogen-bonding network and a rogue cysteine residue in L-cysteine oxidation and crosslink formation in cysteine dioxygenase. This work provides a foundation for the future studies focused on further elucidating the mechanistic roles of key amino acids involved L-cysteine oxidation and crosslink formation.

Chapter Two

A Hydrogen-bonding Network is Vital for Efficient L-cysteine Oxidation and Crosslink Formation in Cysteine Dioxygenase

2.1 Introduction

Cysteine dioxygenase (CDO) is an iron-dependent enzyme that catalyzes the oxidation of L-cysteine to L-cysteine sulfinic acid (CSA).[7–9] The iron center is octahedrally coordinated by three His residues and three water molecules. Neighboring the iron center is a thioether crosslink between a tyrosine (Tyr157) and cysteine residue (Cys93).[82,87,90,101] The crosslink has been identified as a protein-derived cofactor due to an observed 5-fold increase in catalytic activity compared to non-crosslinked CDO.[86] In addition, Tyr157 forms a hydrogen-bonding network with nearby histidine (His155) and serine (Ser153) (**Fig. 2.1**). The function of the hydrogen-bonding network in CDO is of interest due to the involvement of Tyr157 in the network.[84,87,101,102,106,117,137]

The L-cysteine oxidation and crosslink formation mechanisms both require the cysteine substrate, molecular oxygen, and ferrous iron.[83,89–91,138] It is not clear if the crosslink must be formed prior to L-cysteine oxidation. While this has not been fully determined, the proposed mechanisms appear to follow separate chemical steps.[86] Based on three-dimensional structures and sequence alignments, the Tyr157-His155-Ser153 network in CDO is conserved among some

mammalian and bacterial organisms.[87,102,103,117] The network involves Tyr157, a component of the protein-derived thioether crosslink, which has been shown to be important in the catalytic efficiency of CDO.[82,87,93,98,101–103,117] The hydroxyl group of Tyr157 has been proposed to play an important role in catalysis by positioning molecular oxygen for binding to the iron center or acting as a catalytic acid/base during crosslink formation or L-cysteine oxidation.[82,87,89,101,103,117,139] Since His155 is within close proximity to Tyr157, the hydrogen-bonding interaction could stabilize Tyr157 intermediates involved in both mechanisms [93]. This hydrogen-bonding network has been compared to catalytic triads in other enzymes and its conserved nature suggest that it may be involved in L-cysteine oxidation or crosslink

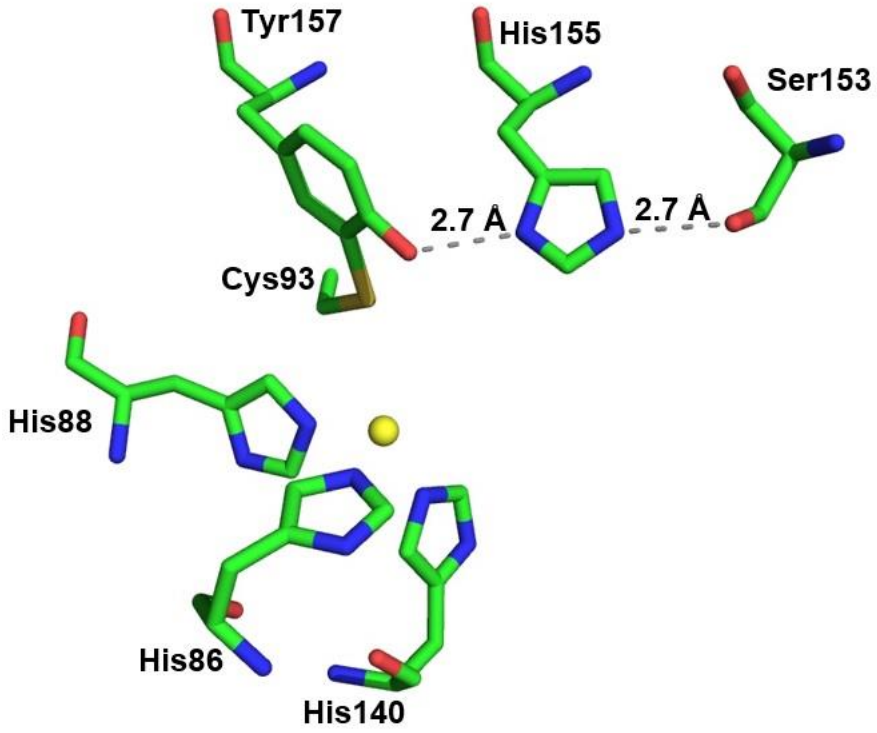


Fig. 2.1 Three-dimensional structure showing that Tyr157, His155, and Ser153 are within 2.7 Å of each other resulting in a hydrogen-bonding network in the active site of CDO. PDB:2B5H. [87]

formation. [84,87,101,102,106,117,137] However, the Ser residue is not universally conserved, and therefore the nature of the hydrogen-bonding network serving as a true catalytic triad may not be valid. [103]

The role of the His155 in both crosslink formation and L-cysteine oxidation was evaluated to establish a potential role for this residue in both mechanisms. Previous studies have investigated the role of the hydrogen-bonding network in L-cysteine oxidation based on a single substitution (H155A CDO). [11,12] In this study, several substitutions of His155 were generated to evaluate specific properties of the His residue in relation to hydrogen-bonding. By constructing these variants, His155 was probed by both kinetic and spectroscopic investigations to examine the role of His155 in acid/base catalysis and/or stabilizing Tyr157 intermediates during both L-cysteine oxidation and crosslink formation. These findings provide another dimension to the role His155 plays in both the L-cysteine oxidation and crosslink formation mechanisms in CDO.

2.2 Material and methods

2.2.1 Materials

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), L-cysteine, L-ascorbate, ampicillin, streptomycin sulfate, lysozyme, potassium chloride (KCl), isopropyl-β-d-thiogalactoside (IPTG), ferrous ammonium sulfate, (5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4,4"-disulfonic acid) ferrozine, and brilliant blue R were purchased from Sigma (St. Louis, MO). Sodium dodecyl sulfate was purchased from Biorad (Hercules, CA). Glycerol and sodium chloride were purchased from Macron Fine Chemicals (Center valley, PA). Pfu Turbo DNA polymerase was purchased from Agilent (La Jolla, CA). Oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA). Macro-Prep® High Q Support was purchased from Bio-Rad

Laboratories (Hercules, CA). Difco-brand Luria–Bertani (LB) media was purchased from Becton, Dickinson and Company (Sparks, MD). Amicon Ultra-4 Centrifugal Filter Devices (10 kDa MWCO) were purchased from Millipore (Billerica, MA).

2.2.2 Protein Expression and Purification

The cDNA gene for rat CDO was cloned into the pET21a expression vector as previously described.[92] The vector containing the CDO gene was used to construct the rat CDO variants (H155A, H155E, and H155Q CDO) by site-directed mutagenesis. Primers for all variants were designed as 27 base oligonucleotides. To generate His155 variants, the His155 codon (CAC) in wild-type CDO was replaced with GCG (Ala), GAA (Glu), and CAG (Gln) codons. To generate the Ser153 to Ala variant, the Ser153 codon (TCT) was replaced with GCG (Ala). Successful substitutions were confirmed by DNA sequence analysis (Eurofins MWG Operon). Each CDO variant was transformed into BL21(DE3) *E. coli* competent cells and stored as glycerol stocks at -80°C.

The cells containing the CDO plasmid were isolated on LB-agar medium containing 0.1 mg/mL ampicillin (LB-amp). A single colony was selected from the medium and grown in 5 mL LB-amp media at 37°C for ~7 hrs. A 1% inoculum was transferred from the 5 mL culture to a 100 mL culture and grown overnight at 37°C. A 2% inoculum from the overnight culture was transferred to four 1 L flasks and grown at 37°C until the cultures reached a A_{600nm} value of 0.4-0.6. The cells were induced with 0.4 mM IPTG and grown for an additional ~6 hrs at 25°C. Cells were harvested, and the pellets were resuspended with 100 mL of buffer containing 10% glycerol and 25 mM HEPES (pH 7.5) and 0.2 mg/mL of lysozyme. Cells were lysed by sonication, and the clarified supernatant was treated with 1.2% streptomycin sulfate and stirred at 4°C for 1 hr. Following centrifugation, the supernatant was then applied to a Macro-Prep® High Q Support

column, and protein was eluted by a linear gradient from 0-150 mM sodium chloride in 10% glycerol and 25 mM HEPES (pH 7.5). Fractions were evaluated by SDS-PAGE and the purest fractions were pooled and iron loaded with a molar ratio of 1:1 ferrous ammonium sulfate:protein. To remove excess iron, samples were dialyzed twice against one liter of 25 mM HEPES (pH 7.5) with 100 mM sodium chloride and 10% glycerol at 4°C. Protein aliquots were flash frozen and stored at -80°C.

The amount of iron present in the protein preparation was determined by a colorimetric assay using an iron chelator, ferrozine, as described previously with modifications. [140] CDO samples were diluted to 10 μM in a final volume of 1 mL using 25 mM HEPES (pH 7.5). A standard curve was generated using iron ICP standard (0-15 μM). Additionally, 25 mM HEPES (pH 7.5) and deionized and distilled water were used as controls. The results obtained were the average of three separate experiments.

2.2.3 Steady-State Kinetic Analyses and Product Formation of Wild-type and His155 CDO Variants

Steady-state kinetic studies on CDO variants were performed using a Clark-type oxygen electrode (Hansatech, Inc., Norfolk, United Kingdom) to measure the rate of dioxygen utilization by each enzyme in the presence of L-cysteine. The concentration range implemented for wild-type CDO was 0.2-5 mM L-cysteine, and 10-500 mM L-cysteine for the His155 CDO variants. Each reaction contained 2 μM protein and 1 mM ascorbate in 25 mM HEPES (pH 7.5). The reactions were initiated by the addition of L-cysteine, and the initial velocities were measured from the linear portion of the trace. The average initial velocities from three separate experiments were plotted against the substrate concentration and the data fit to the Michaelis-Menten equation using KaleidaGraph™ software. The steady-state parameters obtained were the average of three separate

experiments. The rate of dioxygen consumption was correlated with the rate of cysteine sulfinic acid (CSA) formation using LC–MS using the protocol previously described.[86] The values obtained reflected the averages of three separate experiments.

2.2.4 Crosslink Formation Studies of the Wild-type and CDO Variants

Crosslink formation was investigated by varying L-cysteine concentrations. In crosslink formation assays, 5 μM of wild-type or His155 CDO variants were incubated with 1 mM of ascorbic acid and 0-100 mM L-cysteine substrate in 25 mM HEPES (pH 7.5) at 37°C with gentle shaking. Each sample was treated with 2% SDS and 5% 2-mercaptoethanol, heat denatured for 3 minutes, and analyzed by 12% SDS-PAGE. In addition, the ratio of non-crosslinked to crosslinked isoforms was quantified by ImageJ software.[92]

2.2.5 EPR Spectroscopic Analysis of Wild-type and CDO Variants

The oxidation state of the iron center in wild-type CDO and variants was measured by electron paramagnetic resonance spectroscopy (EPR). Samples were prepared by diluting the CDO enzymes to 90 μM in 25 mM HEPES (pH 7.5), 100 mM NaCl, and 10% glycerol in a final volume of 300 μL. When L-cysteine was included, the CDO samples were supplemented with a final concentration of 10 mM L-cysteine in a final volume of 300 μL. Amicon Ultra-4 Centrifugal Filter Devices (10 kDa MWCO) were used for samples where L-cysteine was removed. All EPR spectra were obtained at X-band frequency (9.38 GHz) on a Bruker-EMX spectrometer (Bruker Biospin Corporation, Billerica, MA) at 8 K. Temperature was maintained using an Oxford Instruments ESR 900 flow cryostat and an ITC4 temperature controller. All EPR spectra were obtained using the following settings: microwave frequency: 9.38 GHz, microwave power: 0.2 mW; receiver gain:

2 x 10^4; modulation frequency: 100 kHz; modulation amplitude: 6 G; time constant: 163.85 ms; sweep time: 167.772 s.

2.3 Results

2.3.1 Steady-State Kinetic analysis of wild-type and His155 CDO variants

In order to determine if His155 plays a role in L-cysteine substrate oxidation, His155A, H155E, and H155Q CDO variants were constructed and evaluated by steady-state kinetic studies. In this study, His155 was replaced with an alanine to disrupt interactions with Tyr157. Additionally, His155 was replaced with an uncharged Gln to determine if the hydrogen bonding network could still be maintained. A His155 to Glu substitution introduced a negative charge to replace the basic properties of the His imidazole ring. The ability of the CDO variants to oxidize L-cysteine was monitored to determine if His155 played a role in catalysis. The resulting k_{cat}/K_m value for wild-type CDO was 2233 $mM^{-1}min^{-1}$ (**Table 2.1**). In order to confirm that the dioxygen utilization observed correlated with the conversion of L-cysteine to the CSA product, LC-MS was implemented. Wild-type CDO displayed a ~1:1 ratio of O_2 utilization:CSA production. Initially, the same range of L-cysteine concentrations was used to evaluate the catalytic parameters for the His155 CDO variants. However, the His155 variants showed negligible activity under similar conditions. Increasing L-cysteine concentrations gave k_{cat} values for the His155 CDO variants that were within 3- or 4-fold of the k_{cat} value for wild-type CDO. However, the K_m values for the His155 CDO variants were 380- to 1800-fold higher than the K_m value for wild-type CDO. This resulted in k_{cat}/K_m values of 1.5 $mM^{-1}min^{-1}$ and 0.8 $mM^{-1}min^{-1}$ for H155A and H155Q CDO, respectively. The H155E CDO variant had a k_{cat}/K_m value of 0.3 $mM^{-1}min^{-1}$. Product formation for the His155 variants was not determined due to the lack of appreciable catalytic activity (**Table 2.1**). His155 has also been proposed to be part of a catalytic triad with Tyr157 and Ser153.[87,93,103,117,119,141]

However, substitution of Ser153 with Ala showed comparable activity as wild-type CDO and was not evaluated further in these studies (**Table 2.1**).

Table 2.1 Steady-state kinetic parameters of wild-type CDO and the H155 CDO variants.

	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})	% Fe
Wild-type CDO	134 ± 4	0.06 ± 0.01	2233 ± 378	60 ± 2
S153A CDO	59 ± 2	0.07 ± 0.01	852 ± 135	95 ± 3
H155A CDO	35 ± 2	23 ± 7	1.5 ± 0.5	97 ± 2
H155E CDO	35 ± 2	110 ± 18	0.3 ± 0.1	80 ± 5
H155Q CDO	48 ± 2	58 ± 11	0.8 ± 0.2	84 ± 3

The concentration of L-cysteine was varied from 0.2-5 mM for wild-type CDO and 10-500 mM for the CDO variants.

2.3.2 Analysis of crosslink formation with His155 CDO variants

In order to evaluate the role of His155 in crosslink formation, wild-type and His155 CDO variants were evaluated by crosslink formation studies. Purified wild-type CDO existed as a heterogeneous mixture of non-crosslinked and crosslinked isoforms. The His155 variants yielded various ratios of non-crosslinked to crosslinked isoforms following purification. The H155Q CDO variant existed as a 50:50 mixture of non-crosslinked and crosslinked isoforms analogous to wild-type CDO. Interestingly, H155A and H155E CDO existed predominantly as the non-crosslinked isoform. The H155A CDO variant existed as an 80:20 mixture of non-crosslinked and crosslinked isoforms, and H155E CDO existed as a 95:5 mixture of the isoforms (**Fig. 2.2**).

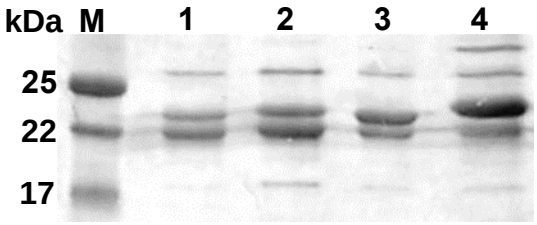


Fig. 2.2 Purification analysis depicting the ratio of non-crosslinked:crosslinked isoforms of wild-type and His155 CDO variants. M: protein standard marker 1. wild-type CDO (50:50) 2. H155Q CDO (50:50) 3. H155A CDO (80:20) 4. H155E CDO (95:5). Samples were treated with 2% 2-mercaptoethanol and 5% SDS and analyzed on 12% SDS-PAGE.

