Cysteine dioxygenase: structure and mechanism

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Cysteine dioxygenase (CDO) catalyzes the oxidation of cysteine to cysteine sulfinic acid, which is the first major step in cysteine catabolism in mammalian tissues. Crystal structures of mouse, rat, human and bacterial CDO have recently become available and provide significant mechanistic insights. Unlike most non-heme Fe(II) dioxygenases, coordination of the Fe in CDO deviates from the 2-His-1-carboxylate facial triad archetype and instead adopts a His_3 facial triad. This change is expected to have an influence on oxygen activation by the catalytic site. The structures also reveal the presence of a cysteinyltyrosine (Tyr157-Cys93) post-translational modification near the active site. Kinetic studies of mutant CDOs reveal that the cysteine residue is less critical than the tyrosine for enzyme activity. Inconsistencies about the details of the active site and the nature of substrate binding exist and are discussed. Herein we review the structural biology along with relevant kinetics studies that have been conducted on CDO for insights into the reaction mechanism of this novel non-heme iron dioxygenase.

1 Introduction

Oxygenases catalyze the addition of molecular oxygen to organic substrates and thus play a pivotal role in a broad range of essential biological reactions that are involved in mammalian metabolism. The best known and probably most studied of these systems are those proteins that contain one or more iron-porphyrin units *e.g.*, indoleamine 2,3-dioxygenase and L-tryptophan 2,3-dioxygenases.[1–4] However, non-heme iron enzymes comprise a large and expanding collection of dioxygenases.[5–8] A prominent structural motif in these dioxygenases is a triad of ligands arranged in a mutually *cis* geometry and comprised of two histidines and a carboxylate side chain (the 2-His-1-carboxylate facial triad) leaving three mutually *cis* sites for coordination of the substrate or cofactor and dioxygen. It has been noted that there are dioxygenases (*e.g.*, diketone dioxygenase and carotenoid oxygenase) that deviate from this paradigm and the impact of those variations has been reviewed.[9] One such non-heme Fe(II) dioxygenase is cysteine dioxygenase (CDO) which converts cysteine to cysteine sulfinic acid (sulfur oxidation).

Recent structural and mechanistic investigations show that CDO has several features that are unique among the non-heme Fe(II) dioxygenases. First, the aforementioned typical structure of the active site consisting of a 2-His-1-carboxylate facial triad is not adopted. Instead in CDO, a third histidine imidazole

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group substitutes the carboxylate ligand but the facial geometry is maintained. The typical non-heme Fe dioxygenase utilizes the iron center and either the substrate or a cofactor to reductively activate dioxygen, which then carries out the catalysis. The substitution of the carboxylate ligand in the 2-His-1-carboxylate facial triad by histidine is expected to have a large effect on the redox potential of the iron center and thus on the reductive activation of oxygen. In the case of CDO, a protein-based cofactor may be present in the form of a cysteinyltyrosine. This protein-based cofactor is found in only one other enzyme, galactose oxidase, where it serves as a one-electron redox center. However, because the substrate (cysteine) readily reacts with dioxygen, it is possible that CDO does not operate by oxygen activation. Instead, CDO may catalyze the reaction by bringing the reactants together and preventing undesired reactions with other biological thiols. In this review we will discuss the role of the His_3 facial triad; the nature of substrate binding to the active site; and the role cysteinyltyrosine group in regards to enzymatic activity and how these differences compare to other non-heme dioxygenases.

2 Biological significance

Cysteine dioxygenase catalyzes the conversion of L-cysteine to L-cysteine sulfinic acid (CSA) (Fig. 1). CDO is found in mammalian cells and is particularly abundant in hepatocytes,[10] where it is regulated by cysteine-mediated degradation.[11,12] CDO is also found in some yeast (*Histoplasma capsulatum*[13]) and in some bacteria.[14] The product, CSA, lies at a metabolic branch-point, with one route leading to taurine biosynthesis and the other pathway producing sulfate.[15] CSA

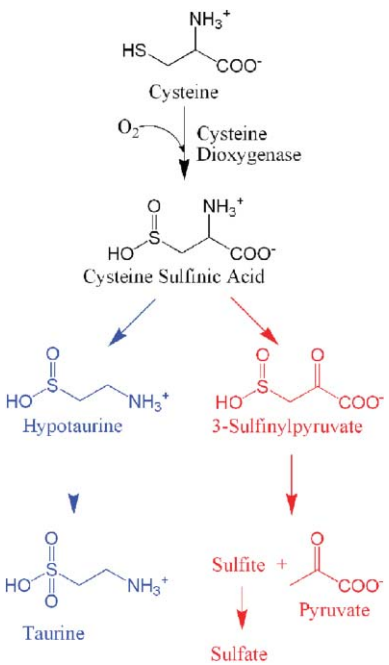

Fig. 1 Cysteine dioxygenase catalyzes the oxidation of cysteine to cysteine sulfinic acid (CSA), which is subsequently metabolized to hypotaurine by cysteine sulfinate decarboxylase or to 3-sulfinylpyruvate by aspartate aminotransferase.

can undergo transamination to form 3-sulfinylpyruvate, which spontaneously decomposes to pyruvate and sulfite, the latter being readily oxidized to sulfate.[16] Alternatively, the pathway leading to taurine formation proceeds *via* decarboxylation of CSA to form hypotaurine (2-aminoethane sulfinate) by the pyridoxal 5′-phosphate-dependent enzyme cysteine sulfinate decarboxylate.[17] Hypotaurine is subsequently oxidized to taurine in a poorly understood process where both enzymatic and nonenzymatic reactions are possibly involved.[18]

Taurine has diverse roles in mammalian organisms including maintaining cardiac functions,[19] protecting neural cells from excitotoxicity and damage induced by ischemia.[20] Taurine has been postulated to be a neurotransmitter, as it is the second most abundant amino (sulfonic) acid in the central nervous system.[21] This amino acid plays a key role in stabilizing mammalian skeletal muscle membrane and based on studies with aged rats, it is hypothesized that changes in muscle function during aging could be associated with a reduction of taurine content.[22] It is also a major ingredient in the energy drink Red Bull™, which touts the antioxidant properties of taurine and its ability to promote the elimination of harmful substances from the body.[23] However, the beneficial role of taurine as a softdrink additive is controversial.[24]

The absence or lack of activity of CDO has been linked to a number of disease states. The substrate of CDO, cysteine, is both toxic (1–2 g kg^{-1}) and readily oxidizes to form the poorly soluble disulfide, cystine (depending on the pH, concentrations above 250 mg L^{-1} will cause it to precipitate forming cystine stones).[25] It has been noted that cysteine elicits excitotoxin behavior, acting on the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor.[26] Other researchers postulate that this amino acid contributes to the formation of reactive oxygen species, oxidizing essential molecules, such as the catecholaminergic neurotransmitter dopamine.[27] High levels of this amino acid have been associated with neurological disorders such as motor neuron disease,[28] Alzheimer's disease and Parkinson's disease.[29] Localized accumulation of cyst(e)ine and decreased activity of CDO has been observed in patients suffering from the rare neurological disorder Hallervorden–Spatz disease.[30] In addition, poor sulfoxidation and the reduced formation of inorganic sulfate are associated with rheumatoid arthritis.[31] Yamaguchi and co-workers reported a lack of CDO activity in malignant tumor cells such as rat hepatoma cell (AH 2440 and AH 109A) and mouse Ehrlich ascites tumor cells,[10] and thus CDO is a member of the long list of enzymes whose expression is altered in cancer cells.

