An Insight into the Mechanism of Human Cysteine Dioxygenase

KEY ROLES OF THE THIOETHER-BONDED TYRISOINE-CYSTEINE COFACTOR

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Cysteine dioxygenase is a non-heme mononuclear iron metalloenzyme that catalyzes the oxidation of cysteine to cysteine sulfonic acid with addition of molecular oxygen. This irreversible oxidative catabolism of cysteine initiates several important metabolic pathways related to diverse sulfate compounds. Cysteine dioxygenase is therefore very important for maintaining the proper hepatic concentration of intracellular free cysteine. Mechanisms for mouse and rat cysteine dioxygenases have recently been reported based on their crystal structures in the absence of substrates, although there is still a lack of direct evidence. Here we report the first crystal structure of human cysteine dioxygenase in complex with its substrate l-cysteine to 2.7 Å, together with enzymatic activity and metal content assays of several single point mutants. Our results provide an insight into a new mechanism of cysteine thiol dioxygenation catalyzed by cysteine dioxygenase, which is tightly associated with a thioether-bonded tyrosine-cysteine cofactor involving Tyr-157 and Cys-93. This cross-linked protein-derived cofactor plays several key roles different from those in galactose oxidase. This report provides a new potential target for therapy of diseases related to human cysteine dioxygenase, including neurodegenerative and autoimmune diseases.

Cysteine dioxygenase (CDO, EC 1.13.11.20) is a non-heme mononuclear iron metalloenzyme that catalyzes the irreversible oxidation of cysteine to cysteine sulfonic acid (CSA) with addition of molecular oxygen (1) (Structure 1). This oxidative catabolism of cysteine initiates several important metabolic pathways related to pyruvate and several sulfurate compounds, including sulfate, hypotaurine, and taurine. CDO is expressed at appreciable levels in the brain, kidney, and lung, with extremely high levels in liver tissue (2–5), where CDO plays an important role in maintaining the hepatic concentration of intracellular free cysteine within a proper narrow range (6). When the levels of cysteine decrease below this range, the increase of CDO ubiquitination rate results in rapid degradation of the ubiquitinated portion by the 26 S proteasome system (7, 8). However, the precise means by which cysteine regulates CDO ubiquitination remain unknown.

Intracellular free cysteine is cytotoxic and neuroexcitotoxic due to oxidative damage via formation of free radicals in the presence of iron (9–11). Elevated cysteine levels were reported previously in relation to several neurodegenerative diseases, including the well known Parkinson and Alzheimer diseases (12–14), and autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (15, 16). CDO is considered to be involved in these diseases due to its function in regulating free cysteine levels.

Sequence alignment classifies CDO as a member of the cupin superfamily (see Fig. 1), whose members possess what may be the most diverse range of functions, encompassing ~18 sub-classes. Nonetheless, neither of the exact characteristic conserved sequences, GX₄HXHX₃₋₄EX₆G or GD₆X₃PXGX₃₋₄HX₃₋₄N (17), is found in CDO, i.e. glutamic acid in Motif 1, glycine, aspartic acid, and proline (highlighted in blue to distinguish from the characteristic conserved residues in red in Fig. 1) in Motif 2 are substituted. Recently reported CDO structures from mouse and rat confirm the cupin fold (18, 19). In fact, recent structural studies of cupin proteins clearly demonstrate that the primary sequences of cupin motifs are not highly conserved, as was first believed (20).

Besides CDO, the cupin superfamily includes several other dioxygenases from all three kingdoms, including pirin that was first characterized structurally by our group (21, 22) and was shown to possess quercetin dioxygenase activity by Adams and colleagues (23). Distinct from the other dioxygenases, CDO catalyzes an unusual oxidation reaction in which two oxygen atoms are added to a single atom without C–C bond cleavage in the substrate.