While wild-type CDO exists as a heterogenous mixture of non-crosslinked and crosslinked isoforms, the fully crosslinked species can be generated by incubating the enzyme with L-cysteine (**Fig. 2.3A**). In order to determine if His155 plays a role in crosslink formation, the His155 CDO variants were incubated with various L-cysteine concentrations (0-100 mM) and compared to wild-type. The H155A CDO variant yielded only 80% of the crosslinked isoform and 20% of the non-crosslinked isoform at 100 mM L-cysteine (**Fig. 2.3B**). Additionally, H155E CDO retained the same ratio of non-crosslinked to crosslinked isoforms as purified even at elevated L-cysteine concentrations (**Fig. 2.3C**). Even though H155Q CDO showed negligible activity, the variant was found to generate the fully crosslinked species at 80-100 mM L-cysteine analogous to wild-type CDO (**Fig. 2.3D**).

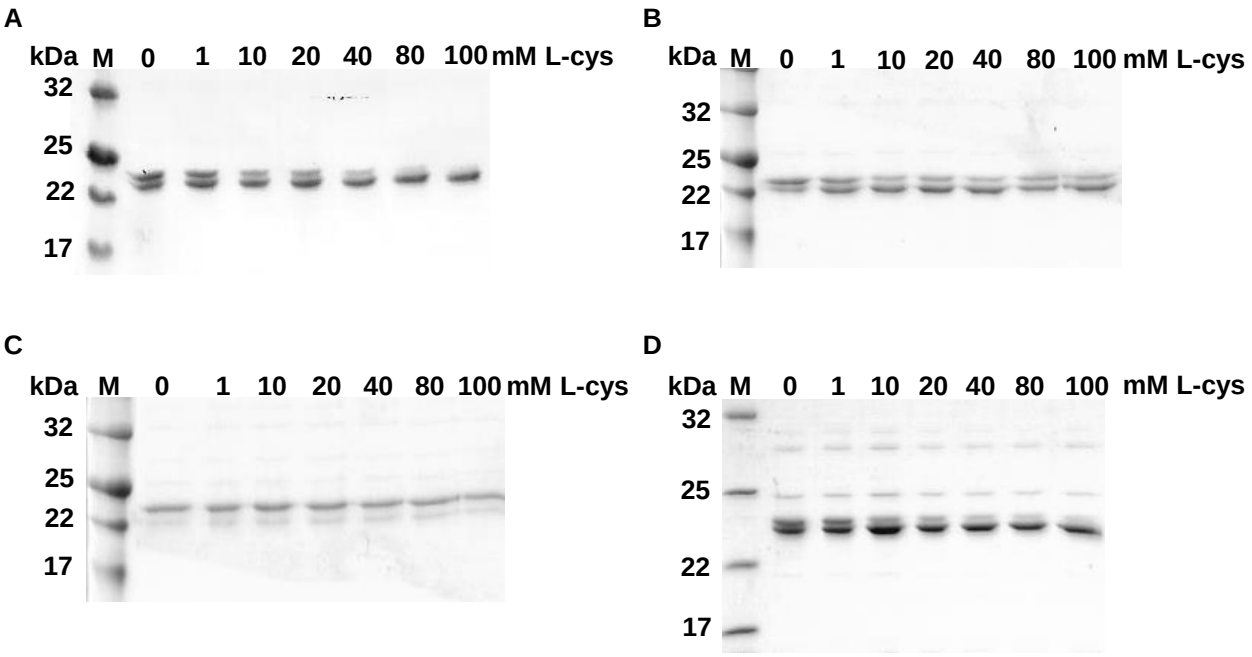


Fig. 2.3 Crosslink formation analyses for the wild-type and His155 CDO variants. A. wild-type CDO B. H155A CDO C. H155E CDO D. H155Q CDO. Wild-type CDO and His155 CDO variants were incubated at 37°C with L-cysteine (1-100 mM) and 1 mM ascorbate in 25 mM HEPES (pH 7.5) with gentle shaking for 30 min. Each sample was treated with 2% 2-mercaptoethanol and 5% SDS and analyzed on 12% SDS-PAGE. The quantity of non-crosslinked and crosslinked species present was quantified using ImageJ software.

2.3.3 Effects of L-cysteine on the iron center in CDO

Because the His155 CDO variants demonstrated minimal crosslink formation and catalytic activity, EPR measurements were performed to determine if the His155 CDO variants altered the environment of the metal center. Wild-type CDO and His155 CDO variants all showed an EPR signal at g=4.3 indicative of the high spin ferric state. [86,92] The His155 CDO variants displayed

broad peaks with lower intensities than wild-type CDO, suggesting that the substitutions influenced the microenvironment surrounding the metal center (**Fig. 2.4**).

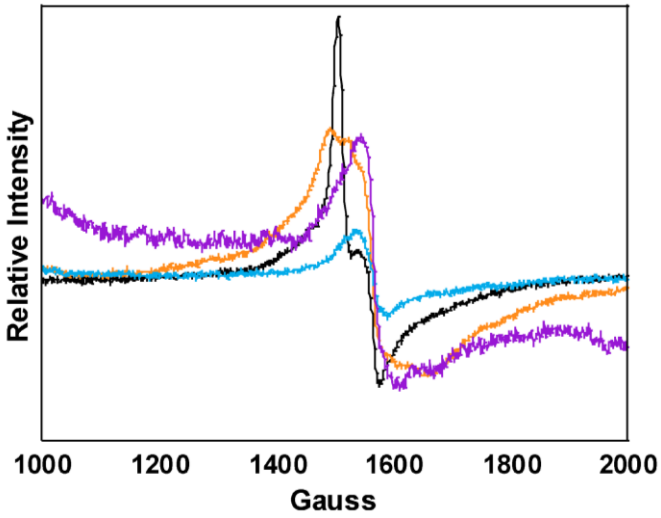


Fig. 2.4 X-band EPR spectra of wild-type CDO and His155 CDO variants. Wild-type CDO (black trace), H155A CDO (purple trace), H155Q CDO (orange trace), and H155E CDO (blue trace). All spectra were measured with 90 μM protein in 25 mM HEPES buffer (pH 7.5) 100 mM NaCl, and 10% glycerol. All spectra were recorded at 9.38 GHz, with a field modulation frequency of 100 kHz and modulation amplitude of 6 G. All spectra were recorded at 8 K.

It has previously been reported that the addition of the L-cysteine substrate to wild-type CDO resulted in a sharper Fe^{3+} signal compared to the ferric signal seen with wild-type CDO in the absence of L-cysteine.[86,92,96] It was proposed that the sharper signal was attributed to the coordination of the L-cysteine substrate to the iron center or formation of the crosslink.[86,96] Further investigation with fully crosslinked wild-type CDO revealed that the sharp signal was due to the formation of the crosslink suggesting that the crosslink changes the microenvironment around the iron center resulting in a more defined signal.[86] Upon addition of L-cysteine, each variant

demonstrated a sharp signal comparable to wild-type CDO (**Fig. 2.5**). The ability of H155Q CDO

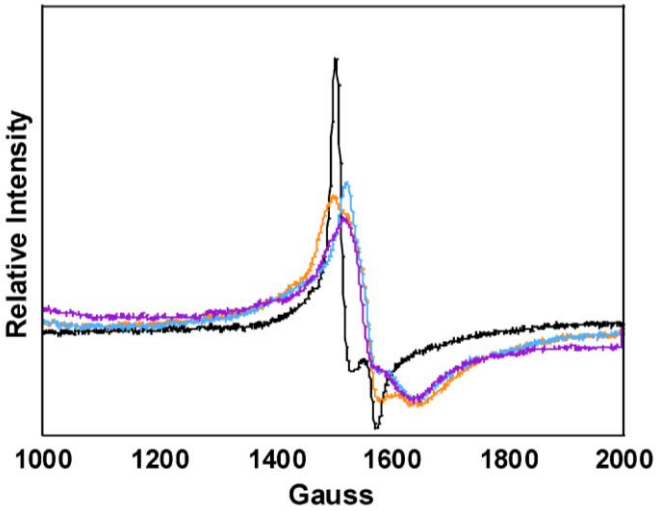


Fig. 2.5 X-band EPR spectra of wild-type CDO and His155 CDO variants with the L-cysteine substrate. Wild-type CDO (black trace), H155A CDO (purple trace), H155Q CDO (orange trace), and H155E CDO (blue trace). All spectra were measured with 90 μM protein and 10 mM L-cysteine in 25 mM HEPES buffer (pH 7.5) 100 mM NaCl, and 10% glycerol. All spectra were recorded at 9.38 GHz, with a field modulation frequency of 100 kHz and modulation amplitude of 6 G. All spectra were recorded at 8 K.

variant to generate 100% of the crosslinked isoform suggested that the formation of the crosslink changes the environment around the iron center even in the absence of His155. However, H155E and H155A CDO are not able to form the crosslink at the concentrations used in EPR experiments but share the same spectrum as wild-type CDO in the presence of the L-cysteine substrate.

In order to determine if the sharp signal was due to crosslink formation or L-cysteine coordination, wild-type, H155Q, and H155E CDO variants were buffer exchanged to remove the L-cysteine substrate. Wild-type and H155Q CDO maintained the sharp EPR signal, while the

H155E CDO variant showed a decrease in the sharp signal (**Fig. 2.6A**). The sharp signal noted for wild-type and H155Q CDO is likely due to crosslink formation. Because H155E CDO is unable to form the crosslink, the decreased signal observed could be attributed to the L-cysteine substrate no longer coordinating to the iron center (**Fig. 2.6B**).

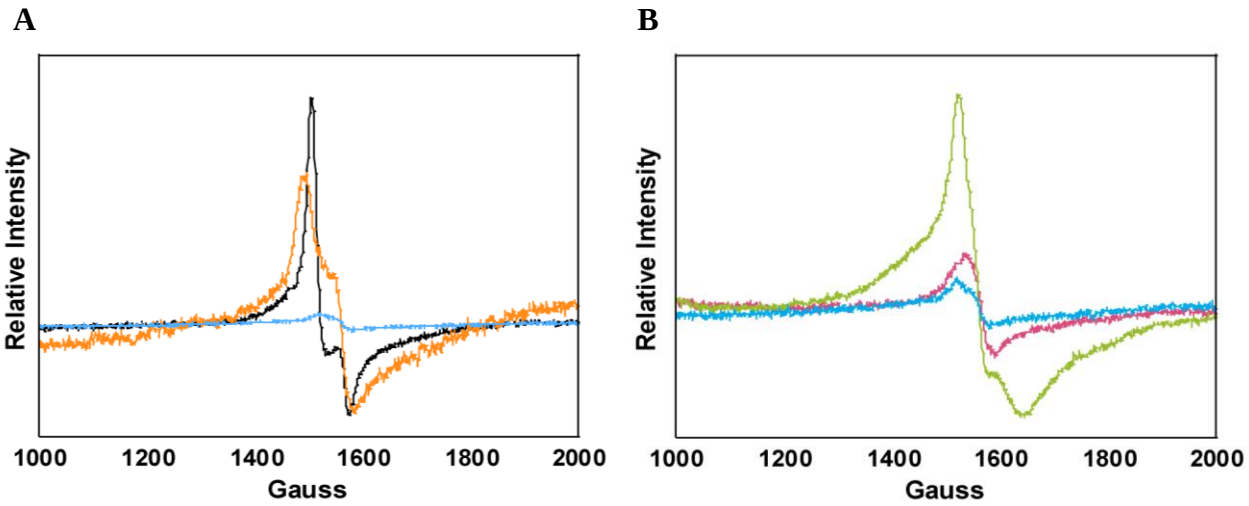


Fig. 2.6 X-band EPR spectra of wild-type CDO and His155 CDO variants with L-cysteine removed. A. Wild-type CDO (black trace), H155Q CDO (orange trace), and H155E CDO (blue trace). All spectra were measured with 90 μM protein in 25 mM HEPES buffer (pH 7.5), 100 mM NaCl, and 10% glycerol. Samples were incubated with 10 mM L-cysteine for 30 min and centrifuged three times with the buffer to remove L-cysteine prior to measurements. B. X-band EPR spectra of H155E CDO with and without L-cysteine. H155E CDO (pink trace), H155E CDO with L-cysteine (green trace), and H155E CDO with L-cysteine removed (blue trace). All spectra were measured with 90 μM protein in 25 mM HEPES buffer (pH 7.5), 100 mM NaCl, and 10% glycerol. Samples were incubated with 10 mM L-cysteine for 30 min. The sample was centrifuged three times with 25 mM HEPES buffer (pH 7.5), 100 mM NaCl, and 10% glycerol to remove L-csyteine. All spectra were recorded at 9.38 GHz, with a field modulation frequency of 100 kHz and modulation amplitude of 6 G. All spectra were recorded at 8 K.

2.4 Discussion

The hydrogen-bonding network is conserved in CDO in select organisms. [87,102,103,117] The network involves Tyr157, a component of the protein-derived thioether crosslink that is critical for the catalytic efficiency of CDO. [82,87,93,98,101–103,117] The hydroxyl group of Tyr157 has been proposed to play a role in catalysis by positioning the substrates for binding the iron center and/or acting as a catalytic acid/base.[5–7,11,14-16] In previous reports, it has been suggested that His155 could be stabilizing Tyr157 through hydrogen-bonding interactions during catalysis due to the proximity of His155 to Tyr157.[11,15] Spectroscopic and computational studies were previously performed with an Ala variant of His155 to evaluate the hydrogen-bonding network of CDO.[11] Substitution of His155 to Ala resulted in a decrease in catalytic activity of nearly 2-orders of magnitude compared to wild-type CDO. Computational models suggested His155 stabilized Fe-oxo intermediates during catalysis through hydrogen-bonding interactions attributing to the abolished activity observed when His155 was substituted. However, this conclusion was unable to be supported by spectroscopic studies.[103] In a separate study, it was suggested that the low catalytic activity seen for H155A CDO was due to a six-coordinate center (H_2O/Cys)-Fe(II)CDO observed by spectroscopic and computational methods, and His155 prevents a water molecule from binding to the iron center maintaining an open site for molecular oxygen to bind during catalysis. [117] Consistent with previous studies, His155 appeared to play a role in assisting Tyr157 to properly bind the dioxygen substrate during catalysis through hydrogen-bonding interactions. [103,117] However, the functional role of His155 in both crosslink formation and L-cysteine oxidation was not evaluated.

Tyr157 in the thioether adduct could play a role in stabilizing the superoxo intermediate that is generated with the activation of dioxygen. [8,18] Therefore, the hydrogen-bonding

interactions of His155 with Tyr157 may assist in the activation of dioxygen. Consistent with previous reports, the H155A CDO variant had negligible catalytic activity compared to wild-type CDO.[103,117] Similarly, the H155E and H155Q CDO variants were unable to oxidize L-cysteine. Wild-type CDO was previously shown to couple O_2 utilization with CSA production in a 1:0.7 ratio suggesting all of the O_2 utilized by wild-type CDO upon the addition of L-cysteine was turned over to the CSA product. Catalytic activity remained low even when the concentrations for L-cysteine was increased in kinetic assays. This concentration range is beyond the physiological concentrations for L-cysteine (90-100 μM), and any CSA produced for the His155 CDO variants would be difficult to quantify.[50] The absence of activity with each variant is likely caused by the inability of these residues to properly position Tyr157 during cysteine oxidation.

In crosslink formation studies, H155Q CDO can form the fully crosslinked species at 80-100 mM L-cysteine comparable to wild-type CDO. Conversely, H155A CDO forms up 80% of the crosslinked species but does not form the fully crosslinked isoform at similar concentrations of L-cysteine used to evaluate crosslink formation with H155Q and wild-type CDO. The H155E CDO variant was not able to form the crosslinked species regardless of the concentration of L-cysteine present. The negative charge on the carboxylate of H155E CDO would likely disrupt the Tyr157 intermediates generated during crosslink formation. However, the H155Q CDO variant was able to generate 100% of the crosslinked species, yet it was unable to oxidize the L-cysteine substrate. The H155Q CDO variant could still be able to properly position Tyr157 during crosslink formation if the hydrogen-bonding interactions are maintained. However, the disruption of catalytic activity seen with this variant suggests it is no longer properly positioned to interact with Tyr157. These results suggest that His155 plays a role in both L-cysteine oxidation and crosslink formation, but the catalytic role is likely different in each mechanism.