3 Early enzymology

The enzyme was first described in the 1960s by demonstrating that crude rat liver extracts contain an enzyme that produced L-cysteine sulfinic acid from L-cysteine.[32–35] Through labeling studies with $^{18}O_2$ and $H_2{}^{18}O$, Lombardini *et al.* showed that the enzyme is a dioxygenase.[36] Early studies of CDO were hampered by the apparent instability of the enzyme during conventional isolation procedures. Cysteine dioxygenase was purified to homogeneity by Yamaguchi *et al.*, but the process yielded an inactive form of the enzyme.[37] The same research group discovered that the metalloenzyme could be reactivated

by pre-incubation with L-cysteine under anaerobic conditions.[38] The activated enzyme was reported to be rapidly and irreversibly inactivated during aerobic assays. The deactivation could be prevented by the presence of a second cytosolic protein, protein-A. The stabilizing protein-A was also purified by the Yamaguchi group,[39] which showed that it has an approximate molecular weight of 78 kDa by exclusion chromatography on a Sephadex G-200 column. However, it showed a single peptide band having an estimated MW of 19.5 kDa by SDS-polyacrylamide gel electrophoresis; suggesting that protein-A is composed of four monomers. According to the authors, protein-A does not participate in the catalytic process, as neither K_m nor the initial reaction velocity is altered by its presence. In addition, the same enzyme activity is observed during anaerobic preincubation in the presence or absence of the stabilizing protein. Their purified rat liver cysteine dioxygenase was characterized as a single subunit protein with a molecular weight of 22.5 kDa, and pI value of 5.5. Atomic absorption analysis indicated 0.8 moles of iron per mole of enzyme.[40] In spite of numerous reports that confirm the previous mass for CDO, a stable protein of 68 kDa was recently observed by Waring and co-workers.[41,42] This protein did not dissociate even in the presence of SDS and reducing agents at boiling temperatures.

Recent work in the author's laboratory[43,44] and two other laboratories,[45–47] using homogeneous preparations of recombinant CDO have clarified some of the confusion in the literature. These studies establish that the functional enzyme is a monomer with a molecular weight of *ca.* 23 kDa.[44] It was also found that the enzyme activity is dependent on ferrous iron and that it is specific for L-cysteine oxidation (*e.g.*, homocysteine is an inhibitor but not a substrate).[44] The recombinant rat CDO prepared in the authors laboratory had a K_m = 2.5(4) mM,[44] in agreement with a range of values of 0.45–5.7 mM measured for CDO from a variety of sources.[11,45,47] Cysteine dioxygenase has a relatively large K_m value compared to most enzymes. However, it is not uncommon for enzymes that utilize sulfur-containing substrates to have K_m values in the millimolar range.[17,44,48–50] Cysteine concentration in hepatic cells range from approximately 0.01 to 0.1 mM,[15] which is below the K_m value calculated for CDO. Having a K_m value greater than intracellular cysteine concentration could allow the enzyme to respond to changes in substrate concentration. The optimum pH is still controversial; a value 7.5 was measured in the author's lab,[44] and is different from another determination of 6.1.[45] Contrary to the earlier reports in the literature, CDO does not require the presence of a secondary protein (protein A) or need anaerobic pre-incubation in order to be active.[44] Unlike many non-heme ferrous iron dioxygenases, CDO does not require α-ketoglutarate (α-KG). In fact, α-KG acts as an inhibitor.[43] The studies of recombinant CDO all note that Fe is present in substoichiometric amounts in as-isolated protein.[44,45,47,51]

4 Structural biology

The recent availability of overexpressed recombinant enzyme has facilitated a number of studies of the structure of CDO, including three crystallographic characterizations[46,47,51] and one study of the Fe site using X-ray absorption spectroscopy from the author's lab.[43] The first crystal structure was of mouse CDO (identical to rat and 91% identical to human) and had a resolution of 1.75 Å.[47] This structure established that CDO has a β-barrel structure that is characteristic of the cupin superfamily (Fig. 2(A)). Members of the cupin family include several non-heme iron dioxygenases that have been crystallographically characterized, including homogentisate dioxygenase,[52] 3-hydroxyanthranilate-3,4-dioxygenase,[53] quercetin 2,3-dioxygenase,[54] and acireductone dioxygenase.[55] Members of the cupin family feature two conserved sequence motifs that provide the ligands for the metal binding site:[56] $G(X)_5HXH(X)_{3,4}$-$E(X)_6G$ and $G(X)_5PXG(X)_2$-$H(X)_3N$ (Fig. 3). The active site is typically formed using a combination of the two His and Glu (or Asp) residues from the first motif and the His residue from the second, more variable motif.[57] In the *Mus musculus* CDO sequence, the glutamate residue in the first sequence motif is replaced by a cysteine (Cys93), while only the histidine and asparagine residues of the second motif are retained. The Cys substitution is a feature of all CDOs from eukaryotes, but is not retained in bacterial enzymes, where it is typically a Gly residue (Fig. 3). The first crystal characterized contained a mixture of metals,[47] and the structure was solved as a Ni(II) complex. The Ni(II) center was found to be six-coordinate with a novel facial arrangement of three His residues (His86, His88, His140) and three solvent (presumably aqua) ligands (Fig. 2(B)). This active site structure is similar to the 2-His-1-carboxylate facial triad found in a number of non-heme Fe(II) enzymes that activate O_2,[6,58,59] where the carboxylate is replaced by the His140 residue from the second conserved motif.

The crystal structure of the Ni(II) complex also revealed that Cys93 is involved in a post-translational modification with Tyr157 to form a cysteinyltyrosine linkage near the active site (Fig. 2(B)). The only enzyme besides CDO featuring this post-translational modification is galactose oxidase.[60] In galactose oxidase, the cofactor forms upon incorporation of Cu(I) and exposure to oxygen.[61] Cu(I) binds to the enzyme *via* the tyrosine in the cysteinyltyrosine. Exposure to oxygen leads to the formation of Cu(II) and a peroxy radical, which reacts with the cysteine to form a cysteinyl radical. The cysteinyl radical attacks the ortho position of the tyrosine and results in the formation of the reduced active site, followed by deprotonation and release of H_2O_2. A similar mechanism for formation of the cysteinyltyrosine in CDO using an Fe(II) in place of Cu(I) can be envisioned.[46]

Although the cysteinyltyrosine is not an iron ligand in CDO (the Fe–O distance: 4.16 Å) it is nearby and the combination could serve as a source of two electrons for the reduction of O_2 to peroxide in analogy with the galactose oxidase site. Several properties are associated with the cysteinyltyrosine protein cofactor.[62] First, it can form protein cross-linkages similar to disulfide bridges; however, this linkage cannot be broken so easily. Second, the modification of the tyrosine lowers its oxidation potential by $\sim$0.5 V relative to phenols and stabilizes the radical by delocalization. Third, the tyrosine-OH group is more acidic by about 1 pH unit relative to phenols. Thus, the group can play a structural role, a redox