CDO has been thoroughly studied functionally since it was first reported by Ewet and colleagues (24). In addition to mammals, CDO was also found in other eukaryotes and prokaryotes by genome analysis (25). Although several mechanisms of CDO have recently been proposed on the basis of structural analysis or x-ray absorption spectroscopy results (18, 19, 26), there is no direct structural evidence to date of the manner by which substrates bind to CDO.
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In the case of eukaryotic CDOs, the highly conserved glutamic acid in cupin motif 1, mentioned above, is substituted by a cysteine, the thiol sulfur of which forms a thioether bond with the Cε of a nearby tyrosine residue (18, 19). This rare Tyr-Cys adduct is termed “cross-linked protein derived cofactor” and was first reported in the crystal structure of galactose oxidase in 1991 (27).

Due to activation by the hydroxyl group on the phenol ring, it is easier for the ortho hydrogen atom to be substituted than in the absence of the hydroxyl group. In known protein structures, lysine and histidine are also found to be covalently bonded to tyrosine at the same ortho position (28–30). Theoretical calculations showed similar chemical properties between different cross-linked protein derived cofactors involving tyrosine (30). Thus, it is reasonable to believe that Tyr-Cys and Tyr-His may share similar functions.

To study the detailed mechanism of CDO, we co-crystallized CDO and its natural substrate, l-cysteine, and solved the structure of this complex. Based on preliminary structural analysis, several mutants of conserved residues were constructed to perform catalytic assays and detection of metal content. This work suggests a new mechanism for CDO activity, and some key roles for the Tyr-Cys cofactor in catalysis.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of Human CDO and Mutants**—Full-length Homo sapiens CDO and mutants, including R60Q, R60A, C93S, C93A, Y157F, C164S, and C164A, were cloned into the pGEX-6p-1 expression vector (GE Healthcare) and expressed in Escherichia coli BL21(DE3) (Novagen). Oxygen mixed in culture by vigorous shaking is beneficial for cell culture but harmful for maintaining the ferrous state of iron. Because ferric ions cannot be coordinated by the CDO active site, we added exogenous iron just before lysis instead of induction as reported in previous studies (18, 31). The harvested pellets were resuspended with 1 mM FeSO₄ and immediately lyzed by sonication to release glutathione S-transferase (GST)-fused CDO and mutants, which were purified following standard GST fusion protein purification protocols. The GST tag was removed by cleavage with PreScission Protease (GE Healthcare) during the purification of the GST-fused CDO. The liberated native CDO and mutants were further purified by gel-filtration chromatography in 20 mM sodium-4-morpholineethanesulfonic (Na-MES), 50 mM NaCl (pH 6.5) and were ultrafiltered to the required concentration for subsequent catalytic assays, detection of metal content, and crystallization. Wild-type CDO and mutants each showed a single peak in gel-filtration chromatography representing a monomeric protein, but Y157F showed an additional peak with an estimated molecular weight equivalent to a CDO dimer. The monomer form and the dimer form of Y157F were separated as two distinguished samples for further analysis.

**Crystallization**—For co-crystallization experiments, l-cysteine was added to the purified wild-type CDO solution (20 mM MES, 50 mM NaCl, pH6.5) to a final concentration of 20 mM, as well as FeSO₄ to 0.25 mM. A 1-µl aliquot of the enzyme solution (10 mg/ml) was mixed with 1 µl of reservoir solution containing 0.1 M Na-cacodylate, 0.2 M MgCl₂, and 17.6% polyethylene glycol 8000 (pH 6.5) and crystallized by the hanging drop, vapor-diffusion method at 16 °C. The crystals grew as rods up to 0.2 × 0.2 × 0.8 mm in 1 week. After soaking in a cryoprotectant containing reservoir solution plus 10% glycerol for 1 min, the crystal was flash-frozen by liquid nitrogen and stored also in liquid nitrogen for future data collection using synchrotron radiation.