The percentage of iron bound to recombinant wild-type CDO is variable among groups studying CDO (10-68%).[138,142] The iron content of all His155 CDO variants were elevated by ~20% compared to wild-type CDO, but the increased iron content did not appear to improve catalysis. In EPR studies, wild-type and all variants showed a ferric signal at g=4.3. The intensities and shapes of the signal for each variant compared to CDO were different suggesting that the microenvironment was altered due to the substitutions. Upon the addition of L-cysteine, all His155 CDO variants displayed sharper signals. The sharp ferric signal observed upon the addition of L-cysteine with wild-type CDO was associated with L-cysteine coordination or crosslink formation.[86,96] Both wild-type and H155Q CDO retained the sharp EPR signal following the removal of L-cysteine. A sharp EPR signal was previously observed for the fully crosslinked CDO isoform in the absence of L-Cys which was attributed to the structural rearrangement in the microenvironment of the iron center.[86] The sharp signal observed with H155Q CDO is likely due to the crosslink, as this variant was able to form the fully crosslinked isoform. Interestingly, H155E CDO returned to the resting state seen before L-cysteine was added. Because this variant could not generate the crosslink as effectively, the sharp signal may be correlated with L-cysteine coordination. Perturbations introduced by negatively charged Glu155 may prevent the crosslink from forming attributing to the change in EPR signal once L-cysteine was unbound.

Previous and current studies suggest that His155 is involved in stabilizing Tyr157 by acting as a proton donor/acceptor.[87,101,117] However, the importance of the Tyr157-His155 interaction is likely different for L-cysteine oxidation and crosslink formation as both mechanisms are proposed to follow separate steps. [8] It has been proposed that Tyr157 undergoes PCET during crosslink formation forming a radical.[86] Since Tyr157 has been proposed to form a tyrosyl radical during crosslink formation, His155 assist in formation of the tyrosyl radical by accepting a proton from

Tyr157. Several enzymes including galactose oxidase (GO), ribonucleotide reductase, and azurin contain a tyrosine residue that undergoes proton coupled electron transfer (PCET) which results in a tyrosyl radical.[143–146] Interestingly, modified azurins in *Pseudomonas aeruginosa* contain a Tyr residue within hydrogen-bonding distance from a His residue. It was shown that fine tuning of the redox potential of the tyrosyl radical was critical for redox chemistry to occur, and that the placement of a basic residue nearby a tyrosine could act as a hydrogen-bond acceptor to form and stabilize the tyrosyl radical. [143,147] Additionally, Tyr157 has been proposed to position oxygen coordination to the iron center during L-cysteine oxidation through hydrogen-bonding.[101] Similar to the role proposed for crosslink formation, His155 could assist in stabilizing Tyr157 through hydrogen-bonding interactions. This stabilization would be critical for stabilizing the iron superoxo intermediate proposed to form during catalysis.[86,103] Based on these studies and the conserved nature of Tyr157-His155-Ser153, the hydrogen-bonding interactions among these residues is crucial for efficient L-cysteine oxidation and crosslink formation in CDO.

Chapter Three

A Rogue Cysteine Residue Mandates L-cysteine Accessibility in Cysteine Dioxygenase

3.1 Introduction

Cysteine Dioxygenase (CDO) is a mononuclear iron-dependent enzyme that catalyzes the oxidation of L-cysteine to L-cysteine sulfinic acid (CSA), which is an important branchpoint in sulfur metabolism forming pyruvate and sulfite.[3,7,9,148] Adjacent to the 3-His (His86, His88, and His140) coordinated iron center is a thioether crosslink that consists of a cysteine residue (Cys93) and a tyrosine residue (Tyr157). [82,87,90,93] Following purification, wild-type CDO exists as a heterogenous mixture of the non-crosslinked and crosslinked isoforms; however, the fully crosslinked species can be generated at increased L-cysteine concentrations. Crosslinked CDO demonstrated enhanced catalytic activity compared to non-crosslinked CDO; therefore, it has been suggested that the crosslink is formed in response to increase catalytic activity when L-cysteine levels are elevated. [98] Both L-cysteine oxidation and crosslink formation require L-cysteine, dioxygen, and ferrous iron.[82,90,91,93,98] It is unclear if these mechanisms occur in a particular order, but they likely follow different chemical steps.[86]

Sequence alignments and three-dimensional structures have also shown conserved residues beyond the active site among mammalian CDO. In the opening of the active site ~8 Å away from the iron center is a cysteine residue (Cys164). The Cys164 residue does not participate in any

intramolecular disulfide bonds and exists as a free thiol.[82,87,107–109] It is unusual that a cysteine residue would reside in the pathway of a redox active site where L-cysteine and dioxygen are utilized as substrates. In addition to L-cysteine, 3-mercaptopropionic acid and mercaptopyruvate have been shown to be utilized as substrates in bacterial CDO. [82,107,109,149] Cys164 is also replaced with Met or Arg residues in CDO of bacterial organisms.[87,110,149] Additionally, some three-dimensional structures have depicted Cys164 interacting with a water molecule which was within hydrogen bonding distance to the L-cysteine substrate.[107] Due to the location of these residues, it has been proposed that Cys164 and the Met and Arg residues in bacterial CDO play a role in substrate specificity by coordinating the substrate. [82,107,109,149]

In multiple three-dimensional structures, an excess electron density was detected at Cys164 which was identified as a cystine involving Cys164 and a free cysteine.[87,106,109] The resulting cystine blocked the opening of the active site and forced conserved residues, Met179 and Arg60, to rotate towards the iron center. Since Arg60 has been shown to play a role in substrate specificity by coordinating the carboxylate group of the L-cysteine substrate, the rotation of this residue towards the iron center would prevent the coordination of the L-cysteine substrate.[87,107] Although some studies have deemed the cystine artifactual, it has also been suggested that Cys164 could be modified and play a role as a regulatory response. [82,109]

Due to the high reactivity of cysteine residues, the conservation, and the location of Cys164 in CDO, Cys164 has multiple proposed roles that have not been adequately evaluated. In this study, we have generated variants of Cys164 in CDO to determine if Cys164 plays a role in substrate specificity or serves as a regulatory switch. In addition, Cys164 CDO variants were evaluated to determine if Cys164 plays a mechanistic role in L-cysteine oxidation or crosslink formation. These studies suggest that Cys164 does not play a direct role in the substrate specificity or regulatory

properties of CDO but is involved in modulating the accessibility of the L-cysteine substrate to the active site for L-cysteine oxidation and crosslink formation.

3.2 Material and Methods

3.2.1 Materials

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), L-cysteine, L-ascorbate, ampicillin, streptomycin sulfate, lysozyme, potassium chloride (KCl), isopropyl-β-d-thiogalactoside (IPTG), ferrous ammonium sulfate, (5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4,4"-disulfonic acid) ferrozine, and brilliant blue R were purchased from Sigma (St. Louis, MO). Sodium dodecyl sulfate was purchased from Biorad (Hercules, CA). Glycerol and sodium chloride were purchased from Macron Fine Chemicals (Center valley, PA). Pfu Turbo DNA polymerase was purchased from Agilent (La Jolla, CA). Oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA). Macro-Prep® High Q Support and gel filtration standard was purchased from Bio-Rad Laboratories (Hercules, CA). Difco-brand Luria–Bertani (LB) media was purchased from Becton, Dickinson and Company (Sparks, MD). Amicon Ultra Centrifugal Filter Devices (10 kDa MWCO) were purchased from Millipore (Billerica, MA).

3.2.2 Protein Expression and Purification

The cDNA gene for rat CDO was cloned into the pET21a expression vector as previously described.[92] The vector containing the CDO gene was used to construct the rat CDO variants (C164A, C164S, C164D, C164M, and C164R CDO) by site-directed mutagenesis. Primers for all variants were designed as 27 base oligonucleotides. To generate Cys164 variants, the Cys164 codon (TGT) in wild-type CDO was replaced with GCG (Ala), AGC (Ser), GAT (Asp), ATG (Met), and CGT (Arg) codons. Successful substitutions were confirmed by DNA sequence analysis

(Eurofins MWG Operon). Each CDO variant was transformed into BL21(DE3) *E. coli* competent cells and stored as glycerol stocks at -80°C.

The cells containing the CDO plasmid were isolated on LB-agar medium containing 0.1 mg/mL ampicillin (LB-amp). A single colony was selected from the medium and grown in 5 mL LB-amp media at 37°C for ~7 hrs. A 1% inoculum was transferred from the 5 mL culture to a 100 mL culture and grown overnight at 37°C. A 2% inoculum from the overnight culture was transferred to four 1 L flasks and grown at 37°C until the cultures reached an A_{600} value of 0.4-0.6. The cells were induced with 0.4 mM IPTG and grown for an additional 6 hrs at 25°C. In preparations where the non-crosslinked isoform was isolated, the cultures were grown until a A_{600} value of 0.6-0.8 was reached. The cells were then treated with 0.1 mM 1,10-phenanthroline and 0.1 mM HCl 15 min prior to induction. Cells were harvested, and the pellets were resuspended with 100 mL of buffer containing 10% glycerol and 25 mM HEPES (pH 7.5) and 0.2 mg/mL of lysozyme. Cells were lysed by sonication, and the clarified supernatant was treated with 1.2% streptomycin sulfate and stirred at 4°C for 1 hr. Following centrifugation, the supernatant was then applied to a Macro-Prep® High Q Support column, and protein was eluted by a linear gradient from 0-150 mM sodium chloride in 10% glycerol and 25 mM HEPES (pH 7.5). Fractions were evaluated by SDS-PAGE and the purest fractions were pooled and iron loaded with a molar ratio of 1:1 ferrous ammonium sulfate:protein. For preparations where the non-crosslinked isoform was isolated, pooled fractions were dialyzed twice against two liters of 25 mM HEPES (pH 7.5), 100 mM sodium chloride and 10% glycerol at 4°C to remove 1,10-phanthroline. To remove excess iron, samples were dialyzed twice against one liter of 25 mM HEPES (pH 7.5), 100 mM sodium chloride, and 10% glycerol at 4°C. Protein aliquots were flash frozen and stored at -80°C.

The amount of iron present in the protein preparation was determined by a colorimetric assay using an iron chelator, ferrozine, as described previously with modifications. [140] CDO samples were diluted to 10 μM in a final volume of 1 mL using 25 mM HEPES (pH 7.5). A standard curve was generated using iron ICP standard (0-15 μM). Additionally, 25 mM HEPES (pH 7.5) and deionized and distilled water were used as controls. The results obtained were the average of three separate experiments.

3.2.3 Thiol Quantification and pK_a Determination of Cysteine Residues in CDO

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was used to quantify free thiols in the folded and denatured states of wild-type, non-crosslinked, C164A, and non-crosslinked C164A CDO. In each reaction, 10 μM protein and 100 μM DTNB were incubated for 15 min in 25 mM HEPES (pH 7.5). In reactions where the samples were denatured, 6 M guanidinium chloride (GuHCl) was incubated with 10 μM enzyme for 1 hr prior to the addition of DTNB. Each reaction was monitored at 412 nm and quantified utilizing the molar absorption coefficient value (ε_{412}=14,150 M^{-1} cm^{-1}). For reactions where GuHCl was utilized, a value of ε_{412}=13,700 M^{-1} cm^{-1} was used for thiol quantification. The results obtained were the average of three separate experiments.

To determine the pK_a of the solvent accessible thiols in wild-type, non-crosslinked, C164A, and non-crosslinked C164A CDO, a protocol was utilized as previously described with minor modifications.[150] The pK_a values were determined by measuring the absorbance at 240 and 280 nm over a pH range of 6.2–10.2 in the appropriate buffer (50 mM MES, pH 6.2 and 6.5; 50 mM potassium phosphate 6.5-7.2; 50 mM Tris–HCl, pH 7.2–9.0; 50 mM glycine, pH 9.0–10.2, and 100 mM NaCl). Each enzyme (10 μM final concentration) was equilibrated in each buffer for 10 min prior to measuring the absorbance values. The final protein concentration in solution was determined using the absorption coefficient of CDO at 280 nm ($\varepsilon_{280} = 2.54 \times 10^4$ M^{-1} cm^{-1}). The

amount of enzyme in solution and A_{240} values were used to calculate the ε_{240} at each pH value. The ε_{240} value for the samples at each pH value was determined using the calculated protein concentration in solution. The ε_{240} values obtained were plotted against the pH values (6.2-10.2) and fit to **Equation 3.1** where *y* is ε_{240} and A is the lower plateau at high pH, and B is the upper plateau at low pH. The pK_a values determined were the average of three separate experiments.

$$y = \frac{[(A\times10^{-pH})+(B\times10^{-pKa})]}{10^{-pKa}+10^{-pH}} \qquad \textbf{Equation 3.1}$$

3.2.4 Oxidative Modifications of wild-type and Cys164 CDO Variants

Oxidative modifications of wild-type, non-crosslinked, C164A, and non-crosslinked C164A variants were generated varying concentrations of hydrogen peroxide (H_2O_2) (0.045-1 mM), L-cysteine (45-450 µM), and 4-chloro-7-nitrobenzofurazan (NBD-Cl) (45-450 µM) with a fixed concentration (45 µM) of protein (**Table 3.1**). Each sample was incubated with each reagent for 30 min at room temperature. All samples were incubated in 50 mM potassium phosphate (pH 7.0) except for samples involving L-cysteine which were incubated in 50 mM HEPES (pH 7.5). If multiple reagents were used, samples were washed with either 50 mM potassium phosphate (pH 7.0) or 50 mM HEPES (pH 7.5) using Amicon Ultrafree-MC Centrifugal Filter Devices in between each incubation to remove any excess reagent.

Any modifications were measured utilizing UV-visible absorption spectroscopy and mass spectrometry when applicable. Samples were prepared for UV-visible absorption spectroscopy by removing any excess reagent with 50 mM potassium phosphate (pH 7.0) using Amicon Ultrafree-MC Centrifugal Filter Devices. Any thiol adducts with NBD were evaluated at 420 nm and quantified utilizing a molar extinction coefficient of 13,000 M^{-1} cm^{-1}, and potential sulfenic acid

adducts were detected at 347 nm and quantified using the molar extinction coefficient of 13,400 M^{-1} cm^{-1}.[151] For direct injection mass spectrometry analysis, a quadrupole time-of-flight mass spectrometer (Q-Tof Premier, Waters) with electrospray ionization (ESI) in ESI^{+}-MS mode was utilized. Samples were prepared by washing with 1 mM ammonium bicarbonate (pH 7.0) using Amicon Ultrafree-MC Centrifugal Filter Devices. Prior to mass spectrometric measurements, the samples included a final concentration of 10 µM protein in 1 mM ammonium bicarbonate (pH 7.0), 50% acetonitrile, and 4% formic acid.

Table 3.1 H_2O_2 (0.045-1.0 mM), NBD-Cl (45-450 µM), and L-cysteine (45-450 µM) utilized to introduce oxidative modifications in CDO at a fixed concentration (45 µM) of protein.

Protein:H_2O_2	Protein:NBD-Cl	Protein:H_2O_2:NBD-Cl	Protein:H_2O_2:L-Cysteine
1:1	1:1	1:1:1	1:1:1
1:2	1:10	1:2:10	1:5:10
1:4		1:4:10	
1:5		1:5:10	
1:10		1:10:10	
1:22			

To determine how these modifications affected L-cysteine oxidation and crosslink formation, modified proteins were then evaluated by steady-state kinetic analyses and crosslink formation studies (Sections 3.2.5 and 3.2.6, *Materials and Methods*). In addition, the oligomeric

states were evaluated to determine if cysteinylation had an effect on the oligomerization of CDO (Section 3.2.8, *Materials and Methods*).

3.2.5 Steady-State Kinetic Analyses and Product Formation of Wild-type and C164 CDO Variants

Steady-state kinetic studies on the CDO variants were performed using a Clark-type oxygen electrode (Hansatech, Inc., Norfolk, United Kingdom) to measure the rate of dioxygen utilization by each enzyme in the presence of L-cysteine and L-cysteine analogs (D-cysteine, cysteamine, 3-mercaptopropionic acid, and 3-mercaptopyruvate). The concentration range implemented for wild-type, C164A, C164S, and C164R CDO variants was 0.2-5 mM L-cysteine, and 1-50 mM L-cysteine for C164D and C164M CDO variants. For reactions where L-cysteine analogs were used, a concentration range of 1-50 mM was utilized. Each reaction contained 2 μM protein and 1 mM ascorbate in 25 mM HEPES (pH 7.5). The reactions were initiated by the addition of L-cysteine or L-cysteine analogs, and the initial velocities were measured from the linear portion of the trace. The average initial velocities from three separate experiments were plotted against the substrate concentration and the data fit to the Michaelis-Menten equation using KaleidaGraph™ software. The steady-state parameters obtained were the average of three separate experiments. The rate of dioxygen consumption was correlated with the rate of cysteine sulfinic acid (CSA) formation by LC–MS using the protocol previously described.[86] The values obtained reflected the averages of three separate experiments.