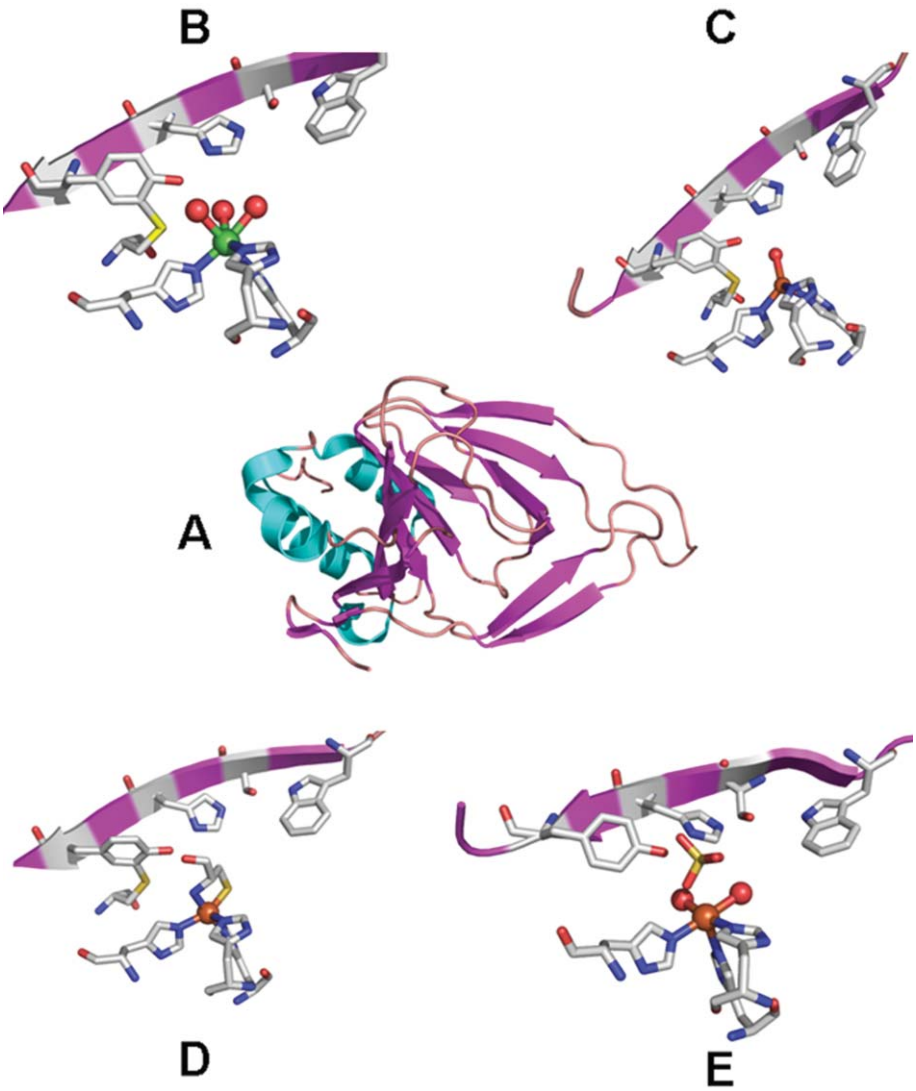

Fig. 2 Crystal structures of CDO. (A) Ribbon diagram illustrating the tertiary structure of CDO including the cupin fold. (B) The $His_3Ni(OH_2)_3$ center and neighbouring cysteinyltyrosine from mouse CDO (2ATF). (C) The $His_3Fe–OH_2$ center and neighbouring cysteinyltyrosine from rat CDO (2B5H). (D) The $His_3Fe(Cys)$ center and neighbouring cysteinyltyrosine from human CDO (2IC1). (E) The $His_3Fe(OH_2)_2$ center with neighbouring sulfate and tyrosine from *Ralstonia eutropha* CDO (2GM6).

role, or serve as a source of protons for protonation of peroxide.

From the standpoint of understanding the structure of the active site of CDO, a weakness of the first crystal structure is that it contained Ni(II) and not Fe(II). This deficiency was addressed by the second reported crystal structure of rat CDO, an identical protein in the identical space group, which was crystallographically characterized at 1.5 Å resolution.[46] This study confirmed the cupin fold and the presence of the cysteinyltyrosine group. The iron site was structurally distinct from the nickel site. While the iron is ligated to the protein by the same three His residues found in the Ni(II) complex, the structure reveals the presence of only one solvent-derived ligand, producing a distorted tetrahedral iron site (Fig. 2(C)). Since both the tetrahedral site and the facial six-coordinate complex have three mutually *cis*-sites available for coordinating substrates, it is unlikely that this structural difference is mechanistically significant. Attempts were made to obtain a structure for a CDO–substrate complex by soaking the crystals with cysteine or cysteamine, or co-crystallizing with cysteine or selenocysteine, but none yielded a well-ordered complex with high occupancy. However, in the crystals prepared with cysteine, changes were consistently observed that suggested the formation of a disulfide between the substrate and Cys164, which lies *ca.* 8 Å from the iron atom. A weakness of this study is that it failed to establish the oxidation state of the iron in the crystal, which was aerobically stored for long periods.

The issue of cysteine binding mode was addressed by a third crystal structure obtained using human CDO. A rather low-resolution structure (2.7 Å) was obtained for the L-cysteine complex (Fig. 2(D)).[51] The active site adopts an unsaturated distorted tetragonal bipyramidal geometry around a ferrous center. The Fe(II) ion is coordinated by the N atoms of His86, His88 and His140; superposition of the two other aforementioned crystal structures show very small r.m.s. deviations for the three conserved histidines and the metal ion. The cysteine

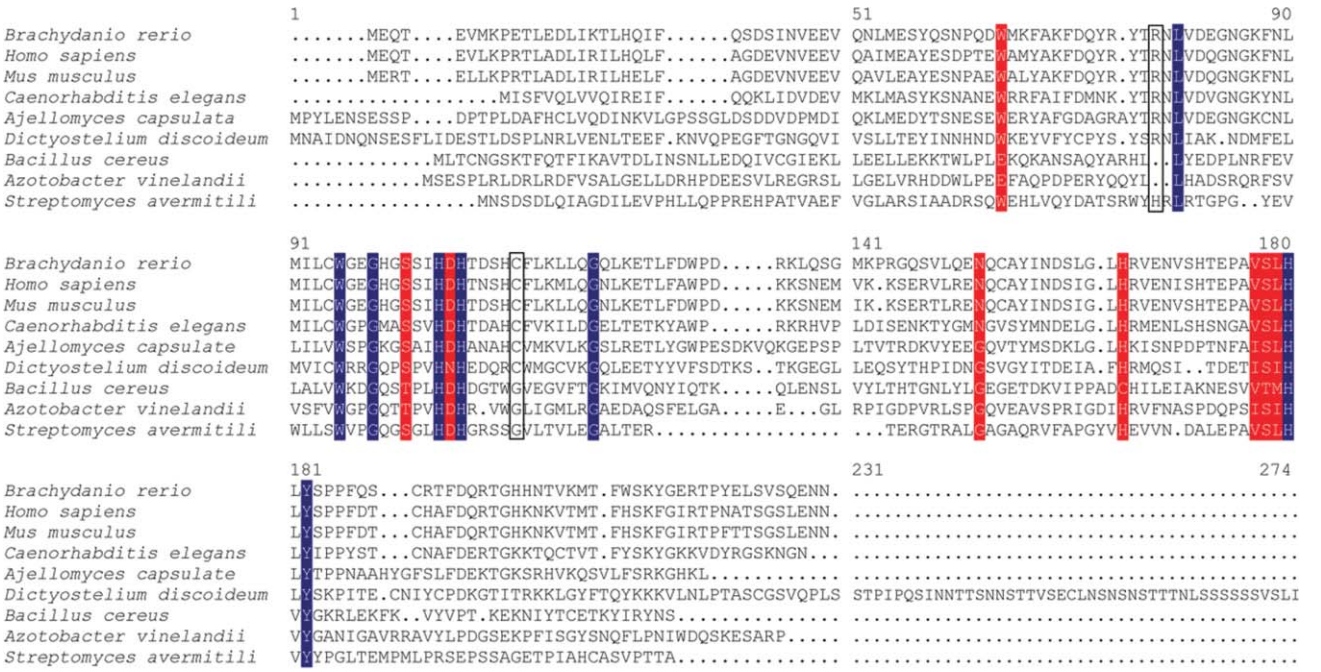

Fig. 3 Sequence alignments of CDO enzymes from eukaryotes and prokaryotes showing invariant residues in blue, conserved in red, and potentially mechanistically important residues are boxed.