**Data Collection, Phasing, and Model Refinement**—Crystallographic data for the complex crystal was collected with an SAC2 (3k × 3k) charge-coupled device detector at beamline BL19-ID of the Advanced Photon Source (Argonne, IL) at 100 K. All x-ray diffraction intensity data were integrated, scaled, and merged using HKL2000 (32). Because other CDO structures with high homology have been reported, molecular replacement performed with CNS (33) was employed for phasing using the crystal structure of mouse CDO as a starting model (Protein Data Bank (PDB) entry 2ATF) (18). The final model was manually adjusted in O (34) and refined with CNS.

**Mass Spectroscopy**—Inductively coupled plasma mass spectrometry was carried out for metal content determination of the wild-type CDO and mutants. Inductively coupled plasma mass spectroscopy experiments were performed at the Analysis Center of Tsinghua University (Beijing, China).

**Catalytic Assay**—l-Cysteine powder was dissolved into the same buffer as used for gel filtration. Freshly prepared human CDO solution and l-cysteine solution were incubated in a 37 °C water bath for 10 min. Appropriate amounts of these two solutions were well mixed to initiate the reaction in a 37 °C water bath. Details of the final concentrations in different experiments are given in Fig. 4. After a specified amount of time, the reaction was terminated by adding 0.5% (v/v) heptfluorobutyric acid. All aliquots were immediately ultrafiltered through Microcon (Millipore, Bedford, MA) 10,000 kDa molecular weight cut-off ultrafiltration tubes. The flow-through was kept in an ice water bath for further high-performance liquid chromatography analysis.

**Determination of the catalysis product**. l-CSA for cysteine were separated as two distinguished samples for further analysis.

Curves of initial reaction velocity versus substrate cysteine starting concentration were fitted to a Hill function with the Hill coefficient designated as 1.0 in OriginPro (OriginLab, Northampton, MA). Values of $K_m$ and $k_{cat}$ for cysteine were subsequently estimated from the curves.
In experiments conducted in the presence of inhibitors, the same reaction conditions were maintained for comparability. We used the same method for the mutagenesis catalytic assays with all eight mutant CDOs, including the Y157F dimer, at the same protein concentration of 0.031 mg/ml as wild-type human CDO.

FIGURE 1. Multiple sequence alignment of CDOs. From top to bottom, the sequences are from *H. sapiens* (NP001792), *Rattus norvegicus* (NP434696), *Mus musculus* (NP149026), *Bos taurus* (ABF37430), *Xenopus tropicalis* (CAJ81896), *Cyprinus carpio* (BAE73112), *Acetobacterium* (ABF41688), *Candidatus Kuenenia stuttgartiensis* (CAJ71302), and *Cupriavidus necator* (AAZ64393). Highly conserved residues are highlighted in red; similar residues are in green; the special residues Cys-93 and Cys-164 are in blue. The secondary structure of human CDO is indicated in the top line, as well as the cupin superfamily motifs in the bottom line. Among the characteristic conserved residues of cupin motifs, conserved ones are colored in red, whereas substituted ones are colored in blue. Sequences were aligned using ClustalW, and the alignment was represented using ESPript.

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Table 1

| Crystallographic data and refinement statistics |
|-----------------------------------------------|
| **Crystalllographic data**                    |
| Space group                                   | P6₃            |
| Unit cell                                     |               |
| a (Å)                                        | 131.0         |
| b (Å)                                        | 131.0         |
| c (Å)                                        | 34.2          |
| Wavelength (Å)                                | 0.97932       |
| Resolution range (Å)                         | 50-2.7        |
| Computed reflections                         | 50,235        |
| Unique reflections                            | 9,575         |
| Redundancy                                    | 5.3           |
| Rmerge                                        | 0.150 (0.355) |
| Mean l(r)/()                                  | 11.7 (2.68)   |

| Refinement statistics                         |
| Reflections in working set                   | 8,161         |
| Reflections in test set                      | 901           |
| R cryst                                      | 0.178         |
| Rfree                                        | 0.215         |
| r.m.s.d. bonds (Å)                            | 0.016         |
| r.m.s.d. angles ()                            | 1.9           |
| Average B-factor (Å²)                         | 297.0         |