3.2.6 Crosslink Formation Studies of the Wild-type and CDO Variants

Crosslink formation was investigated by varying L-cysteine concentrations. In crosslink formation assays, 5 μM wild-type, non-crosslinked wild-type, or Cys164 CDO variants were

incubated with 1 mM of ascorbic acid and 0-100 mM L-cysteine substrate or analogs in 25 mM HEPES (pH 7.5) at 37°C with gentle shaking. Each sample was treated with 2% SDS and 5% 2-mercaptoethanol, heat denatured for 3 minutes, and analyzed by 12% SDS-PAGE. The ratio of non-crosslinked to crosslinked isoforms was quantified by ImageJ software.[92]

3.2.7 EPR Spectroscopic Analysis of Wild-type and CDO Variants

The oxidation state of the iron center in wild-type CDO and variants was measured by electron paramagnetic resonance spectroscopy (EPR). Samples were prepared by diluting the CDO enzymes to 90 µM in 25 mM HEPES (pH 7.5), 100 mM NaCl, and 10% glycerol in a final volume of 300 µL. When L-cysteine was included, the CDO samples were supplemented with a final concentration of 10 mM L-cysteine in a final volume of 300 µL. All EPR spectra were obtained at X-band frequency (9.38 GHz) on a Bruker-EMX spectrometer (Bruker Biospin Corporation, Billerica, MA) at 8 K. Temperature was maintained using an Oxford Instruments ESR 900 flow cryostat and an ITC4 temperature controller. All EPR spectra were obtained using the following settings: microwave frequency: 9.38 GHz, microwave power: 0.2 mW; receiver gain: 2×10^4; modulation frequency: 100 kHz; modulation amplitude: 6 G; time constant: 163.85 ms; sweep time: 167.772 s.

3.2.8 Oligomeric States of Wild-type and Cys164 CDO Variants

The oligomeric states of wild-type, non-crosslinked wild-type, crosslinked wild-type, C164A, non-crosslinked C164A CDO, and crosslinked C164A CDO were determined utilizing a protocol previously described with a few modifications.[152] Analytical size-exclusion chromatography of the CDO enzymes were performed on an Agilent 1260 Infinity GPC/SEC chromatograph. The molecular weights of each enzyme (50 µM) were determined using a Waters

BioSuite HR SEC, 7.8 mm × 300 mm, 250 Å column. The enzymes were eluted from the column with 150 mM sodium phosphate (pH 7.0) and 100 mM NaCl with a flow rate of 0.2 mL/min monitored at 280 nm. A Bio-Rad gel filtration standard with a molecular weight range from 1.35 to 670 kDa was used to generate a curve to fit the molecular weights based on retention times. The standard curve (log of the molecular weight vs retention time) was generated on the basis of the elution time monitored at 280 nm. A standard curve of known molecular weights (1.35-670 kDa) was generated based on retention times utilizing a Bio-Rad gel filtration standard. In experiments where the L-cysteine substrate was used, a 1:1 ratio of protein:substrate was added in each sample.

3.3 Results

3.3.1 Thiol Quantification and pK_a Determination of Wild-type and Cys164 CDO Variants

There are four cysteine residues in CDO. Cys93 is a component of the thioether crosslink, and Cys164 exists as a free thiol in three-dimensional structures in the opening of the active site (**Fig. 3.1**). In addition, there are two other cysteine residues (Cys76 and Cys130) in CDO. In three-dimensional structures, Cys76 appears to be buried in CDO and is located ~15 Å away from the iron center. The thiol of Cys76 is not involved with any interactions with other residues and is facing away from the iron center. Located ~12 Å away from the iron center, Cys130 also exists as a free thiol at the surface of CDO with the thiol group facing the iron center. To determine the likelihood of Cys164 being modified in CDO, the accessibility and reactivity of cysteine residues in CDO was determined by quantifying free thiols and determining the pK_a values of any free thiols present.

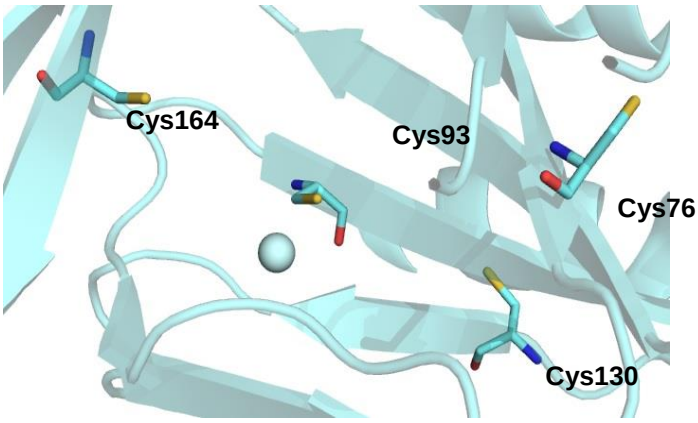


Fig. 3.1 Three-dimensional structure depicting the cysteine residues in CDO where Cys76 is at the surface of CDO facing away from the iron, Cys93 is a component of the thioether crosslink, Cys130 is buried and facing the iron center, and Cys164 is at the opening of the active site.

Results obtained from the DTNB assays revealed 0.5 free thiols in wild-type and C164A CDO indicating that the Cys93 residue is likely detected in the folded states due to the non-crosslinked and crosslinked isoforms existing as a 50:50 ratio in these enzymes (**Table 3.2**). For non-crosslinked CDO, one free thiol was accessible which is likely Cys93 since it is not involved in the crosslink. Only 0.5 thiols were detected in the non-crosslinked C164A CDO enzyme. Some of the free thiols may not be detected due to changes in the overall structure induced by the mutation and absence of the crosslink restricting access of DTNB. In the denatured state, wild-type and C164A CDO had one free thiol indicating that another thiol has been detected although it does not appear to be fully accessible. In the denatured non-crosslinked enzymes, two free thiols were exposed which were likely Cys93 and either Cys76 or Cys130. In addition, the calculated pK_a of any free thiols present in the enzymes were evaluated, and it was determined that all of the enzymes had one pK_a value ranging from 9.1-9.6 (**Table 3.3**).

Table 3.2 Free thiols present in the folded and denatured states of wild-type, C164A, non-crosslinked, and non-crosslinked C164A CDO.

	Free Thiols	
Enzyme	Folded	Denatured
wild-type CDO	0.50 ± 0.03	1.00 ± 0.03
C164A CDO	0.50 ± 0.03	1.00 ± 0.02
non-crosslinked CDO	1.0 ± 0.1	2.00 ± 0.01
non-crosslinked C164A CDO	0.50 ± 0.03	2.00 ± 0.05

Table 3.3 Calculated pK_a values in wild-type, C164A, non-crosslinked, and non-crosslinked C164A CDO.

Enzyme	pK_a
wild-type CDO	9.6 ± 0.3
C164A CDO	9.3 ± 0.2
non-crosslinked CDO	9.1 ± 0.1
non-crosslinked C164A CDO	9.4 ± 0.1

3.3.2 Analysis of Oxidative Modifications of Cysteine Residues in CDO

To determine if Cys164 plays a regulatory role in CDO by oxidative modifications, varying ratios of H_2O_2, NBD-Cl, and L-cysteine were incubated with a fixed concentration of protein to

target cysteine residues in wild-type, non-crosslinked, C164A, and non-crosslinked C164A CDO as described previously (3.2.4, *Materials and Methods*).

H_2O_2 and L-cysteine were incubated with the CDO enzyme to determine if CDO could undergo cysteinylation at Cys164 for regulatory purposes. Utilizing mass spectrometry, no modifications were detected in any of the CDO enzymes. In addition, high performance liquid chromatography coupled with size-exclusion chromatography (HPLC-SEC) was used to evaluate any potential oligomeric state changes induced by L-cysteine. All of the enzymes, including enzymes incubated with L-cysteine, existed as monomers.

Wild-type, C164A, non-crosslinked, and non-crosslinked C164A CDO were incubated with H_2O_2 and NBD-Cl. H_2O_2 could modify Cys164 to a sulfenic, sulfinic, or sulfonic acid. If Cys164 was modified to a sulfenic acid, NBD-Cl would be utilized to trap the intermediate before it was able to react further. The CDO enzymes were incubated with various equivalents of H_2O_2 with ratios of NBD-Cl adjusted accordingly. The CDO enzymes were then evaluated by mass spectrometry and UV-visible spectroscopy. Results obtained from mass spectrometry did not indicate any Cys-S-O-NBD CDO adducts. In addition, no absorption peaks were identified at 347 nm suggesting that none of the enzymes were modified with the Cys-S-O-NBD adduct. Wild-type, non-crosslinked wild-type, C164A, and non-crosslinked C164A CDO were also incubated with H_2O_2 and NBD-Cl separately. Utilizing mass spectrometry, it was determined that none of the enzymes were modified by any oxygen atoms suggesting that CDO was not susceptible to any oxidative modifications in the presence of H_2O_2. However, wild-type, non-crosslinked, C164A, and non-crosslinked C164A CDO were modified by NBD-Cl alone. The UV-visible absorption spectra for all of the enzymes showed a peak at 420 nm indicative of a thiol adduct with NBD. The

number of thiols in each enzyme modified with NBD-Cl was quantified and was comparable to the number of free thiols determined when DTNB was utilized (**Table 3.2**).

The NBD-modified CDO enzymes were then evaluated by steady-state kinetic analyses to determine if the cysteine residue that was labeled could be involved in catalytic activity. None of the labeled enzymes had any detectable kinetic activity. In addition, crosslink formation studies were performed with the NBD-modified CDO enzymes, and none of the enzymes could form the crosslinked species.

3.3.3 Steady-state Kinetic Analysis on Wild-type and Cys164 CDO Variants

In order to determine if Cys164 plays a role in catalysis, additional Cys164 CDO variants were constructed and evaluated. An alanine substitution was generated to eliminate any potential interactions by the thiol of Cys164, while a serine residue would maintain the polarity of Cys164. Aspartic acid was selected to resemble a possible oxidative modification at the Cys164 position. Since Arg and Met have been proposed to dictate substrate specificity in Gln-type and Arg-type bacterial CDO, Cys164 was also replaced with arginine and methionine residues.

To determine if Cys164 plays a role in catalysis, the rates of dioxygen utilized by wild-type CDO and each variant to convert L-cysteine to cysteine sulfinic acid were obtained. Wild-type CDO contained 60% iron and had a k_{cat}/K_m value of 2233 $mM^{-1}min^{-1}$. Initially, the concentration range implemented for C164D and C164M CDO variants was 0.2-5 mM analogous to wild-type CDO. However, these variants did not reach substrate saturation until a concentration range of 1-50 mM was applied. Interestingly, all of the variants displayed diminished catalytic activity compared to wild-type CDO despite having an iron content of nearly 100% (**Table 3.4**). O_2 utilization coupled with product formation was determined to ensure that the rates observed

reflected cysteine sulfinic acid production (**Table 3.5**). A ratio of ~1:1 dioxygen utilization to product formation was obtained for wild-type CDO suggesting that all of the dioxygen utilized was converted to product. In contrast, the C164A and C164S CDO variants had ratios of ~1:0.5 demonstrating only half of the dioxygen utilized was converted to product.

Table 3.4 Steady-state kinetic studies on wild-type and Cys164 CDO variants.

Enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})	% Fe
wild-type CDO	134 ± 4	0.06 ± 0.01	2233 ± 378	60 ± 2
C164A CDO	6.0 ± 0.1	0.19 ± 0.03	31 ± 5	99 ± 1
C164S CDO	19 ± 1	0.33 ± 0.05	58 ± 9	100 ± 2
C164D CDO	143 ± 2	9 ± 1	7 ± 1	97 ± 4
C164R CDO	13 ± 1	0.14 ± 0.03	92 ± 22	100 ± 3
C164M CDO	40 ± 2	6 ± 1	7 ± 1	100 ± 2

The concentration of L-cysteine was varied from 0.2-5 mM for wild-type and C164A, C164S, and C164R CDO and 1-50 mM for C164D and C164M CDO.

Since Cys164 has been proposed to dictate substrate specificity, steady-state kinetic studies were performed with wild-type, C164A, and C164S CDO and various L-cysteine analogs (D-cysteine, cysteamine, and 3-mercaptopropionic acid). C164A and C164S CDO variants were utilized since the Ala variant would disrupt any electrostatic interactions, and Ser could maintain similar properties as Cys164. A concentration range of 0.1-50 mM was implemented for each

Table 3.5 O_2 utilization coupled with CSA production of wild-type, C164A, and C164S CDO.

Enzyme	O_2 Utilization (nmol/min/mg)	CSA Production (nmol/min/mg)
wild-type CDO	4612 ± 212	3318 ± 153
C164A CDO	247 ± 20	120 ± 21
C164S CDO	580 ± 20	232 ± 67

analog. Wild-type, C164A, and C164S CDO did not show any catalytic activity with 3-mercaptopropionic acid. In addition, wild-type CDO displayed nominal activity with D-cysteine with a k_{cat}/K_m value of 2 $mM^{-1}min^{-1}$. However, both C164A and C164S CDO were unable to reach saturation with D-cysteine. Wild-type CDO displayed the highest catalytic activity with cysteamine giving a k_{cat}/K_m value of 8 $mM^{-1}min^{-1}$ comparable to both the C164A and C164S CDO variants (**Table 3.6**). The Cys164 CDO variants had comparable activity to wild-type CDO with the L-cysteine analogs; however, they showed diminished activity with L-cysteine compared to wild-type CDO suggesting that Cys164 plays a role in L-cysteine oxidation although it may not play a role in substrate specificity.

Table 3.6 Steady-state kinetic analysis on wild-type, C164A, and C164S CDO with cysteamine.

Enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})
wild-type CDO	32 ± 1	4.0 ± 0.6	8 ± 1
C164A CDO	114 ± 22	13 ± 6	9 ± 4
C164S CDO	111 ± 14	15 ± 5	7.0 ± 0.1

Since Cys164 has been proposed to play a role in substrate specificity in CDO, the Met and Arg residues found in the comparable position as Cys164 in bacteria have been anticipated to influence the substrate specificity in bacterial CDO. Because 3-mercaptopropionic acid and 3-mercaptopyruvate exhibited catalytic activity with bacterial CDO, steady-state kinetic studies of wild-type, C164M, and C164R CDO were performed with 3-mercaptoprionic acid and 3-mercaptopyruvate to determine if Cys164 partitions the substrate preference of CDO. Comparable

to wild-type CDO, C164M and C164R CDO did not show any catalytic activity with either substrate.

3.3.4 Crosslink Formation Analysis on Wild-type and Cys164 CDO Variants

To determine if Cys164 plays a role in crosslink formation, wild-type and Cys164 CDO variants were evaluated by crosslink formation studies. Following purification, wild-type and Cys164A CDO variants existed in a heterogeneous mixture of non-crosslinked and crosslinked isoforms. Wild-type and C164A CDO existed as a 50:50 mixture while Cys164S CDO was 60% crosslinked. However, C164D, C164M, and C164R CDO were all predominately in the non-crosslinked isoform existing only as 10%, 7%, and 6% crosslinked, respectively (**Table 3.7**).

Table 3.7 Crosslink formation studies on wild-type and the Cys164 CDO variants with L-cysteine.

	% crosslink formed						
Enzyme	0 mM	1 mM	10 mM	20 mM	40 mM	80 mM	100 mM
wild-type CDO	50	58	71	70	97	99	100
C164A CDO	50	92	100	100	100	100	100
C164S CDO	60	63	61	71	70	72	75
C164D CDO	10	10	25	24	29	53	50
C164M CDO	7	18	53	57	78	79	74
C164R CDO	6	36	53	55	60	62	60

Wild-type is able to generate the fully crosslinked species at 100 mM L-cysteine. Interestingly, C164A CDO is able to form the crosslink like a champ by achieving the fully crosslinked isoform at only 10 mM L-cysteine despite having low catalytic activity. Similar to wild-type and C164A CDO, C164S and C164M CDO can form 75% of the fully crosslinked species. However, C164D and C164R CDO can only attain a ~50:50 ratio of the non-crosslinked to crosslinked isoforms.