substrate in the active site coordinates Fe(II) *via* its amino nitrogen (*trans* to His140) and thiolate sulfur (*trans* to His88). Although no stable water molecule or dioxygen molecule are found in the active site cavity, the bottom hydrophobic space *trans* to His86 remains open and available for another ligand. This structure also features the cysteinyltyrosine found in the other two structures.

It should be noted that there is a fourth crystal structure of CDO that was recently deposited in the PDB database but it is currently unpublished (Fig. 2(E)). The structure is that of *Ralstonia eutropha* CDO, a prokaryotic CDO. In this structure, the iron center adopts a pseudo-octahedral geometry with a sulfate ion hydrogen-bonded to the hydroxyl group of the conserved Tyr. This structure lacks the cysteinyl tyrosine found in eukaryotic CDOs due to the substitution of cysteine by glycine, a deviation found in all known prokaryotic CDOs (Fig. 3). Superposition of this structure with that of human CDO shows an overall r.m.s. deviation of 4.96 Å, lending support for the hypothesis that the cysteinyltyrosine leads to the distorted coordination found in eukaryotic CDOs.[51]

The interaction of rat CDO with active site ferrous iron and with cysteine was addressed in the author's laboratory using XAS.[43] These studies concluded that the ferrous center present in the resting enzyme was six-coordinate in frozen solution, and was consistent with ligation by three His residues and three aqua ligands, in agreement with the Ni(II) structure. Structural changes were observed upon anaerobic addition (to prevent turnover) of cysteine (enough to saturate the binding site), but no evidence for the presence of a S-donor ligand was observed. These results are summarized in Fig. 4 and show that the Fe(II) center in the resting enzyme is six-coordinate with three histidine ligands and three other O/N-donor ligands. This characterization implies that cysteine does not bind to iron *via* sulfur. However, EXAFS is dependent on the correlated motion of the absorbing and scattering atoms, and a weak interaction can cause a loss of the EXAFS arising from the disordered ligand. Such is the case for the weak Cu–S-Met92 interaction in plastocyanin.[63] In the crystal structure of the cysteine complex of CDO, the cysteine ligand exhibits a large *B* value (46.36 for the cysteine substrate, larger than the overall *B* value of 29.90), suggesting that it may not be tightly bound or that the site is only partially occupied.

Studies using NO as an oxygen mimic to probe the non-heme center in cysteine dioxygenase have provided evidence about the ability of O_2 to bind the remaining coordination position.[64] In these studies, an NO–CDO complex is formed in the presence of cysteine. EPR shows the presence of a low-spin iron nitrosyl, $[FeNO]^7$ (S = 1/2), complex with a strong resemblance to model systems[65] (Fig. 5). Based on these spectra and subsequent DFT studies, it is believed that the cysteine is bound in a bidentate fashion to the iron–nitrosyl CDO active site, consistent with the crystal structure of the cysteine complex. Similar studies have been used to provide important information about the exogenous ligands surrounding the Fe(II) center in other dioxygenases.[66,67] A number of these studies have been characterized crystallographically with NO serving as a surrogate for O_2.[68–70] An EPR study showed that the spectra of the nitrosyl adducts of 2, 3-catechol 2,3-dioxygenase was perturbed when substrates or inhibitors are added, demonstrating the formation of ES or EI nitrosyl complexes.[71] This was later confirmed by a crystal structure depicting the substrate coordinating in a bidentate fashion with the nitric oxide occupying the possible oxygen site,[70] similar to the geometry hypothesized in NO-CDO.

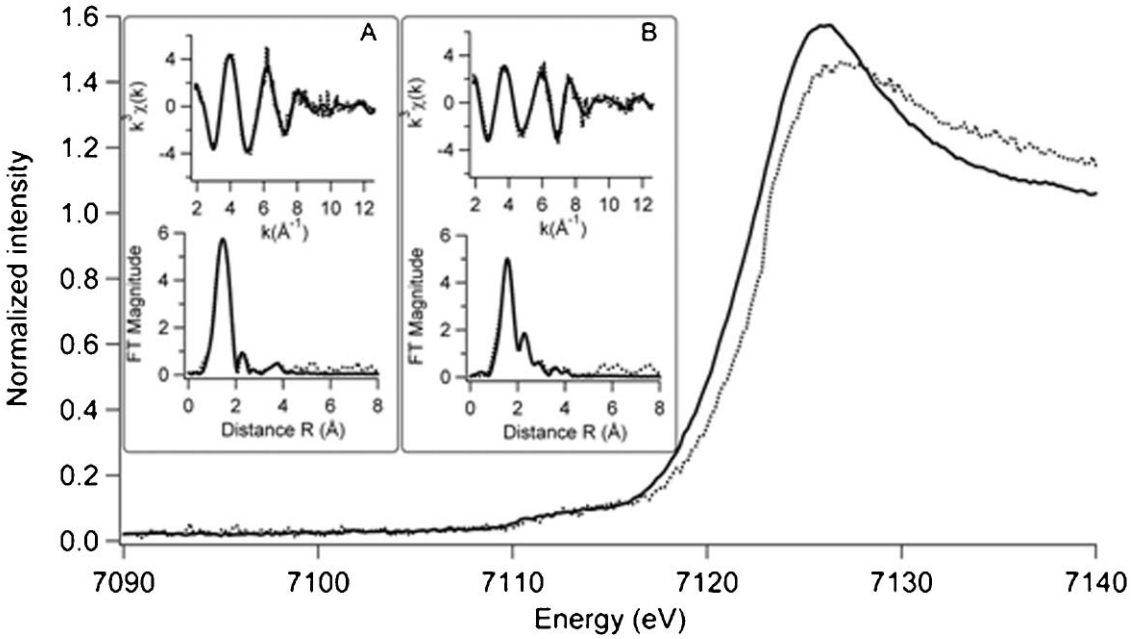

Fig. 4 Iron K-edge XANES of the resting CDO (dashed line) and ES complex (solid line). Inset (A): Unfiltered (top) and Fourier-transformed (bottom) EXAFS spectra (dashed line) of resting CDO. The fit (solid line) shown corresponds to 3 Fe–His (Fe–N 2.08 Å) + 3 Fe–O/N 1.88 Å. Inset (B): Unfiltered (top) and Fourier-transformed (bottom) EXAFS spectra (dashed line) of the CDO ES complex. The fit (solid line) shown corresponds to 3 Fe–His (Fe–N 2.17 Å) + 3 Fe–O/N 2.04 Å.