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RESULTS

Structure Determination and Refinement—Initial analysis of the collected x-ray diffraction data indicated alternative possibilities of space groups P6₃ and P6₃. Molecular replacement calculations confirmed the latter space group, P6₃, as the correct one for the crystal of human CDO in complex with the substrate cysteine. The resulting electron-density map was continuous and interpretable. This space group differs from the space group of the mixed β-barrel of the CDO crystal in the PDB (entry 2C1B).

Overall Structure—Human CDO comprises four helices (blue in Fig. 2A), including two α-helices (α1 and α2), one single-turn 310 helix (α4), one mixed helix with half α-helix and half 310 helix (α3), and thirteen β-strands that form two antiparallel β-sheets (yellow and brown in Fig. 2A) and one mixed β-sheet (red in Fig. 2A). As a member of the cupin superfamily, CDO possesses two β-strands, β3 and β4, corresponding, respectively, to βC and βD of cupin motif 1, as well as β7 and β8 to βG and βH of cupin motif 2, respectively. The structurally conserved cupin β-barrel is constituted by all three β-sheets, by which the active center at the chelated Fe²⁺ is surrounded. Helices α1 and α2 are parallel to each other, and both lie perpendicular to α3. These three helices pack against the outside of the mixed β-sheet of the CDO β-barrel.

Distinct from the general antiparallel β-barrel, with retinol-binding protein as a representative example (34), cupins share a barrel with an axis perpendicular to the direction of the β-strands that construct this barrel. For CDO, a cavity is formed along this axis from the protein surface to the coordinated ferrous ion at the active site (Fig. 2B). Calculated electrostatic potential distribution around the cavity shows a positively charged outer surface and a negatively charged inner surface.

A high structural similarity among human CDO, mouse CDO, and rat CDO was observed. The latter two share the same amino acid sequence. With a primary sequence identity of 92%, crystal structures of human CDO and mouse CDO (PDB entry: 2ATF) share an r.m.s.d. of 0.74 Å for main-chain Cα atoms from Val-6 to Pro-190, as well as 0.72 Å between human and rat CDO (PDB entry: 2B5H) for the same region. Superposition of the backbones of human, mouse, and rat CDO reveals that the core regions around the active sites are more similar than the peripheral regions (Fig. 2C). Additionally, all heterogeneous residues among the three CDOs are distributed away from the active sites.

Besides the cysteine substrate at the active site, another solution cysteine is included in the final model, which lies under the bottom of the β-barrel and is hydrogen-bonded to Arg-123, Gln-129, Tyr-132, and several waters. High B-factors for this cysteine molecule, resulting from a loose interaction with the enzyme surface, suggests its non-functionality in the thiol sulfur oxidation catalyzed by CDO.

Active Site—The active site of human CDO is located at the Fe²⁺ ion coordinated by the Ne atoms of His-86, His-88, and His-140. All four atoms share similarly low B-factors (17–20 Å²), indicating high occupancy of the ferrous ion and stable conformation of this region. By contrast, the cysteine substrate in this active cavity, which coordinates Fe²⁺ via its amino nitrogen (trans to His-140) and thiol sulfur (trans to His-88), exhibits comparatively high B-factors (40–45 Å²). However, a clear difference Fourier electron-density map close to the metal center.
offers strong support for the reliability of this cysteine substrate that is additionally hydrogen-bonded to Arg-60, Tyr-58, Tyr-157, and His-155 in addition to coordination to the ferrous ion (Fig. 3A).