Crosslink formation studies were also performed with L-cysteine analogs to determine if Cys164 influenced substrate specificity for crosslink formation. Wild-type, C164A, and C164S CDO were evaluated with 3-mercaptopropionic acid, D-cysteine, and cysteamine. The percentage of crosslinked species was the same as the initially purified enzymes with 3-mercaptopropionic acid. However, both wild-type and C164A CDO were able to generate ~80% of the crosslinked isoform utilizing D-cysteine to C164S CDO (**Table 3.8**). Wild-type CDO was able to generate ~80% of the crosslinked isoform utilizing cysteamine; however, neither of the variants formed any additional crosslinked species (**Table 3.9**).

Table 3.8 Crosslink formation studies on wild-type, C164A, and C164S CDO with D-cysteine.

	% crosslinked formed						
Enzyme	0 mM	1 mM	10 mM	20 mM	40 mM	80 mM	100 mM
wild-type CDO	50	72	77	74	78	81	78
C164A CDO	50	50	55	75	76	73	75
C164S CDO	60	58	63	53	60	61	63

Table 3.9 Crosslink formation studies on wild-type, C164A, and C164S CDO with cysteamine.

	% crosslink formed						
Enzyme	0 mM	1 mM	10 mM	20 mM	40 mM	80 mM	100 mM
wild-type CDO	50	59	76	86	79	85	81
C164A CDO	50	50	50	52	51	54	55
C164S CDO	60	54	62	61	62	60	63

3.3.5 Evaluation of the Oxidative States of the Iron Center in Wild-type, Non-crosslinked, and Cys164 CDO Variants

Because the Cys164 CDO variants had an impact on L-cysteine oxidation and crosslink formation, the variants were evaluated by EPR spectroscopy to evaluate the metal center. Wild-type, C164A, and C164S CDO all exhibited a ferric signal with *g* values of 4.3 indicative of the high-spin ferric state (**Fig. 3.2**). [92] The ferric peaks of wild-type, C164A, and C164S CDO all maintained the same overall shape; however, C164S CDO showed a slightly lower intensity than wild-type and C164A CDO suggesting that the C164S CDO variant may have induced subtle changes in the microenvironment around the iron center.

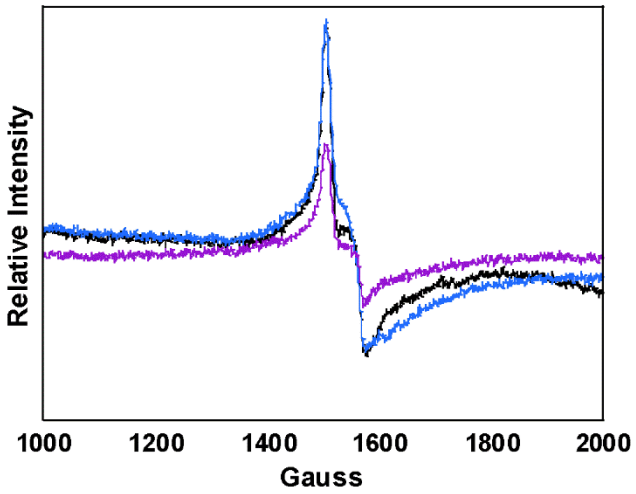


Fig. 3.2 X-band EPR spectra of wild-type CDO and Cys164 CDO variants. Wild-type CDO (black trace), C164A CDO (blue trace), C164S CDO (purple trace). All spectra were measured with 90 μM protein in 25 mM HEPES buffer (pH 7.5) 100 mM NaCl, and 10% glycerol. All spectra were recorded at 9.38 GHz, with a field modulation frequency of 100 kHz and modulation amplitude of 6 G. All spectra were recorded at 8 K.

Upon the addition of the L-cysteine substrate, wild-type and the Cys164 variants all displayed sharp ferric signals (**Fig. 3.3**). The ferric peak obtained for C164S CDO became sharper

when L-cysteine was added; however, the signal was not as sharp as wild-type and C164A CDO. The sharp signal could be attributed mostly to L-cysteine coordination rather than the formation of the crosslink. Since C164S CDO does not form the crosslink as readily as wild-type or C164A CDO, the sharp signal observed may be attributed to L-cysteine coordination.

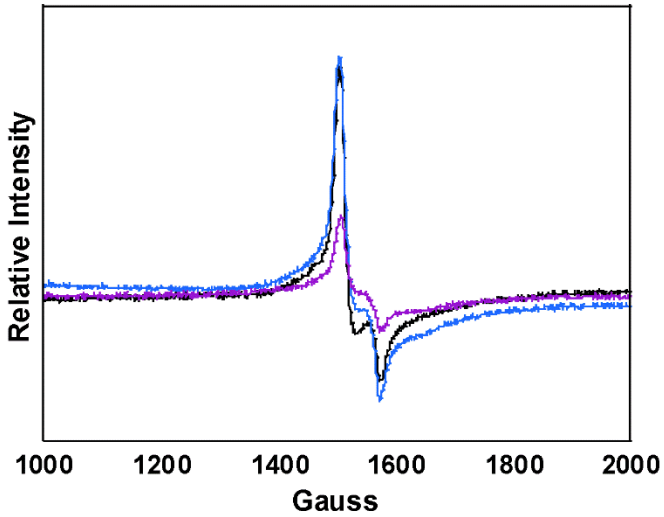


Fig. 3.3 X-band EPR spectra of wild-type CDO and Cys164 CDO variants with the L-cysteine substrate. Wild-type CDO (black trace), C164A CDO (blue trace), and C164S CDO (purple trace). All spectra were measured with 90 μM protein and 10 mM L-cysteine in 25 mM HEPES buffer (pH 7.5) 100 mM NaCl, and 10% glycerol. All spectra were recorded at 9.38 GHz, with a field modulation frequency of 100 kHz and modulation amplitude of 6 G. All spectra were recorded at 8 K.

3.4 Discussion

Cys164 is located at the opening of the CDO active site and is conserved among mammalian systems. Interestingly, Cys164 is surrounded by mostly hydrophobic residues and does not appear to be involved in any intramolecular disulfide bonds and exists as a free

thiol.[82,87,107–109] Cys164 first received attention from the modification formed with a free cysteine in multiple three-dimensional structures. [87,106,109] It was determined that Cys164 was modified by a free cysteine resulting in a cystine which caused Met179, and Arg60 to rotate preventing L-cysteine coordination. [87,106,109] Cys164 was then identified as a potential regulatory switch that becomes modified inhibiting L-cysteine oxidation at increased L-cysteine concentrations [82,109] Cys164 was then substituted with Ser and Ala residues to if determine L-cysteine oxidation could occur at increased L-cysteine concentrations in the absence of cysteinylated Cys164. However, C164A and C164S CDO resulted in a 20% decrease in specific activity compared to wild-type CDO suggesting that cysteinylation of Cys164 may not be responsible for the inhibition of L-cysteine oxidation, or Cys164 may not be cysteinylated at increased L-cysteine concentrations during catalysis.[82] In a separate study, Cys164 was within hydrogen-bonding distance with a water molecule which appeared to be coordinating the amine group of the L-cysteine substrate in a three-dimensional structure. Therefore, Cys164 was anticipated to play a role in substrate specificity. [107] In addition, Arg or Met residues occupy the position that Cys164 maintains in bacterial CDO. Since bacterial CDO has been shown to utilize other substrates such as 3-meraptopropionic acid and mercaptopyruvate, it has been speculated that these residues play a role in the substrate specificity of CDO.[105,149]

Contrary to previous reports, Cys164 does appear to influence L-cysteine oxidation and crosslink formation. In this study, we evaluated multiple Cys164 CDO variants in steady-state kinetic and crosslink formation studies. In addition, we evaluated if Cys164 is involved in oxidative modifications and if they play a role in oligomerization, catalysis, or formation of the crosslink. We also investigated the proposed substrate specificity in CDO among mammalian and bacterial systems by designing mutations in mammalian CDO to resemble bacterial CDO.

Prior to introducing oxidative modifications in CDO, the solvent accessible and buried free thiols were quantified and the pK_a values for the free thiols were determined. Half of a free thiol was detected in both wild-type and C164A CDO in the native form. Because wild-type and C164A CDO purify as heterogenous mixtures of the non-crosslinked and crosslinked isoforms, DTNB is likely modifying Cys93 in the non-crosslinked isoforms. One free thiol was identified in non-crosslinked CDO which was likely Cys93 since it is not involved in the thioether crosslink. However, non-crosslinked C164A CDO exhibited 0.5 free thiols. This could be due to a subtle conformational change induced by the non-crosslinked isoform combined with the mutation of Cys164 influencing access of DTNB to the active site. In the denatured states of wild-type and C164A CDO, one free thiol was identified indicating an accessible cysteine or other cysteine residues could be partially accessible thereby contributing to the free thiol count. Additionally, non-crosslinked wild-type CDO and non-crosslinked C164A CDO had two free thiols suggesting an additional thiol was detected. One pK_a value was identified for all enzymes, and the pK_a values were similar for all enzymes. These results suggest that the only reactive free thiol available in the folded state was Cys93, and that Cys164 is not solvent accessible in the folded or intact states.

Cysteine residues in multiple enzymes have been shown to undergo oxidative modifications primarily for regulatory purposes. In certain enzymes such as superoxide dismutase (SOD), a cysteine residue at the dimer interface of the protein can form a short-lived cysteine sulfenic acid intermediate which then resolves to a disulfide bond with another cysteine. Cysteinylation of a cysteine residue in one monomer has been shown to protect the catalytically active monomer during oxidative stress in SOD.[48] Since Cys164 appeared to be cysteinylated in wild-type CDO three-dimensional structures, it was then speculated the Cys164 could play a regulatory role in CDO *via* cysteinylation. [82,109] To determine if Cys164 was susceptible to

oxidative damage, wild-type, C164A, non-crosslinked wild-type, non-crosslinked C164A CDO were targeted with various ratios of H_2O_2. Interestingly, none of the CDO enzymes were oxidized even at a protein:H_2O_2 ratio of 1:200 indicating that CDO could be protected from oxidation. In addition, no cysteine sulfenic acid-NBD adducts were detected suggesting that none of the cysteine residues in CDO undergo any cysteine sulfenic acid modifications. Thiol-NBD adducts were detected by UV-visible spectroscopy and mass spectrometry. The number of free thiols detected with NBD match the number of free thiols detected with DTNB. Also, the NBD-modified enzymes were evaluated by steady-state kinetics and crosslink formation, and none of the enzymes could form the fully crosslinked species and had nominal catalytic activity. Taken together, these results support that NBD modified the Cys93 residue in CDO. The L-cysteine substrate was also incubated with H_2O_2 and the CDO enzymes; however, no cysteinylation was detected. Based on these results, it does not appear that Cys164 is modified during oxidative stress. The disulfide between Cys164 and a free cysteine observed in three-dimensional structures was likely an artifact of crystallization when the CDO enzyme was soaked or co-crystallized with the L-cysteine substrate; therefore, cysteinylation of Cys164 may not be physiologically relevant.

In steady-state kinetic studies utilizing L-cysteine, wild-type CDO had a k_{cat}/K_m value of 2233 $mM^{-1}min^{-1}$. However, all Cys164 CDO variants had diminished activity compared to wild-type CDO suggesting that Cys164 plays a role in catalysis despite its distance from the active site. To determine if Cys164 plays a role in substrate specificity, C164A and C164S CDO were selected for steady-state kinetic analysis with L-cysteine analogs and compared to wild-type CDO. In addition, C164M and C164R CDO were selected for steady-state kinetic studies involving 3-mercaptoproponic acid and mercaptopyruvate which are proposed substrates for bacterial CDO. Wild-type CDO displayed nominal catalytic activity with D-cysteine with a k_{cat}/K_m value of 2 mM^{-}

$^{1}min^{-1}$ whereas C164A and C164S CDO did not show any catalytic activity. For studies utilizing cysteamine, wild-type, C164A, and C164S CDO all had low catalytic activity compared to studies utilizing L-cysteine. Additionally, wild-type, C164A, C164M, C164R, and C164S CDO did not have any catalytic activity with 3-mercaptopriopionic acid or mercaptopyruvate which are proposed substrates for bacterial CDO. The absence of activity with the Cys164 variants suggests that Cys164 plays a role in catalysis. Cys164 may play a role in the substrate specificity of CDO; however, it is not likely to play a role in substrate coordination as previously proposed due to the distance of Cys164 to the L-cysteine substrate.

In crosslink formation studies, wild-type CDO can form the fully crosslinked species at 80-100 mM L-cysteine. C164S and C164M CDO can form ~80% of the crosslinked species at 100 mM L-cysteine. However, C164D and C164R CDO can only form about half of the crosslinked species. Interestingly, C164A CDO can generate the fully crosslinked species at 10 mM L-cysteine; however, it has diminished catalytic activity. The negative charge of Asp and the positive charge of Arg at the opening of the active site appear to interfere with crosslink formation which may be due to the charges introduced by these residues. Since the Ser variant is a conserved substitution, the conservation of the thiolate properties of Cys164 by the Ser variant may be adequate to generate most of the crosslinked species. The C164M CDO variant is able to generate ~80% of the crosslinked species. The polarity of the Met residue is unlikely to cause any perturbations in the active site; however, the size of the Met residue may hinder access of the L-cysteine substrate. The Ala variant readily forms the crosslink at only 10 mM L-cysteine. This could be attributed to the hydrophobicity and size of Ala allowing L-cysteine access to the CDO active site.

Wild-type, C164A, and C164S CDO were also evaluated with L-cysteine analogs to determine if Cys164 plays a role in substrate specificity for crosslink formation. Wild-type and C164A CDO could form ~80% of the crosslink with D-cysteine while C164S CDO only formed about 50% of the fully crosslinked species. However, wild-type could form the fully crosslinked species with cysteamine while both Cys164 CDO variants could not generate any additional crosslinked species suggesting that Cys164 does not play a role in substrate specificity for crosslink formation.

The iron center of wild-type, C164A, and C164S CDO was evaluated to determine if Cys164 has an effect on the oxidation state of the iron center in CDO. Minor changes were observed with wild-type CDO and the variants. In the resting state, C164A and C164S CDO displayed ferric signals that maintained the same overall shape as wild-type CDO. When L-cysteine was added, all of the peaks became more intense and defined. The sharp signal has been observed previously with wild-type CDO and the L-cysteine substrate which was correlated with either L-cysteine coordination or formation of the crosslink modifying the iron center.[86] In addition, results from previous EPR studies with crosslinked CDO showed a sharp signal, but the addition of L-cysteine to crosslinked CDO did not affect the appearance of the peak suggesting that the sharp signal observed was likely attributed to the formation of the crosslink.[86] C164S CDO did not have as intense of a peak as wild-type or C164A CDO; however, this was likely attributed to C164S CDO forming less of the crosslinked species than wild-type or C164A CDO. Substitution of Cys164 with the Ser residue may have affected L-cysteine coordination to the iron center resulting in less of the crosslink formed and a less intense EPR signal.

It is not entirely clear why some of the Cys164 CDO variants can form most or all of the fully crosslinked species but are catalytically inefficient. As mentioned, L-cysteine oxidation and

crosslink formation both require L-cysteine, dioxygen, and ferrous iron. The fully crosslinked species can be generated at elevated L-cysteine concentrations which increases catalytic activity ~5-fold compared to non-crosslinked CDO. While these mechanisms would seem to coincide, it is unclear if they are ordered or concerted. However, they likely follow separate chemical steps which is evident based on these studies. C164A CDO can form the fully crosslinked species at 10 mM L-cysteine which is more efficient than wild-type CDO. However, the absence of Cys164 results in diminished activity. Therefore, Cys164 could be responsible for regulating the accessibility of the L-cysteine substrate for L-cysteine oxidation.