5 Proposed reaction mechanisms

A great deal of mechanistic information is available regarding non-heme iron dioxygenases and it has been extensively reviewed.[5,6,58,59,72] Non-heme dioxygenases are grouped into two classes by whether they activate the substrate for attack by O_2 or activate O_2 for attack on the substrate. Dioxygenases employing substrate activation involve coordination of the substrate to Fe(III) (*e.g.* intradiol dioxygenases such as protocatechuate 3,4-dioxygenase, and lipoxygenases), whereas those employing O_2 activation utilize Fe(II) and produce Fe(III)-peroxy species,[73] which may produce Fe(IV)=O intermediates following O–O bond cleavage.[74] In addition, the His_3 facial arrangement of protein ligands found in all four CDO crystal structures is a variation on the 2-His-1-carboxylate facial triad that is a common theme among non-heme Fe(II) dioxygenases. This protein ligand arrangement leaves three mutually *cis* positions available on the Fe(II) center for coordination of substrates, cofactors, and dioxygen. The Fe(II) dioxygenases can be further subdivided between those that require redox cofactors and those that have redox active substrates to provide electrons for O_2 activation. Among enzymes in the first group are the α-ketoglutarate (KG)-dependent enzymes (*e.g.*, taurine dioxygenase, TauD, Fig. 6).[74,75] These enzymes coordinate α-KG in a bidentate fashion *via* the keto and neighboring carboxylate groups to form a five-membered chelate ring and activate it for oxidative decarboxylation. The substrate is not bound to the Fe(II) center but is bound nearby and displaces a water molecule, thus opening a coordination site for O_2 activation. Examples of enzymes that employ redox active substrates and require no additional redox cofactor include extradiol-cleaving catechol dioxygenases (*e.g.*, catechol 2,3-dioxygenase) and isopenicillin

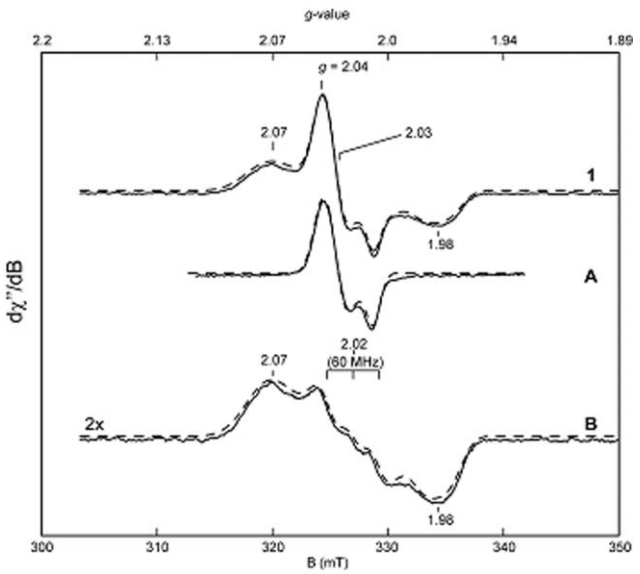

Fig. 5 Spectrum 1 (solid line) is the X-band EPR spectrum observed at 4.2 K from a pre-formed complex of CDO and L-cysteine upon NO addition. Spectrum A (solid line) is a control showing the $S = 1/2$ $[Fe(NO)_2(L)_2]$ signal ($g_{av} = 2.03$) from Fe(II), L-cysteine and NO, obtained in the absence of CDO. This control is used to differentiate between CDO's true spectra and a contaminating species commonly observed during NO additions to ferrous iron in the presence of small thiol ligands (*e.g.* cysteine). A quantitative simulation of A (dashed line) is overlaid on the data. Spectrum B (solid line) is obtained after subtracting signal A from spectra 1. This rhombic $S = 1/2$ signal is assigned to the CDO ternary complex, [Fe(Cys)(NO)]. A quantitative simulation for B (dashed line) is overlaid on the subtraction. The dashed line in 1 is a sum of simulated spectra A and B.

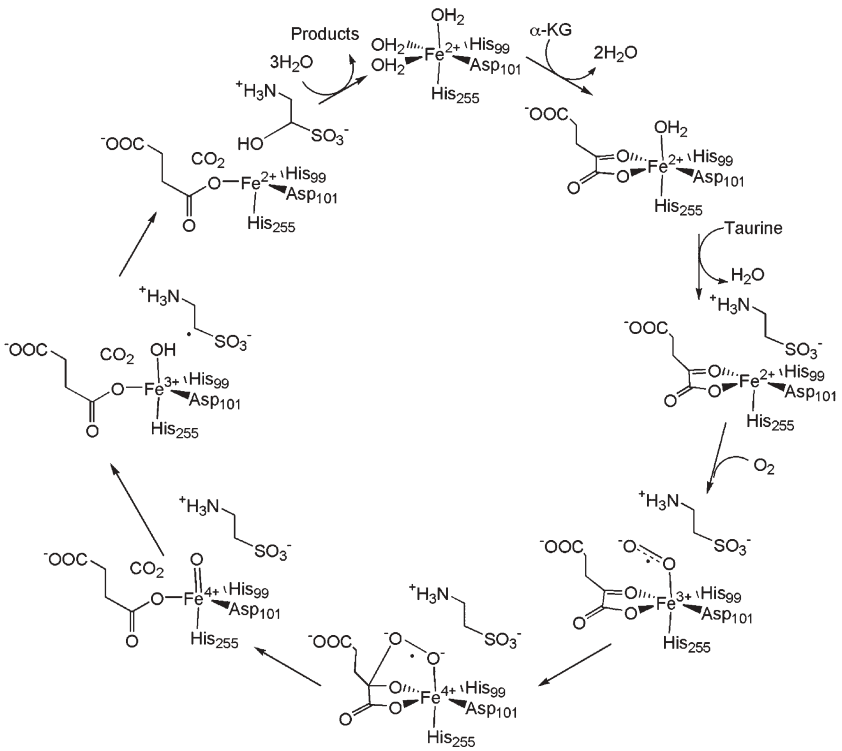

Fig. 6 Proposed reaction mechanism for TauD, adapted from ref. 74.

N synthase (IPNS), which has a substrate containing a thiol (L-α-aminoadipoyl)-L-cysteinyl-D-valine) that binds to Fe(II) as a thiolate ligand (Fig. 7).[68] The oxygen-dependent reaction of IPNS involves hydrogen atom abstraction and deprotonation to perform the bicyclization using a proposed Fe(IV)-oxo species and does not oxidize the S atom or produce a sulfoxy intermediate. The common theme that emerges is that an ordered mechanism is followed, where O_2 is activated at a five-coordinate Fe(II) center only after the substrate and cofactor (if required) are in place.

CDO does not fit into these classifications. Although it clearly does not require an additional redox cofactor, one could view the presence of a cysteinyltyrosine as an internal redox cofactor, as in the case of galactose oxidase,[60] which supplies the additional electron to produce an Fe(III)-peroxy species for catalysis. Such a possibility is reminiscent of Rieske dioxygenases,[73] where the reduced Rieske Fe_2S_2 cluster provides a second electron for the formation of the Fe(III)-peroxy intermediate. Although the cysteine involved in the cysteinyltyrosine is conserved only in eukaryotic CDOs, the tyrosine is invariant and could serve as a source of one-electron *via* formation of a tyrosyl radical. Alternatively, the substrate (cysteine) is clearly a redox active substrate and capable of providing an electron for formation of an Fe(III)-peroxy species. This could be facilitated by coordination of the cysteinate thiolate S-donor.