The whole active site is sunk in a deep cavity under the protein surface. To clearly represent this cavity space, a cutaway stereo view from opposite directions is shown in Fig. 3 (B and C). In our crystal structure of human CDO in complex with cysteine, no stable water molecules or dioxygen molecules are found in the active cavity, although the bottom hydrophobic space of the cavity, trans to His-86-Ne, remains open and available for another ligand. In our proposed CDO mechanism, this site is to be occupied by the second substrate, molecular dioxygen.

Distinct from all metal ion coordination modes of CDOs reported previously (18, 19), our work uncovered an unsaturated distorted tetragonal bipyramidal ferrous center with a vacant coordination site. To assess the differences in coordination geometry between human CDO, mouse CDO, and rat CDO, we measured the bond angles between the coordination bonds involving His-86, His-88, and His-140. The results showed that the bond angles are similar, especially between human CDO and rat CDO (9°), both of which possess iron as the bound metal. In addition, the bond angles deviate from either octahedral or tetrahedral geometry. All of this evidence implies an alternate coordination mode at the active site of CDO.

After preliminary refinement of our model, abnormally strong difference Fourier electron density was observed in two regions close to the active site. One region lies between Cys-93-S and Tyr-157-C, and suggests a covalent bond between these two atoms, which is consistent with the observations by Simmons and McCoy in the rat CDO and mouse CDO structures (18, 19). The bond angles around the sp² carbon in the thioether bond were loosely restricted to 120° during refinement, and the C–S bond distance was restricted to 1.8 Å based on the thioether bond in galactose oxidase (27).

Another region is located close to the thiol group of Cys-164 and indicates a covalent modification involving Cys-164 (Fig. 3A). Because of its triangular shape, cysteine sulfenic acid, the product of the reaction catalyzed by CDO, was used to substitute Cys-164 to match this strong density. However, subsequent refinement failed due to unreasonably high B-factors of the two additional carboxylic oxygen atoms. Therefore, we had...
no alternative but to leave this region un-modeled in our final structure. Similarly undefined strong density against Cys-164-Sγ was reported in the rat CDO structure, which was co-crystallized together with cysteine (19).

Activity Assay at the Level of Seconds—Several different methods for assaying CDO activity have been reported (1, 18, 31, 35, 36), all of which determined the enzymatic activity over a time of minutes. Our efforts to accurately control catalytic time improved the activity assay level to the order of seconds. The results show a typical enzyme catalytic reaction progress curve with a limit of ~0.2 mM (Fig. 4A), indicating an obvious decline of substrate after ~60 s from the start of the reaction. In our reaction system for CDO, the cysteine substrate is at saturation concentration, whereas the oxygen substrate is not. The aqueous solubility of oxygen at 37 °C is ~0.2 mM, which is close to the limit of the CDO reaction progress curve. Because the cysteine thiol oxidation catalyzed by CDO is a dual substrate reaction, the Michaelis constant $K_m$ for one substrate (cysteine) should be determined at the condition at which the other substrate (oxygen) is saturated. Therefore, the data collected within the initial 60 s were used to calculate the initial velocity (Fig. 4C). We determined a $K_m$ of 3.1 ± 0.3 mM with a $k_{cat}$ of 1.7 s$^{-1}$ that is remarkably higher than the $k_{cat}$ for mouse CDO (0.06 s$^{-1}$) or rat CDO (0.72 s$^{-1}$) reported previously (18, 31, 35).