Mechanistically, it remains unclear how Cys164 would regulate the L-cysteine substrate. In our studies, we did not observe any cysteinylation which had induced the rotation of Met179 and Arg60 preventing access of the L-cysteine substrate in the three-dimensional structures. However, a similar phenomenon has been identified in three-dimensional structures of non-crosslinked and crosslinked CDO where cysteinylation was not a factor. Three-dimensional structures of non-crosslinked and crosslinked CDO have depicted the Met179 and Arg60 shift (**Fig. 3.4**). In the non-crosslinked structure, Arg60 is 4 Å away from Met179 which is 7 Å away from Cys164 (**Fig. 3.4A**). In the crosslinked structure, Arg60-Met179-Cys164 are within 4 Å of each other (**Fig. 3.4B**). The shift of these residues induces conformational changes at the opening of the active site. In the non-crosslinked CDO three-dimensional structure, the residues appear to be more exposed to the surface of CDO. However, in the crosslinked isoforms, these same residues appeared slightly less exposed. When CDO is expressed in response to increased L-cysteine concentrations, the exposure of these residues in the non-crosslinked isoform may act as a switch allowing the L-cysteine substrate access to the active site. Once the crosslink is formed, these residues move away from the active site. The Arg60 residue is then in position to coordinate the

carboxylate group of the L-cysteine substrate. In addition, the shift of these residues changes the overall conformation of the active site. The Cys164 residue in CDO could initiate these conformational changes and therefore regulate the accessibility of the L-cysteine substrate to the active site during catalysis. However, it is mechanistically unclear how Cys164 would initiate these changes and would need to be further explored.

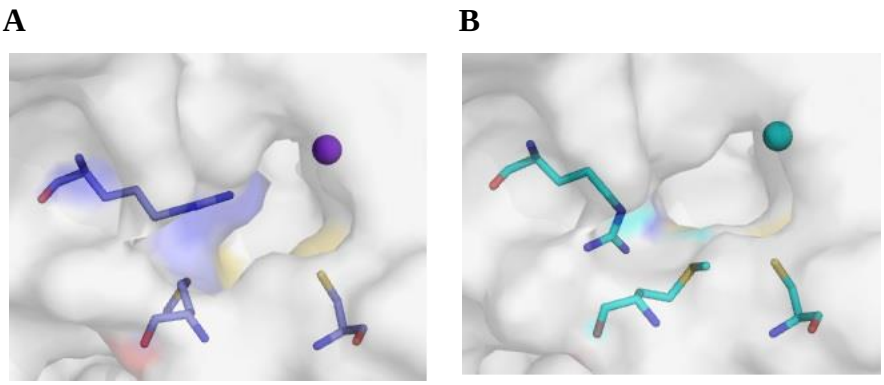


Fig. 3.4 Three-dimensional structures depicting Cys164, Met179, and Arg 60 in the active site of non-crosslinked wild-type CDO and crosslinked wild-type CDO. A. Cys164, Met179, and Arg60 residues are more exposed and shifted towards the iron center in non-crosslinked CDO. B. Cys164, Met179, and Arg60 residues are less exposed and facing away from the iron center in crosslinked wild-type CDO.

Chapter Four

Summary

CDO catalyzes the oxidation of L-cysteine to L-cysteine sulfinic acid. CDO contains a 3-His coordinated iron center, and adjacent to the iron center is a thioether crosslink which includes Cys93 and Tyr157. The crosslink is a post-translational modification that increases catalytic activity ~5-fold compared to non-crosslinked CDO. Wild-type CDO exists as a heterogeneous mixture of the non-crosslinked and crosslinked isoforms; however, the fully crosslinked species can be generated by the addition of the L-cysteine substrate. While both L-cysteine oxidation and crosslink formation mechanisms require L-cysteine, dioxygen, and ferrous iron, it remains unknown if the L-cysteine oxidation and crosslink formation mechanisms are ordered or concerted. The mechanisms likely follow different chemical steps given the overall differences in the reactions they catalyze. The overall goals of these studies were to determine if conserved amino acids play a specific role in L-cysteine oxidation and crosslink formation. The main objectives of these studies were to determine the role that these amino acids play in L-cysteine oxidation and crosslink formation.

In addition to the crosslink, Tyr157 is also involved in a hydrogen-bonding network which includes nearby residues His155 and Ser153. In three-dimensional structures, Tyr157-His155-Ser153 are located 2.7 Å from each other. Based on sequence alignments, these residues are

conserved in CDO among eukaryotic systems. Only the Tyr157 and His155 residues are conserved in CDO in all prokaryotic organisms, whereas Ser153 is partially conserved in prokaryotes. This hydrogen-bonding network has been of interest in CDO due the conservation of these residues in different enzymes, the location of the network in CDO, and the involvement of Tyr157 which is a component of the crosslink. Initially, this network was proposed to behave like a catalytic triad as seen in various enzymes such as serine proteases; however, this network contains distinct differences in comparison to established catalytic triads. Due to the location of the hydrogen-bonding network and the structural involvement of Tyr157 in the crosslink, one of the main objectives in this work was to identify the role of this hydrogen-bonding network in both L-cysteine oxidation and crosslink formation mechanisms.

To determine the role of His155 in L-cysteine oxidation, substitutions of His155 were evaluated with the L-cysteine substrate. The His155 CDO variants were unable to effectively oxidize the L-cysteine substrate. Tyr157 is proposed to stabilize Fe oxo intermediates during catalysis through hydrogen-bonding interactions (**Fig.4.1**). Since ferrous iron is required for both mechanisms, EPR studies were utilized to evaluate the influence His155 has on the metal center in CDO. All of the His155 CDO variants displayed a ferric signal analogous to wild-type CDO. The signal became sharper after the addition of the L-cysteine substrate indicating that L-cysteine was coordinating the iron center. Since, the L-cysteine oxidation mechanism follows an ordered sequential mechanism with L-cysteine as the obligate substrate, and CDO appears to maintain L-cysteine coordination in the absence of His155, the diminished activity observed with the His155 CDO variants could be due to the lack of dioxygen activation. This further suggests that His155 assists in stabilization of Tyr157 when Fe oxo intermediates are formulated during catalysis.

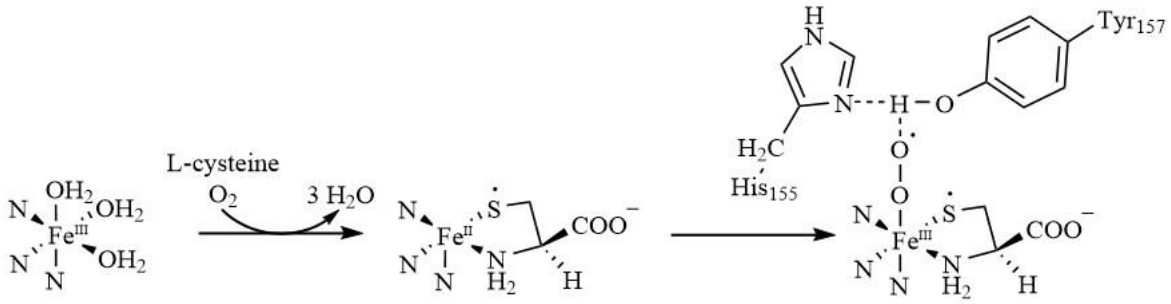


Fig. 4.1 First three steps of the proposed L-cysteine oxidation mechanism where Tyr157 is stabilizing an iron oxo intermediate. His155 is within hydrogen-bonding distance from Tyr157 and could provide additional stabilization for these intermediates.

To determine the role His155 plays in crosslink formation, His155 variants were evaluated with the L-cysteine substrate, and the percentage of crosslink formed was determined. While the His155 CDO variants are catalytically inactive, H155A and H155Q CDO were able to generate 80-100% of the crosslinked species suggesting that His155 may not play a major role in crosslink formation. His155Q CDO was also able to maintain a sharp ferric signal after the L-cysteine substrate was unbound from the iron center further suggesting that generation of the crosslink was able to proceed due to dioxygen activation. His155 does not appear to play a key role in crosslink formation. In three-dimensional structures of non-crosslinked CDO, His155 is over 3 Å away from Tyr157. Therefore, crosslink formation may not dependent on the hydrogen-bonding interaction between His155 and Tyr157. These results further suggest that L-cysteine oxidation and crosslink formation may follow separate mechanisms.

Located in the opening of the CDO active site ~8 Å away from the iron center is a cysteine residue. Cysteine residues can play numerous roles in proteins such as stability, redox signaling, and regulation. These cysteine residues are often modified, but Cys164 exists as a free thiol and does not appear to be involved in any electrostatic interactions with any residues in CDO.

Therefore, the location of this cysteine residue is unusual since CDO utilizes L-cysteine and dioxygen as substrates. Cys164 is also highly conserved in CDO among eukaryotes. In these studies, the role of Cys164 in L-cysteine oxidation and crosslink formation was investigated.

To determine the role of Cys164 in L-cysteine oxidation, variants of Cys164 were evaluated with the L-cysteine substrate. All Cys164 variants demonstrated minimal catalytic activity and product formation compared to wild-type CDO. In addition, Cys164 did not appear to be solvent accessible or undergo any oxidative modifications indicating that Cys164 is not involved in directly regulating enzyme activity. Interestingly, C164A CDO formed the crosslink more efficiently and at lower L-cysteine substrate concentrations than wild-type CDO. Based on these observations, Cys164 appears to play a role in L-cysteine oxidation and crosslink formation by regulating the accessibility of the L-cysteine substrate due to conformational changes noted in three-dimensional structures.

Three-dimensional structures of crosslinked and non-crosslinked wild-type CDO have notable conformational changes (**Fig. 4.2**). In the non-crosslinked CDO structure, the residues Cys164, Met179, and Arg60 are more exposed in the active site where the Met179 and Arg60 residues are shifted towards the iron center (**Fig.4.2 A**). However, the Cys164, Met179, and Arg60 residues are within 4 Å of each other and are less exposed facing away from the iron center in crosslinked wild-type CDO (**Fig. 4.2 B**). Arg60 has been shown to play a role in substrate

specificity by coordinating the carboxylate of the iron coordinated L-cysteine substrate. Based on the minimal catalytic activity observed with the Cys164 CDO variants, Cys164 could play a role in substrate specificity by regulating the access of the L-cysteine substrate to the active site in CDO by signaling the shift of these residues for substrate coordination. However, it remains unclear how Cys164 initiates this change.

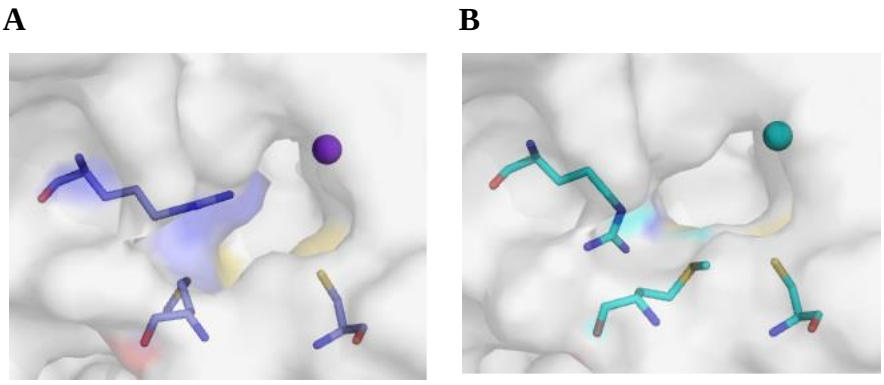


Fig. 4.2 Three-dimensional structures depicting Cys164, Met179, and Arg 60 in the active site of non-crosslinked wild-type CDO and crosslinked wild-type CDO. A. Cys164, Met179, and Arg60 residues are more exposed and shifted towards the iron center in non-crosslinked CDO. B. Cys164, Met179, and Arg60 residues are less exposed and facing away from the iron center in crosslinked wild-type CDO.

The results from these studies provide a foundation for future mechanistic studies in CDO. The L-cysteine oxidation and crosslink formation mechanisms are likely separate and distinct. Some of these amino acids such as His155 and Cys164 in CDO play a pivotal role in L-cysteine oxidation, while their role in crosslink formation is minimal. H155Q and C164A CDO variants generate the crosslink yet have no appreciable catalytic activity. Even though generation of the crosslink enhances catalytic activity and both mechanisms require L-cysteine, dioxygen, and iron,

it is still not known whether or not L-cysteine oxidation and crosslink formation occur simultaneously. These substitutions could be evaluated to further elucidate these mechanisms. The crosslink mechanism could be evaluated without little interference from catalysis. In addition, the conformational changes noted for the non-crosslinked and crosslinked enzymes could be further evaluated to determine if Cys164 regulates the access of L-cysteine by initiating the conformational change observed.

References:

(1) Jakob, U., and Reichmann, D. (Eds.). (2013) Oxidative Stress and Redox Regulation. Springer Netherlands.

(2) Stipanuk, M. H. (2004) Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu. Rev. Nutr. 24*, 539–577.

(3) Stipanuk, M. H., and Ueki, I. (2011) Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. *J Inherit Metab Dis 34*, 17–32.

(4) Belalcázar, A. D., Ball, J. G., Frost, L. M., Valentovic, M. A., and Wilkinson, J. (2013) Transsulfuration Is a Significant Source of Sulfur for Glutathione Production in Human Mammary Epithelial Cells. *International Scholarly Research Notices*. Research article.

(5) Lobo, V., Patil, A., Phatak, A., and Chandra, N. (2010) Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev 4*, 118–126.

(6) Brass, E. P. (1994) Overview of coenzyme A metabolism and its role in cellular toxicity. *Chemico-Biological Interactions 90*, 203–214.

(7) Ewetz, L., and Sörbo, B. (1966) Characteristics of the cysteinesulfinate-forming enzyme system in rat liver. *Biochimica et Biophysica Acta (BBA) - Enzymology and Biological Oxidation 128*, 296–305.

(8) Wainer, A. (1965) The production of cysteinesulfinic acid from cysteine in vitro. *Biochimica et Biophysica Acta (BBA) - General Subjects 104*, 405–412.

(9) Yamaguchi, K., Hosokawa, Y., Kohashi, N., Kori, Y., Sakakibara, S., and Ueda, I. (1978) Rat liver cysteine dioxygenase (cysteine oxidase): Further purification, characterization, and analysis of the activation and inactivation. *J. Biochem. 83*, 479–491.

(10) Jong, C. J., Azuma, J., and Schaffer, S. (2012) Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production. *Amino Acids 42*, 2223–2232.

(11) Papanikolaou, G., and Pantopoulos, K. (2005) Iron metabolism and toxicity. *Toxicology and Applied Pharmacology 202*, 199–211.

(12) Hancock, J. T., Desikan, R., and Neill, S. J. (2001) Role of reactive oxygen species in cell signalling pathways. *Biochem. Soc. Trans. 29*, 345–350.

(13) Lee, J.-I., Londono, M., Hirschberger, L. L., and Stipanuk, M. H. (2004) Regulation of cysteine dioxygenase and γ-glutamylcysteine synthetase is associated with hepatic cysteine level. *The Journal of Nutritional Biochemistry 15*, 112–122.

(14) Stipanuk, M. H., Coloso, R. M., Garcia, R. A. G., and Banks, M. F. (1992) Cysteine Concentration Regulates Cysteine Metabolism to Glutathione, Sulfate and Taurine in Rat Hepatocytes. *J Nutr 122*, 420–427.

(15) Biswas, S., Chida, A. S., and Rahman, I. (2006) Redox modifications of protein-thiols: emerging roles in cell signaling. *Biochem. Pharmacol. 71*, 551–564.

(16) Beeby, M., O'Connor, B. D., Ryttersgaard, C., Boutz, D. R., Perry, L. J., and Yeates, T. O. (2005) The genomics of disulfide bonding and protein stabilization in thermophiles. *PLoS Biol. 3*, e309.

(17) Sanchez, R., Riddle, M., Woo, J., and Momand, J. (2008) Prediction of reversibly oxidized protein cysteine thiols using protein structure properties. *Protein Sci. 17*, 473–481.

(18) Betteridge, D. J. (2000) What is oxidative stress? *Metab. Clin. Exp. 49*, 3–8.

(19) Veeralakshmi, M., Thangapandian, V., Mahalakshmi, G., Kuralarasi, R., and Lingakumar, K. (2017) CHANGES IN ANTI OXIDANT ENZYME ACTIVITIES IN Pseudomonas syringae pv syringae (BACTERIAL BROWN SPOT) INFECTED SEEDLINGS OF Vigna radiata L. *6*, 5.

(20) Fomenko, D. E., and Gladyshev, V. N. (2012) Comparative Genomics of Thiol Oxidoreductases Reveals Widespread and Essential Functions of Thiol-based Redox Control of Cellular Processes. *Antioxid Redox Signal 16*, 193–201.

(21) Holmgren, A. (1985) Thioredoxin. *Annual Review of Biochemistry 54*, 237–271.