Site-directed mutagenesis studies were conducted to elucidate the impact of altering residues close to the catalytic center.[51] In these studies, Tyr157 was mutated to phenylalanine and resulted in diminished activity ($\sim$5% of wild type); indicating the importance of Tyr157's role in the catalytic mechanism. Mutation of Arg60 (to either glutamine or alanine) or Cys93 (to either serine or alanine) also reduced the enzymatic activity but only to $\sim$30% and $\sim$50%, respectively. It was speculated that Arg60 mutants alter the hydrogen bonding network around the active site causing instability of the cysteine during coordination. Although the Cys93 does not directly form any hydrogen bonds with cysteine, mutation of the residue would lead to the inability to form a thioether linkage, *i.e.* the cysteinyltyrosine, thus indicating the importance of the post-translational modification for activity. Recent work in the author's lab using recombinant rat CDO confirm the results obtained using the human enzyme.[76] Mutation of Cys93 (to serine) or Tyr157 (to phenylalanine) reduced activity to $\sim$57% and $\sim$8%, respectively. However, the C93S mutant's K_m (1.6 mM) is similar to that found in wild type CDO (1.8 mM) while the Y157F mutant's K_m (0.17 mM) is an order of magnitude lower than that of wild type (Table 1). The invariance in C93S's K_m indicates that the perturbation alters both the dissociation and association of substrate to a similar degree. However, the dramatic decrease in Y157F's K_m indicates that substrate dissociation, most likely due to decreased turnover to product, is being impeded by the removal of the highly conserved tyrosine, echoing the importance of Tyr157's role in the catalytic mechanism.

The use of cysteine as a redox active substrate for the formation of an Fe(III)-peroxy intermediate has given rise to four mechanistic proposals in the literature. In the first mechanism (Fig. 8),[47] the resting enzyme contains Fe(II), which binds cysteine in a bidentate fashion *via* the thiolate and the amine groups, displacing two aqua ligands. Amine ligation was favored because the carboxylate could then interact with Arg60, the only charged residue in the active site. Except for an

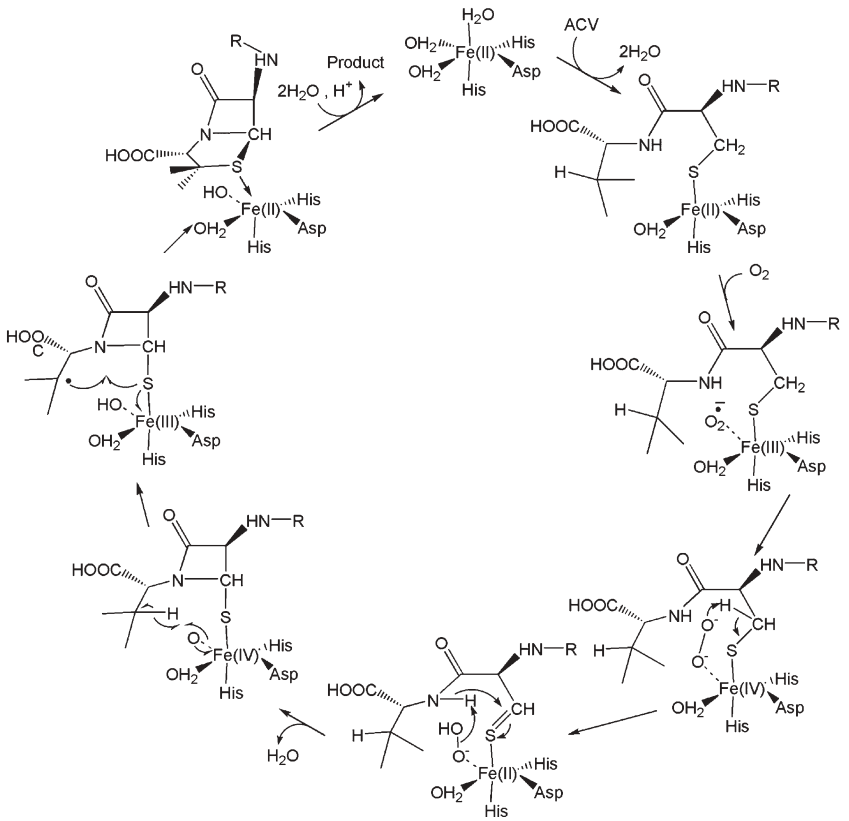

Fig. 7 Proposed reaction mechanism for Isopenicillin N synthase, adapted from ref. 75: ACV = (L-α-aminoadipoyl)-L-cysteinyl-D-valine.

extra coordinating water on the metal, this is essentially the cysteine interaction subsequently revealed by the human CDO crystal structure.[51] This requires deprotonation of the ammonium group of cysteine at neutral pH, which was proposed to be facilitated by His155 and the cysteinyltyrosine and/or by water/hydroxide. Oxidation of Fe(II) to Fe(III) accompanies oxygen binding and reduction to superoxide. Reduction of Fe(III) by the thiolate promotes reaction with the superoxide ligand to form a cyclic peroxo complex. Homolytic cleavage of the O–O bond forms a sulfoxy cation and an activated oxygen atom. This intermediate then reacts with the sulfur to form the sulfinate group. The catalytic cycle is completed by ligand substitution of cysteine sulfinate by water. Although this mechanism is consistent with a lot of structural data available, its reliance on Arg60 is a potential problem, since although it is a common residue in or near this position, it is not a completely conserved residue among the known CDO sequences and is not essential for catalysis (Fig. 3).

Table 1 Activities were determined by quantitatively measuring product formation, CSA, by HPLC employing two C18 columns, as described in ref. 44. The samples were dissolved in the mobile phase that consisted of 99.4 : 0.6 (v/v) water–methanol solution spiked with 0.3% heptafluorobutyric acid. K_m constants were found by determining activities over a range of 0–23 mM cysteine

	Activity/mol min^{-1} mol Fe^{-1}	Activity (%)	K_m/mM
WT	41.8	100	1.8
C93S	23.9	57.2	1.6
Y157F	3.4	8.1	0.17

A second mechanism does not involve coordination of the substrate to Fe (Fig. 9).[46] It is proposed that Fe(II) serves to activate oxygen by reducing it to superoxide. The Fe(III)-superoxo complex, which is stabilized by a H-bonding interaction with Tyr157, is subsequently reduced by oxidation of

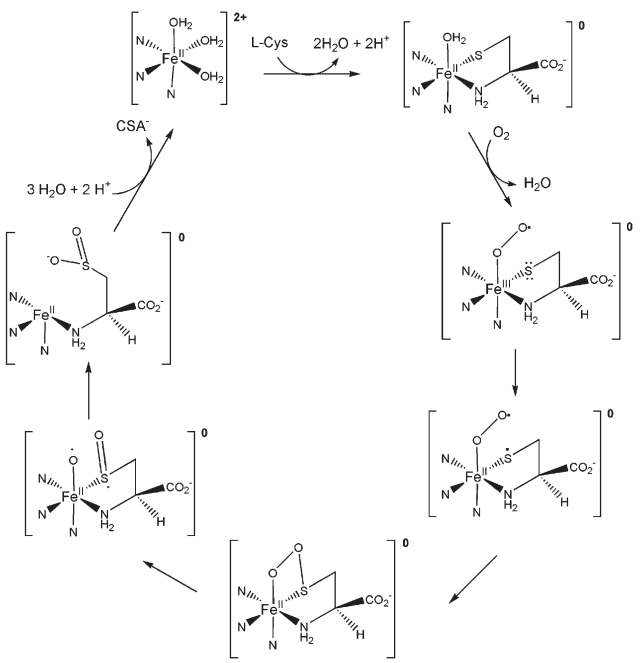

Fig. 8 Proposed CDO mechanism A, adapted from ref. 47.