(22) Wollman, E. E., d'Auriol, L., Rimsky, L., Shaw, A., Jacquot, J. P., Wingfield, P., Graber, P., Dessarps, F., Robin, P., and Galibert, F. (1988) Cloning and expression of a cDNA for human thioredoxin. *J. Biol. Chem. 263*, 15506–15512.

(23) Kalinina, E. V., Chernov, N. N., and Saprin, A. N. (2008) Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes. *Biochemistry Mosc. 73*, 1493–1510.

(24) Fomenko, D. E., and Gladyshev, V. N. (2002) CxxS: fold-independent redox motif revealed by genome-wide searches for thiol/disulfide oxidoreductase function. *Protein Sci. 11*, 2285–2296.

(25) Nagarajan, N., Oka, S., and Sadoshima, J. (2017) Modulation of signaling mechanisms in the heart by thioredoxin 1. *Free Radic. Biol. Med. 109*, 125–131.

(26) Kuster, G. M., Siwik, D. A., Pimentel, D. R., and Colucci, W. S. (2006) Role of Reversible, Thioredoxin-Sensitive Oxidative Protein Modifications in Cardiac Myocytes. *Antioxidants & Redox Signaling 8*, 2153–2159.

(27) Guzik, T. J., West, N. E. J., Pillai, R., Taggart, D. P., and Channon, K. M. (2002) Nitric oxide modulates superoxide release and peroxynitrite formation in human blood vessels. *Hypertension 39*, 1088–1094.

(28) Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium... - PubMed - NCBI.

(29) Hashemy, S. I., and Holmgren, A. (2008) Regulation of the Catalytic Activity and Structure of Human Thioredoxin 1 via Oxidation and *S* -Nitrosylation of Cysteine Residues. *Journal of Biological Chemistry 283*, 21890–21898.

(30) S-nitrosylation of thioredoxin mediates activation of apoptosis signal-regulating kinase 1 - ScienceDirect.

(31) Tao, L., and English, A. M. (2004) Protein S-Glutathiolation Triggered by Decomposed S-Nitrosoglutathione. *Biochemistry 43*, 4028–4038.

(32) Yamamoto, Y., Ritz, D., Planson, A.-G., Jönsson, T. J., Faulkner, M. J., Boyd, D., Beckwith, J., and Poole, L. B. (2008) Mutant AhpC peroxiredoxins suppress thiol-disulfide redox deficiencies and acquire deglutathionylating activity. *Mol Cell 29*, 36–45.

(33) Jönsson, T. J., and Lowther, W. T. (2007) The peroxiredoxin repair proteins. *Subcell. Biochem. 44*, 115–141.

(34) Chae, H. Z., Robison, K., Poole, L. B., Church, G., Storz, G., and Rhee, S. G. (1994) Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc. Natl. Acad. Sci. U.S.A. 91*, 7017–7021.

(35) Wood, Z. A., Schröder, E., Robin Harris, J., and Poole, L. B. (2003) Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci. 28*, 32–40.

(36) Wu, C., Dai, H., Yan, L., Liu, T., Cui, C., Chen, T., and Li, H. (2017) Sulfonation of the resolving cysteine in human peroxiredoxin 1: A comprehensive analysis by mass spectrometry. *Free Radic. Biol. Med. 108*, 785–792.

(37) Lim, J. C., Choi, H.-I., Park, Y. S., Nam, H. W., Woo, H. A., Kwon, K.-S., Kim, Y. S., Rhee, S. G., Kim, K., and Chae, H. Z. (2008) Irreversible Oxidation of the Active-site Cysteine

of Peroxiredoxin to Cysteine Sulfonic Acid for Enhanced Molecular Chaperone Activity. *J. Biol. Chem. 283*, 28873–28880.

(38) Hall, A., Karplus, P. A., and Poole, L. B. (2009) Typical 2-Cys peroxiredoxins – structures, mechanisms and functions. *The FEBS Journal 276*, 2469–2477.

(39) Teixeira, F., Castro, H., Cruz, T., Tse, E., Koldewey, P., Southworth, D. R., Tomás, A. M., and Jakob, U. (2015) Mitochondrial peroxiredoxin functions as crucial chaperone reservoir in Leishmania infantum. *Proc Natl Acad Sci U S A 112*, E616–E624.

(40) Biteau, B., Labarre, J., and Toledano, M. B. (2003) ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulphiredoxin. *Nature 425*, 980–984.

(41) McCord, J. M., and Fridovich, I. (1968) The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem. 243*, 5753–5760.

(42) Fukai, T., and Ushio-Fukai, M. (2011) Superoxide Dismutases: Role in Redox Signaling, Vascular Function, and Diseases. *Antioxid Redox Signal 15*, 1583–1606.

(43) Valentine, J. S., Doucette, P. A., and Zittin Potter, S. (2005) Copper-Zinc Superoxide Dismutase and Amyotrophic Lateral Sclerosis. *Annual Review of Biochemistry 74*, 563–593.

(44) Culotta, V. C., Klomp, L. W., Strain, J., Casareno, R. L., Krems, B., and Gitlin, J. D. (1997) The copper chaperone for superoxide dismutase. *J. Biol. Chem. 272*, 23469–23472.

(45) Multiple Protein Domains Contribute to the Action of the Copper Chaperone for Superoxide Dismutase.

(46) Banci, L., Bertini, I., Cantini, F., Kozyreva, T., Massagni, C., Palumaa, P., Rubino, J. T., and Zovo, K. (2012) Human superoxide dismutase 1 (hSOD1) maturation through interaction with human copper chaperone for SOD1 (hCCS). *Proc. Natl. Acad. Sci. U.S.A. 109*, 13555–13560.

(47) Fetherolf, M. M., Boyd, S. D., Taylor, A. B., Kim, H. J., Wohlschlegel, J. A., Blackburn, N. J., Hart, P. J., Winge, D. R., and Winkler, D. D. (2017) Copper-zinc superoxide dismutase is activated through a sulfenic acid intermediate at a copper-ion entry site. *J. Biol. Chem.* jbc.M117.775981.

(48) Auclair, J. R., Johnson, J. L., Liu, Q., Salisbury, J. P., Rotunno, M. S., Petsko, G. A., Ringe, D., Brown, R. H., Bosco, D. A., and Agar, J. N. (2013) Post-Translational Modification by Cysteine Protects Cu/Zn-Superoxide Dismutase from Oxidative Damage. *Biochemistry 52*, 6137–6144.

(49) Fujiwara, N., Nakano, M., Kato, S., Yoshihara, D., Ookawara, T., Eguchi, H., Taniguchi, N., and Suzuki, K. (2007) Oxidative modification to cysteine sulfonic acid of Cys111 in human copper-zinc superoxide dismutase. *J. Biol. Chem. 282*, 35933–35944.

(50) Kabil, O., and Banerjee, R. (2014) Enzymology of H2S Biogenesis, Decay and Signaling. *Antioxid Redox Signal 20*, 770–782.

(51) Dominy, J. J., Hirschberger, L. L., Coloso, R. M., and Stipanuk, M. H. (2006) Regulation of cysteine dioxygenase degradation is mediated by intracellular cysteine levels and the ubiquitin-26 S proteasome system in the living rat., Regulation of cysteine dioxygenase degradation is mediated by intracellular cysteine levels and the ubiquitin–26 S proteasome system in the living rat. *Biochem J 394*, *394*, 267, 267–273.

(52) Stipanuk, M. H., Dominy, J. E., Lee, J.-I., and Coloso, R. M. (2006) Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. *J. Nutr. 136*, 1652S-1659S.

(53) Garcia, R. A. G., and Stipanuk, M. H. (1992) The Splanchnic Organs, Liver and Kidney have Unique Roles in the Metabolism of Sulfur Amino Acids and Their Metabolites in Rats. *J Nutr 122*, 1693–1701.

(54) Fukagawa, N. K., Ajami, A. M., and Young, V. R. (1996) Plasma methionine and cysteine kinetics in response to an intravenous glutathione infusion in adult humans. *Am. J. Physiol. 270*, E209-214.

(55) Stipanuk, M. H., Coloso, R. M., Garcia, R. A. G., and Banks, M. F. (1992) Cysteine Concentration Regulates Cysteine Metabolism to Glutathione, Sulfate and Taurine in Rat Hepatocytes. *J Nutr 122*, 420–427.

(56) Sakamoto, A., Nishimura, Y., Ono, H., and Sakura, N. (2002) Betaine and homocysteine concentrations in foods. *Pediatrics International 44*, 409–413.

(57) Lee, J.-I., Londono, M., Hirschberger, L. L., and Stipanuk, M. H. (2004) Regulation of cysteine dioxygenase and γ-glutamylcysteine synthetase is associated with hepatic cysteine level. *The Journal of Nutritional Biochemistry 15*, 112–122.

(58) Bella, D. L., Hahn, C., and Stipanuk, M. H. (1999) Effects of nonsulfur and sulfur amino acids on the regulation of hepatic enzymes of cysteine metabolism. *Am. J. Physiol. 277*, E144-153.

(59) Lauterburg, B. H., and Mitchell, J. R. (1987) Therapeutic doses of acetaminophen stimulate the turnover of cysteine and glutathione in man. *Journal of Hepatology 4*, 206–211.

(60) Gordon, N. (2002) Pantothenate kinase-associated neurodegeneration (Hallervorden–Spatz syndrome). *European Journal of Paediatric Neurology 6*, 243–247.

(61) Park, E., Park, S. Y., Dobkin, C., and Schuller-Levis, G. (2014) Development of a Novel Cysteine Sulfinic Acid Decarboxylase Knockout Mouse: Dietary Taurine Reduces Neonatal Mortality. *J Amino Acids 2014*.

(62) Braunstein, A. E., Goryachenkova, E. V., Tolosa, E. A., Willhardt, I. H., and Yefremova, L. L. (1971) Specificity and some other properties of liver serine sulphhydrase: Evidence for its

identity with cystathionine β-synthase. *Biochimica et Biophysica Acta (BBA) - Enzymology 242*, 247–260.

(63) Stipanuk, M. H., and Beck, P. W. (1982) Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. *Biochemical Journal 206*, 267–277.

(64) Stipanuk, M. H. (1986) Metabolism of Sulfur-Containing Amino Acids. *Annu. Rev. Nutr. 6*, 179–209.

(65) Bella, D. L., and Stipanuk, M. H. (1995) Effects of protein, methionine, or chloride on acid-base balance and on cysteine catabolism. *Am. J. Physiol. 269*, E910-917.

(66) Tanaka, H., Takahashi, K., and Ogura, M. (1993) Metabolic Fate of Cysteine Sulfur in Growing Rats at Various Dietary Protein Levels. *J Nutr Sci Vitaminol, Journal of Nutritional Science and Vitaminology 39*, 507–516.

(67) Tomozawa, M., Yukihiro, K., Yao, W. B., Abe, T., Ohta, J., and Ubuka, T. (1998) Excretion of taurine and sulfate in rats fed with a low protein diet. *Acta Med. Okayama 52*, 77–81.

(68) Yukihiro, K., Tomozawa, M., Abe, T., Yao, W. B., Ohta, J., and Ubuka, T. (1998) Excretion of sulfate and taurine in rats fed with a high protein diet. *Acta Med. Okayama 52*, 71–75.

(69) Bella, D. L., Kwon, Y.-H., Hirschberger, L. L., and Stipanuk, M. H. (2002) Post-Transcriptional Regulation of Cysteine Dioxygenase in Rat Liver, in *Taurine 4: Taurine and Excitable Tissues* (Della Corte, L., Huxtable, R. J., Sgaragli, G., and Tipton, K. F., Eds.), pp 71–85. Springer US, Boston, MA.

(70) Stipanuk, M. H., Hirschberger, L. L., Londono, M. P., Cresenzi, C. L., and Yu, A. F. (2004) The ubiquitin-proteasome system is responsible for cysteine-responsive regulation of cysteine dioxygenase concentration in liver. *American Journal of Physiology-Endocrinology and Metabolism 286*, E439–E448.

(71) Khuri, S., Bakker, F. T., and Dunwell, J. M. (2001) Phylogeny, Function, and Evolution of the Cupins, a Structurally Conserved, Functionally Diverse Superfamily of Proteins. *Mol Biol Evol 18*, 593–605.

(72) Lane, B. G., Dunwell, J. M., Ray, J. A., Schmitt, M. R., and Cuming, A. C. (1993) Germin, a protein marker of early plant development, is an oxalate oxidase. *J. Biol. Chem. 268*, 12239–12242.

(73) Oxalate oxidases and differentiating surface structure in wheat: germins | Biochemical Journal.

(74) Dunwell, J. M., and Gane, P. J. (1998) Microbial Relatives of Seed Storage Proteins: Conservation of Motifs in a Functionally Diverse Superfamily of Enzymes. *J Mol Evol 46*, 147–154.

(75) Dunwell, J. M. (1998) Cupins: A New Superfamily of Functionally Diverse Proteins that Include Germins and Plant Storage Proteins. *Biotechnology and Genetic Engineering Reviews 15*, 1–32.

(76) Woo, E. J., Dunwell, J. M., Goodenough, P. W., Marvier, A. C., and Pickersgill, R. W. (2000) Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat. Struct. Biol. 7*, 1036–1040.

(77) Gane, P. J., Dunwell, J. M., and Warwickr, J. (1998) Modeling based on the structure of vicilins predicts a histidine cluster in the active site of oxalate oxidase. *J Mol Evol 46*, 488–493.

(78) Dunwell, J. M., Purvis, A., and Khuri, S. (2004) Cupins: the most functionally diverse protein superfamily? *Phytochemistry 65*, 7–17.

(79) Agarwal, G., Rajavel, M., Gopal, B., and Srinivasan, N. (2009) Structure-Based Phylogeny as a Diagnostic for Functional Characterization of Proteins with a Cupin Fold. *PLOS ONE 4*, e5736.

(80) Dunwell, J. M., Khuri, S., and Gane, P. J. (2000) Microbial Relatives of the Seed Storage Proteins of Higher Plants: Conservation of Structure and Diversification of Function during Evolution of the Cupin Superfamily. *Microbiol. Mol. Biol. Rev. 64*, 153–179.

(81) Dunwell, J. M., Culham, A., Carter, C. E., Sosa-Aguirre, C. R., and Goodenough, P. W. (2001) Evolution of functional diversity in the cupin superfamily. *Trends in Biochemical Sciences 26*, 740–746.

(82) Ye, S., Wu, X., Wei, L., Tang, D., Sun, P., Bartlam, M., and Rao, Z. (2007) An Insight into the Mechanism of Human Cysteine Dioxygenase KEY ROLES OF THE THIOETHER-BONDED TYROSINE-CYSTEINE COFACTOR. *J. Biol. Chem. 282*, 3391–3402.

(83) McCoy, J. G., Bailey, L. J., Bitto, E., Bingman, C. A., Aceti, D. J., Fox, B. G., and Phillips, G. N. (2006) Structure and mechanism of mouse cysteine dioxygenase. *PNAS 103*, 3084–3089.

(84) Dominy, J. E., Simmons, C. R., Karplus, P. A., Gehring, A. M., and Stipanuk, M. H. (2006) Identification and Characterization of Bacterial Cysteine Dioxygenases: a New Route of Cysteine Degradation for Eubacteria. *J. Bacteriol. 188*, 5561–5569.

(85) Stipanuk, M. H., Simmons, C. R., Karplus, P. A., and Dominy, J. E. (2011) Thiol Dioxygenases: Unique Families of Cupin Proteins. *Amino Acids 41*, 91–102.

(86) Njeri, C. W., and Ellis, H. R. (2014) Shifting redox states of the iron center partitions CDO between crosslink formation or cysteine oxidation. *Archives of Biochemistry and Biophysics 558*, 61–69.

(87) Simmons, C. R., Liu, Q., Huang, Q., Hao, Q., Begley, T. P., Karplus, P. A., and Stipanuk, M. H. (2006) Crystal Structure of Mammalian Cysteine Dioxygenase A NOVEL MONONUCLEAR IRON CENTER FOR CYSTEINE THIOL OXIDATION. *J. Biol. Chem. 281*, 18723–18733.

(88) Dominy, J. E., Simmons, C. R., Karplus, P. A., Gehring, A. M., and Stipanuk, M. H. (2006) Identification and Characterization of Bacterial Cysteine Dioxygenases: a New Route of Cysteine Degradation for Eubacteria. *Journal of Bacteriology 188*, 5561–5569.

(89) Dominy, J. E., Hwang, J., Guo, S., Hirschberger, L. L., Zhang, S., and Stipanuk, M. H. (2008) Synthesis of Amino Acid Cofactor in Cysteine Dioxygenase Is Regulated by Substrate and Represents a Novel Post-translational Regulation of Activity. *J. Biol. Chem. 283*, 12188–12201.

(90) Simmons, C. R., Krishnamoorthy, K., Granett, S. L., Schuller, D. J., Dominy, J. E., Begley, T. P., Stipanuk, M. H., and Karplus, P. A. (2008) A Putative Fe2+-Bound Persulfenate Intermediate in Cysteine Dioxygenase. *Biochemistry 47*, 11390–11392.

(91) Kumar, D., Thiel, W., and de Visser, S. P. (2011) Theoretical Study on the Mechanism of the Oxygen Activation Process in Cysteine Dioxygenase Enzymes. *J. Am. Chem. Soc. 133*, 3869–3882.

(92) Imsand, E. M., Njeri, C. W., and Ellis, H. R. (2012) Addition of an external electron donor to in vitro assays of cysteine dioxygenase precludes the need for exogenous iron. *Archives of Biochemistry and Biophysics 521*, 10–17.

(93) McCoy, J. G., Bailey, L. J., Bitto, E., Bingman, C. A., Aceti, D. J., Fox, B. G., and Phillips, G. N. (2006) Structure and mechanism of mouse cysteine dioxygenase. *PNAS 103*, 3084–3089.

(94) Blaesi, E. J., Fox, B. G., and Brunold, T. C. (2014) Spectroscopic and Computational Investigation of Iron(III) Cysteine Dioxygenase: Implications for the Nature of the Putative Superoxo-Fe(III) Intermediate. *Biochemistry 53*, 5759–5770.

(95) Chai, S. C., Bruyere, J. R., and Maroney, M. J. (2006) Probes of the Catalytic Site of Cysteine Dioxygenase. *J. Biol. Chem. 281*, 15774–15779.

(96) Crawford, J. A., Li, W., and Pierce, B. S. (2011) Single turnover of substrate-bound ferric cysteine dioxygenase with superoxide anion: Enzymatic reactivation, product formation, and a transient intermediate. *Biochemistry 50*, 10241–10253.

(97) Tchesnokov, E. P., Wilbanks, S. M., and Jameson, G. N. L. (2012) A Strongly Bound High-Spin Iron(II) Coordinates Cysteine and Homocysteine in Cysteine Dioxygenase. *Biochemistry 51*, 257–264.

(98) Dominy, J. E., Hwang, J., Guo, S., Hirschberger, L. L., Zhang, S., and Stipanuk, M. H. (2008) Synthesis of Amino Acid Cofactor in Cysteine Dioxygenase Is Regulated by Substrate and Represents a Novel Post-translational Regulation of Activity. *J. Biol. Chem. 283*, 12188–12201.

(99) Dominy, J. E., Hirschberger, L. L., Coloso, R. M., and Stipanuk, M. H. (2006) Regulation of cysteine dioxygenase degradation is mediated by intracellular cysteine levels and the ubiquitin–26 S proteasome system in the living rat. *Biochemical Journal 394*, 267–273.

(100) Stipanuk, M. H., Londono, M., Hirschberger, L. L., Hickey, C., Thiel, D. J., and Wang, L. (2004) Evidence for expression of a single distinct form of mammalian cysteine dioxygenase. *Amino Acids 26*, 99–106.

(101) Driggers, C. M., Kean, K. M., Hirschberger, L. L., Cooley, R. B., Stipanuk, M. H., and Karplus, P. A. (2016) Structure-Based Insights into the Role of the Cys–Tyr Crosslink and

Inhibitor Recognition by Mammalian Cysteine Dioxygenase. *Journal of Molecular Biology 428*, 3999–4012.

(102) Li, W., and Pierce, B. S. (2015) Steady-state substrate specificity and O2-coupling efficiency of mouse cysteine dioxygenase. *Archives of Biochemistry and Biophysics 565*, 49–56.

(103) Li, W., Blaesi, E. J., Pecore, M. D., Crowell, J. K., and Pierce, B. S. (2013) Second-Sphere Interactions between the C93–Y157 Cross-Link and the Substrate-Bound Fe Site Influence the O2 Coupling Efficiency in Mouse Cysteine Dioxygenase. *Biochemistry 52*, 9104–9119.

(104) Driggers, C. M., Hartman, S. J., and Karplus, P. A. (2015) Structures of Arg- and Gln-type bacterial cysteine dioxygenase homologs. *Protein Science 24*, 154–161.

(105) Bruland, N., Wübbeler, J. H., and Steinbüchel, A. (2009) 3-Mercaptopropionate Dioxygenase, a Cysteine Dioxygenase Homologue, Catalyzes the Initial Step of 3-Mercaptopropionate Catabolism in the 3,3-Thiodipropionic Acid-degrading Bacterium Variovorax paradoxus. *J. Biol. Chem. 284*, 660–672.

(106) Driggers, C. M., Cooley, R. B., Sankaran, B., Hirschberger, L. L., Stipanuk, M. H., and Karplus, P. A. (2013) Cysteine Dioxygenase Structures from pH4 to 9: Consistent Cys-Persulfenate Formation at Intermediate pH and a Cys-Bound Enzyme at Higher pH. *Journal of Molecular Biology 425*, 3121–3136.

(107) Fellner, M., Siakkou, E., Faponle, A. S., Tchesnokov, E. P., de Visser, S. P., Wilbanks, S. M., and Jameson, G. N. L. (2016) Influence of cysteine 164 on active site structure in rat cysteine dioxygenase. *J Biol Inorg Chem 21*, 501–510.

(108) Siakkou, E., Rutledge, M. T., Wilbanks, S. M., and Jameson, G. N. L. (2011) Correlating crosslink formation with enzymatic activity in cysteine dioxygenase. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1814*, 2003–2009.

(109) Kleffmann, T., Jongkees, S. A. K., Fairweather, G., Wilbanks, S. M., and Jameson, G. N. L. (2009) Mass-spectrometric characterization of two posttranslational modifications of cysteine dioxygenase. *J Biol Inorg Chem 14*, 913–921.

(110) Tchesnokov, E. P., Fellner, M., Siakkou, E., Kleffmann, T., Martin, L. W., Aloi, S., Lamont, I. L., Wilbanks, S. M., and Jameson, G. N. L. (2015) The Cysteine Dioxygenase Homologue from Pseudomonas aeruginosa is a 3-Mercaptopropionate Dioxygenase. *J. Biol. Chem.* jbc.M114.635672.

(111) Tchesnokov, E. P., Fellner, M., Siakkou, E., Kleffmann, T., Martin, L. W., Aloi, S., Lamont, I. L., Wilbanks, S. M., and Jameson, G. N. (2015) The Cysteine Dioxygenase Homologue from Pseudomonas aeruginosa Is a 3-Mercaptopropionate Dioxygenase. *J.Biol.Chem. 290*, 24424–24437.

(112) Whittaker, J. W. (2005) The radical chemistry of galactose oxidase. *Arch. Biochem. Biophys. 433*, 227–239.

(113) Whittaker, M. M., and Whittaker, J. W. (2003) Cu(I)-dependent biogenesis of the galactose oxidase redox cofactor. *J. Biol. Chem. 278*, 22090–22101.

(114) Firbank, S. J., Rogers, M. S., Wilmot, C. M., Dooley, D. M., Halcrow, M. A., Knowles, P. F., McPherson, M. J., and Phillips, S. E. (2001) Crystal structure of the precursor of galactose oxidase: an unusual self-processing enzyme. *Proc. Natl. Acad. Sci. U.S.A. 98*, 12932–12937.

(115) Fellner, M., Aloi, S., Tchesnokov, E. P., Wilbanks, S. M., and Jameson, G. N. L. (2016) Substrate and pH-Dependent Kinetic Profile of 3-Mercaptopropionate Dioxygenase from Pseudomonas aeruginosa. *Biochemistry 55*, 1362–1371.

(116) Davies, C. G., Fellner, M., Tchesnokov, E. P., Wilbanks, S. M., and Jameson, G. N. L. (2014) The Cys-Tyr Cross-Link of Cysteine Dioxygenase Changes the Optimal pH of the Reaction without a Structural Change. *Biochemistry 53*, 7961–7968.

(117) Blaesi, E. J., Fox, B. G., and Brunold, T. C. (2015) Spectroscopic and Computational Investigation of the H155A Variant of Cysteine Dioxygenase: Geometric and Electronic Consequences of a Third-Sphere Amino Acid Substitution. *Biochemistry 54*, 2874–2884.

(118) Stipanuk, M. H., Ueki, I., Dominy, J. E., Simmons, C. R., and Hirschberger, L. L. (2009) Cysteine Dioxygenase: A Robust System for Regulation of Cellular Cysteine Levels. *Amino Acids 37*, 55–63.

(119) Dodson, G., and Wlodawer, A. (1998) Catalytic triads and their relatives. *Trends in Biochemical Sciences 23*, 347–352.

(120) Buller, A. R., and Townsend, C. A. (2013) Intrinsic evolutionary constraints on protease structure, enzyme acylation, and the identity of the catalytic triad. *Proc. Natl. Acad. Sci. U.S.A. 110*, E653-661.

(121) Berg, J. M., Tymoczko, J. L., Stryer, L., Berg, J. M., Tymoczko, J. L., and Stryer, L. (2002) Biochemistry 5th ed. W H Freeman.

(122) Dixon, G. H., Kauffman, D. L., and Neurath, H. (1958) AMINO ACID SEQUENCE IN THE REGION OF DIISOPROPYL PHOSPHORYL BINDING IN DIP-TRYPSIN. *J. Am. Chem. Soc. 80*, 1260–1261.

(123) Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967) Three-dimensional Structure of Tosyl-α-chymotrypsin. *Nature 214*, 652–656.

(124) Walsh, K. A., and Neurath, H. (1964) TRYPSINOGEN AND CHYMOTRYPSINOGEN AS HOMOLOGOUS PROTEINS. *Proc. Natl. Acad. Sci. U.S.A. 52*, 884–889.

(125) de Haën, C., Neurath, H., and Teller, D. C. (1975) The phylogeny of trypsin-related serine proteases and their zymogens. New methods for the investigation of distant evolutionary relationships. *J. Mol. Biol. 92*, 225–259.

(126) Lesk, A. M., and Fordham, W. D. (1996) Conservation and variability in the structures of serine proteinases of the chymotrypsin family. *J. Mol. Biol. 258*, 501–537.

(127) Gupta, S. P. (2017) Viral Proteases and Their Inhibitors. Academic Press.

(128) McGrath, M. E., Wilke, M. E., Higaki, J. N., Craik, C. S., and Fletterick, R. J. (1989) Crystal structures of two engineered thiol trypsins. *Biochemistry 28,* 9264–9270.

(129) Polgár, L., and Asbóth, B. (1986) The basic difference in catalyses by serine and cysteine proteinases resides in charge stabilization in the transition state. *J. Theor. Biol. 121*, 323–326.

(130) Ekici, O. D., Paetzel, M., and Dalbey, R. E. (2008) Unconventional serine proteases: variations on the catalytic Ser/His/Asp triad configuration. *Protein Sci. 17,* 2023–2037.

(131) Berg, J. M., Tymoczko, J. L., and Stryer, L. (2002) Proteases: Facilitating a Difficult Reaction. *Biochemistry. 5th edition.*

(132) Damblon, C., Raquet, X., Lian, L. Y., Lamotte-Brasseur, J., Fonze, E., Charlier, P., Roberts, G. C., and Frère, J. M. (1996) The catalytic mechanism of beta-lactamases: NMR titration of an active-site lysine residue of the TEM-1 enzyme. *Proc. Natl. Acad. Sci. U.S.A. 93*, 1747–1752.

(133) Neurath, H. (1994) Proteolytic enzymes past and present: the second golden era. Recollections, special section in honor of Max Perutz. *Protein Sci. 3*, 1734–1739.

(134) Shafee, T. (2014, February 4) Evolvability of a viral protease: experimental evolution of catalysis, robustness and specificity. Thesis, University of Cambridge.

(135) Sanchez-Pulido, L., and Ponting, C. P. (2016) Vasohibins: new transglutaminase-like cysteine proteases possessing a non-canonical Cys-His-Ser catalytic triad. *Bioinformatics 32*, 1441–1445.

(136) Shin, S., Yun, Y. S., Koo, H. M., Kim, Y. S., Choi, K. Y., and Oh, B.-H. (2003) Characterization of a novel Ser-cisSer-Lys catalytic triad in comparison with the classical Ser-His-Asp triad. *J. Biol. Chem. 278*, 24937–24943.

(137) Aluri, S., and de Visser, S. P. (2007) The Mechanism of Cysteine Oxygenation by Cysteine Dioxygenase Enzymes. *J. Am. Chem. Soc. 129*, 14846–14847.

(138) Ye, S., Wu, X., Wei, L., Tang, D., Sun, P., Bartlam, M., and Rao, Z. (2007) An Insight into the Mechanism of Human Cysteine Dioxygenase KEY ROLES OF THE THIOETHER-BONDED TYROSINE-CYSTEINE COFACTOR. *J. Biol. Chem. 282*, 3391–3402.

(139) McCoy, J. G., Bailey, L. J., Bitto, E., Bingman, C. A., Aceti, D. J., Fox, B. G., and Phillips, G. N. (2006) Structure and mechanism of mouse cysteine dioxygenase. *PNAS 103*, 3084–3089.

(140) Vanoni, M. A., Edmondson, D. E., Zanetti, G., and Curti, B. (1992) Characterization of the flavins and the iron-sulfur centers of glutamate synthase from Azospirillum brasilense by absorption, circular dichroism, and electron paramagnetic resonance spectroscopies. *Biochemistry 31*, 4613–4623.

(141) Kraut, J. (1977) Serine Proteases: Structure and Mechanism of Catalysis. *Annu. Rev. Biochem. 46*, 331–358.

(142) Chai, S. C., Jerkins, A. A., Banik, J. J., Shalev, I., Pinkham, J. L., Uden, P. C., and Maroney, M. J. (2005) Heterologous Expression, Purification, and Characterization of Recombinant Rat Cysteine Dioxygenase. *J. Biol. Chem. 280*, 9865–9869.

(143) Rhile, I. J., Markle, T. F., Nagao, H., DiPasquale, A. G., Lam, O. P., Lockwood, M. A., Rotter, K., and Mayer, J. M. (2006) Concerted Proton−Electron Transfer in the Oxidation of Hydrogen-Bonded Phenols. *J. Am. Chem. Soc. 128*, 6075–6088.

(144) (2012) Redox properties of tyrosine and related molecules. *FEBS Letters 586*, 596–602.

(145) Whittaker, J. W. (2003) Free Radical Catalysis by Galactose Oxidase. *Chem. Rev. 103*, 2347–2364.

(146) Rogers, M. S., Hurtado-Guerrero, R., Firbank, S. J., Halcrow, M. A., Dooley, D. M., Phillips, S. E. V., Knowles, P. F., and McPherson, M. J. (2008) Cross-Link Formation of the Cysteine 228−Tyrosine 272 Catalytic Cofactor of Galactose Oxidase Does Not Require Dioxygen. *Biochemistry 47*, 10428–10439.

(147) (2012) Redox properties of tyrosine and related molecules. *FEBS Letters 586*, 596–602.

(148) Wainer, A. (1965) The production of cysteinesulfinic acid from cysteine in vitro. *Biochimica et Biophysica Acta (BBA) - General Subjects 104*, 405–412.

(149) Driggers, C. M., Hartman, S. J., and Karplus, P. A. (2015) Structures of Arg- and Gln-type bacterial cysteine dioxygenase homologs. *Protein Science 24*, 154–161.

(150) Carpenter, R. A., Zhan, X., and Ellis, H. R. (2010) Catalytic role of a conserved cysteine residue in the desulfonation reaction by the alkanesulfonate monooxygenase enzyme. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1804*, 97–105.

(151) Novel Application of 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole To Identify Cysteine Sulfenic Acid in the AhpC Component of Alkyl Hydroperoxide Reductase - Biochemistry (ACS Publications).

(152) Musila, J. M., L. Forbes, D., and Ellis, H. R. (2018) Functional Evaluation of the π-Helix in the NAD(P)H:FMN Reductase of the Alkanesulfonate Monooxygenase System. *Biochemistry 57*, 4469–4477.