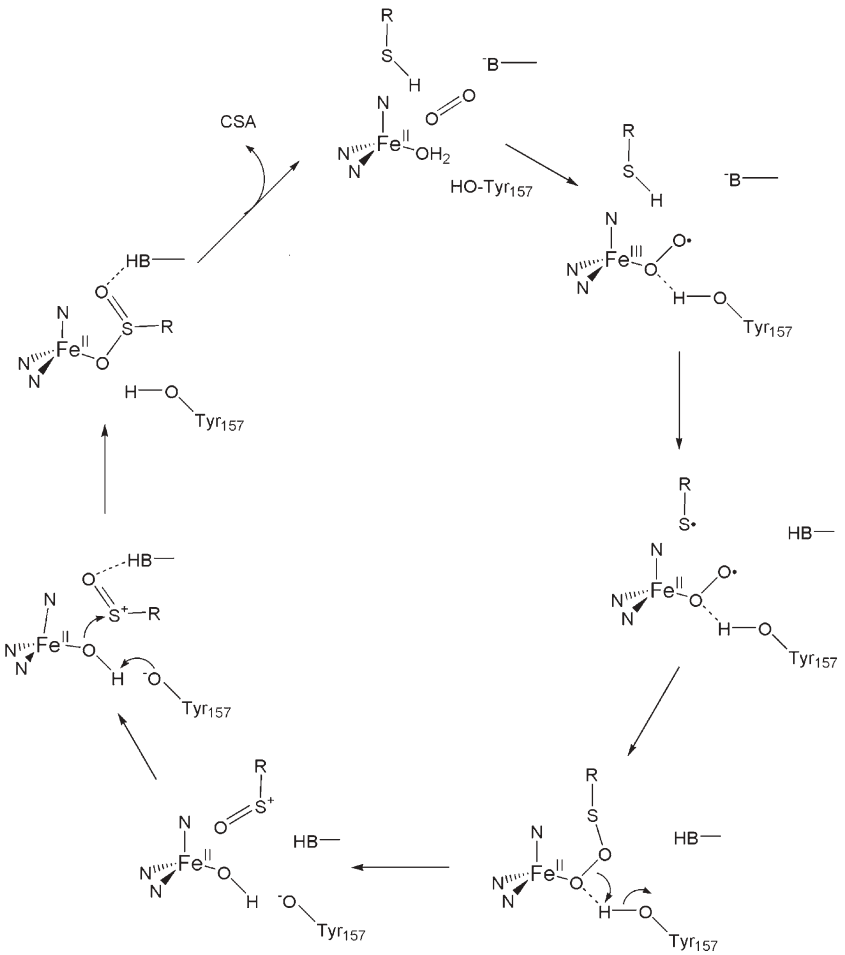

Fig. 9 Proposed CDO mechanism B, adapted from ref. 46.

the cysteine thiolate group bound nearby. A radical coupling reaction between the thiyl radical and the superoxo group bound to Fe(II) produces a sulfenyl cation and coordinated hydroxide. The sulfenyl cation undergoes nucleophilic attack by the hydroxide to yield a sulfinate complex. The catalytic cycle is completed by ligand substitution with water. This mechanism does not utilize the structure of the enzyme–substrate complex found in human CDO; does not involve deprotonation of the ammonium group of cysteine; and must rely on active site reorganization to explain the structural changes in the iron site observed upon cysteine binding by XAS. An attractive feature of this mechanism is the proposed specific role for Tyr157, the residue that is part of the cysteinyltyrosine group, which is required for significant enzyme activity.[51]

A third mechanism was postulated after the crystal structure of human CDO was available (Fig. 10).[51] In this mechanism, the enzyme's resting active site consists of a ferrous ion coordinated by His86, His88 and His140 and an oxygen atom from water, similar to the geometry found in the rat CDO crystal structure.[47] The hydroxyl group of Tyr157 is hydrogen bonded to the coordinated water. Upon addition of substrate, the water molecule is displaced by the thiol group of cysteine. The cysteine also binds the ferrous ion through its amino group. This binding geometry allows the carboxyl group of cysteine to participate in the hydrogen bonding network formed by the second coordination sphere (namely the highly conserved Tyr157, Tyr58 and His155). The dioxygen co-substrate binds in an "end-on" fashion to the vacant position on the Fe(II) center. Homolytic cleavage of the O–O bond then occurs in tandem with abstraction of a hydrogen atom from the Tyr157, forming a tyrosyl radical. The electron in the O–O bond is used to form a bond with the iron center resulting in an oxyferryl species, Fe(IV)=O. The phenoxyl radical then abstracts a hydrogen atom from cysteine's thiol. The ferryl species, a powerful oxidizing agent, can attack the lone pair on cysteine's sulfur, forming a single S–O bond. This intermediate under goes reductive elimination to form S=O and Fe(II). The sulfinic acid group is deprotonated and finally, L-CSA is released from the active site. The deprotonation of the amino group (pK_a = 10.25) in the presence of a thiol (pK_a = 8.00) is a curious aspect of this mechanism. It does, however, draw strongly on the crystal structure data and postulates a role for the required Tyr 157.

The reaction mechanisms described above have the common feature that they all utilize the Fe(II) to activate O_2. Unlike the C–H bond activations catalyzed by Fe(II) dioxygenases that involve high-valent activated oxygen species, cysteine sulfur readily reacts with O_2 in the absence of metal ions.[77] Although the product in aqueous solution is mostly the disulfide

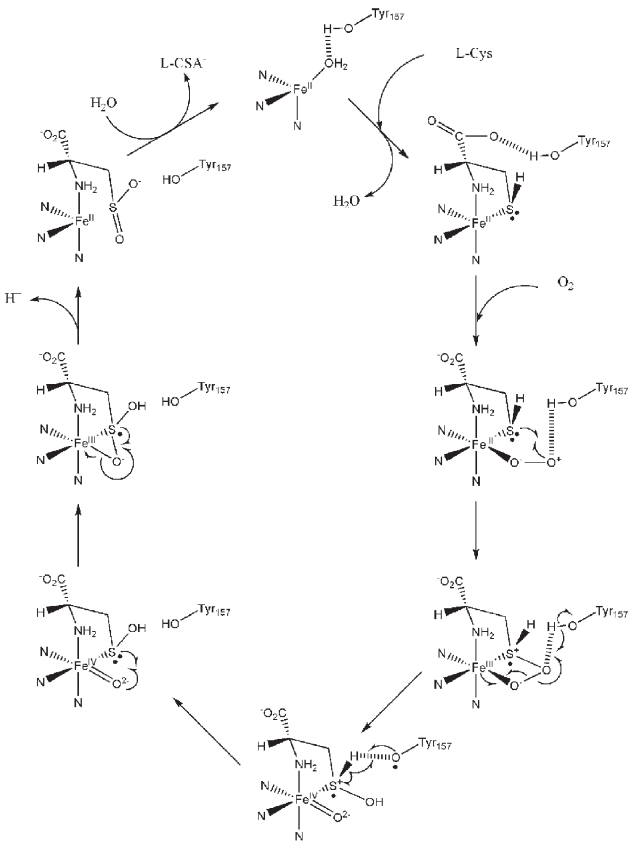

Fig. 10 Proposed CDO mechanism C, adapted from ref. 51.

(cystine), sulfur oxygenates are also formed.[78] Such species also form in transition metal complexes where disulfide formation might be impeded,[79] and in proteins.[80] The persulfoxide intermediate is produced by nucleophilic attack of the thiolate on O_2 and is in equilibrium with a dioxythiirane intermediate. This situation is similar to intermediates found in the oxidation of thioethers by $^1O_2$ and consistent with other thiolate oxidations,[81] including those of a number of Ni(II) and Zn(II) complexes.[79,82,83] Although the S-center that is oxidized is coordinated to the metals in these systems, the metals (Ni(II) and Zn(II)) are not able to reduce oxygen to superoxide. The initial product observed in the oxidation of thiolates coordinated to Ni(II) is typically the sulfinate,[79] and studies of the reaction using isotopically labeled O_2 demonstrate that the sulfinate oxygen atoms are derived from the same O_2 molecule.[81,84] Therefore, it may not be necessary to activate oxygen at the Fe center in CDO.

This situation is reminiscent of acireductone dioxygenase (ARD), where substrate oxidation is observed in the absence of the enzyme and yields the same products (keto acid and formate) as the Fe-containing form of ARD.[55] Thus, activation of oxygen is unnecessary for reaction. ARD functions with either Ni(II) or Fe(II) bound, and gives different oxidation products depending on the metal. Both metals are bound by a triad of His96, His98 and Glu102; plus His140; and two water molecules.[55] Since this Ni(II) complex is unlikely to generate reactive oxygen species, this is further evidence for the lack of O_2 activation. It appears that the protein serves to bring the reactants together and to control the regiochemistry of the oxidation.[55] The proposed mechanisms for acireductone oxidation involve substrate binding in a bidentate fashion to the metals, displacing one His ligand, followed by direct attack by dioxygen.[55,85,86]

In analogy with Fe-ARD, it is also possible that CDO operates to bring the reactants together in a state of deprotonation where they will react without much O_2 activation. In this regard, it is noteworthy that XAS shows a six-coordinate Fe(II) center in the ES complex, indicating that the complex is not poised to activate O_2 *via* a five-coordinate iron center, as in other Fe(II) dioxygenases that utilize redox-active substrates. A fourth mechanism that does not employ radical coupling to produce a thioperoxide is shown in Fig. 11. This mechanism features cysteine initially binding to the iron center by its amine and thiolate groups, a structure consistent with the crystal structure of the CDO–cysteine complex. Although this is not consistent with EXAFS data, which is devoid of evidence of sulfur ligation, it should be reiterated that a weak interaction can cause a loss of the EXAFS arising from the disordered ligand, sulfur in this case. This weak interaction is exploited in the next step where S-donor is displaced in the oxygen complex with the aid of Tyr157, initially serving as a stabilizing residue *via* hydrogen bonding. Oxidation of the S atom then occurs *via* nucleophilic attack on the O_2 ligand. In this mechanism, the iron center is proposed to be principally a site for organizing the reactants and breaking down the spin-forbidden nature of the reaction with O_2 (a ground state triplet) rather than playing a major role in activating oxygen by reducing it to superoxide.[87] This is suggested by the fact

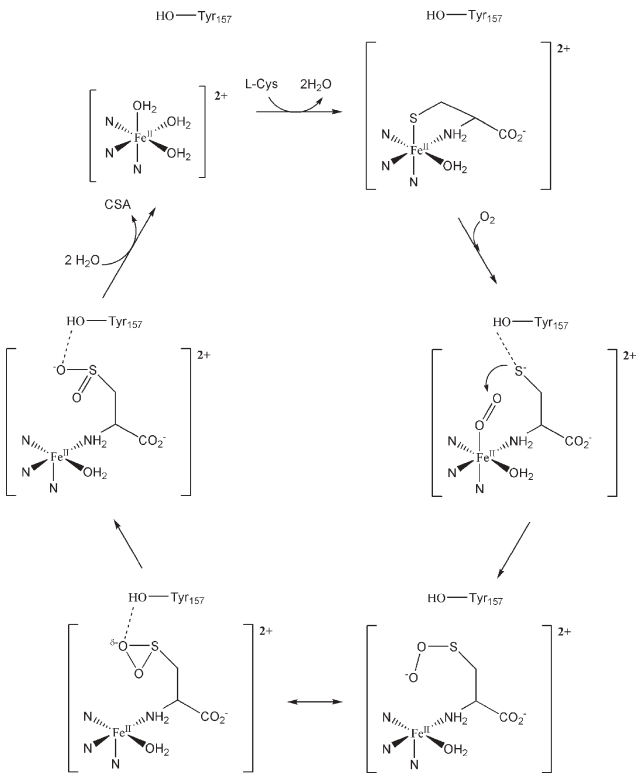

Fig. 11 Proposed CDO mechanism involving direct oxidation of sulfur.

that Fe(II) in CDO with all histidine ligation and a positive overall charge is less electron rich than the Fe(II) centers in many other non-heme Fe(II) dioxygenases, or for that matter in hemoglobin/myoglobin, and thus is expected to be harder to oxidize to Fe(III) to produce superoxide.

Another shortcoming of the mechanisms shown in Figs. 8–10 is that none of them can account for the dramatic increase in activity (*ca.* 20 fold) observed in the presence of cysteamine (2-aminoethanethiol). CDO is specific for cysteine oxidation, and no other known thiol has been shown to be a substrate.[43] Cysteamine was found to greatly *enhance* cysteine oxidation while not being a substrate (no oxidation product from cysteamine was detected by HPLC).[43] One possibility is that the complex with an Fe–S bond revealed by the crystal structure of human CDO is not the complex that undergoes oxidation, rather the thiolate S atom must be unbound to react with O_2 as in the mechanism shown in Fig. 11. Addition of cysteamine might provide an additional ligand for the Fe(II) center which would allow a higher concentration of unbound cysteine thiolate.

A curious feature of the mechanism shown in Fig. 11 is why a known redox cofactor (cysteinyltyrosine) would be used as an H-bond donor. It is noteworthy that neither the cysteine nor the tyrosine residues are absolutely required for activity, as might be expected for a redox cofactor. However, mutation of Tyr157 leads to a major deactivation. Furthermore, as already noted, the cysteinyltyrosine is not universally conserved. In addition, the same concerns about the deprotonation of cysteine (in this case the carboxylate, ammonium group and thiol would all be deprotonated at pH 7) raised in regard to the mechanisms shown in Fig. 8 and 10 apply as well to the mechanism shown in Fig. 11. In this regard, it is also possible that cysteine would coordinate as a thiol ligand, which would further weaken the Fe–S interaction, but require deprotonation to generate a base of sufficient strength to attack the O_2 ligand.

6 Conclusions

CDO's iron center plays an essential role in catalysis and despite its departure from the 2-His-1-carboxylate paradigm found in other non-heme Fe(II) dioxygenases, it is still able to facilitate the oxidation of cysteine efficiently. Structural biological techniques, such as X-ray crystallography and XAS, have been critical tools for obtaining molecular level insights into the active site geometry for a catalytic center that does not contain many spectroscopic handles. Although these structures have given rise to differing proposed mechanisms, it is clear from kinetic studies that the role of Tyr157 is an important one. The lack of conservation of Cys93 in many bacterial CDOs and the reduced activity found when this residue is mutated suggests that the thioether linkage, although beneficial for catalysis, is not crucial for activity. Discrepancies still exist about the nature of substrate binding and its elucidation will be essential to disprove contradictory mechanisms.

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