To compare with previous work on CDO catalytic activity, assays over a period of minutes were also carried out and resulted in the linear plot in Fig. 4B. The data represent a steady state arising from two processes involving dioxygen molecules. One is the consuming velocity related to sulfur dioxygenation. The other is dissolving velocity related to air condition and liquid surface area exposed to air. For different experimental environments, the latter velocity may be significantly different, leading to different steady states with different reaction velocities. However, for a series of experiments with the same conditions, a limited dissociation constant for CDO-cysteine can still be measured, as has been reported previously. To distinguish between the real $K_m$ and $k_{cat}$, a prime mark was used as follows: $K_m'$ 8.4 ± 0.3 mM and $k_{cat}'$ 0.19 s$^{-1}$ (Fig. 4D). Both values are worse than those determined under saturating oxygen conditions but comparable with values reported before (18, 31, 35).
Inhibition of human CDO activity by homocysteine and EDTA was also studied and led to the curves in Fig. 4 (E and F, respectively). As a competitive inhibitor, homocysteine cannot efficiently block the active site, and so the CDO activity was reduced by 50% only when the molar ratio of homocysteine:CDO reached $\frac{1}{30,000}$:1. By contrast, an EDTA:CDO molar ratio of $\frac{1}{1,000}$:1 abolished CDO enzymatic activity. This is consistent with the importance of the ferrous ion in the catalytic mechanism.

Metal Incorporation of Wild-type Human CDO—CDO is a non-heme iron-containing metalloenzyme whose activity depends on proper coordination of the ferrous ion in the active center. Our inductively coupled plasma mass spectroscopy analysis of purified recombinant wild-type human CDO yielded an iron incorporation of $\sim 68\%$, which is much higher than 10% or 25% reported by other groups (18, 19, 31, 35). Besides iron, other metal elements were detected in purified recombinant CDO, including zinc (18.1%) and trace amounts of magnesium (0.41%) and manganese (0.25%) (Fig. 5A). Nickel was not detected in our sample. The active centers of the remaining 13.4% CDO molecules are occupied by other unknown metals or are simply vacant.

Catalytic Efficiency and Metal Incorporation of Mutants—To study the functional importance of amino acid residues located around the putative active center, seven single point mutations in human CDO were made, including R60Q, R60A, C93S, C93A, Y157F, C164S, and C164A, to examine their role in enzymatic activity and metal incorporation. Because mutations of the same amino acid residue (e.g. R60Q and R60A) gave similar results either in catalytic analysis or in metal detection, only data for the R60Q, C93S, Y157F, and C164S mutants, together with the wild type, are shown in Fig. 5B. Because both the monomer and dimer of Y157F displayed very similar results, only the data for Y157F monomer are represented in Fig. 5B.

Among the seven mutants, mutation of Tyr-157 to phenylalanine diminished enzymatic activity to the lowest level ($\sim 5\%$), indicating that Tyr-157 plays very important roles in the catalytic process. Mutation of Arg-60 or Cys-93 also remarkably reduced the activity to $\sim 30\%$ and $\sim 50\%$, respectively. In the case of Arg-60, its absence may cause instability of the cysteine substrate binding and a consequent reduction in enzyme activity. In contrast, Cys-93 does not directly form covalent, coordination, or hydrogen bonds with the substrate or metal center. The decreased activity of Cys-93 mutants must therefore be related to the lack of thioether bond formation involving Cys-93.

Simmons and colleagues (19) proposed that Cys-164 may inhibit CDO activity via its adduct with an unknown molecule. Although similar electron density appears against the thiol...
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A.

- Metal content of wild-type human CDO: Iron occupies 67.8%, which is higher than previously reported.
- Activity ratio of wild-type (WT) human CDO and mutants.
- Iron and zinc content and relative activity of wild-type (WT) human CDO and mutants, including R60Q, C93S, Y157F, and C164S.

B.

- Metal incorporation over WT.
- Activity of wild-type (WT) human CDO and mutants.

Group of Cys-164 in our human CDO structure in complex with cysteine, mutation of Cys-164 showed a ~20% abatement of enzymatic activity, instead of enhancement.

Mutation of Arg-60, Cys-93, and Tyr-157, the residues close to the catalytic center, also affected the metal incorporation of CDO, whereas Cys-164 did not. Much higher levels of other divalent metals, mostly zinc, were detected in the purified CDO mutants, especially those with substitutions of Cys-93 or Tyr-157, which share similar zinc contents of ~45%.

In the case of Cys-93, the specific activity of Cys-93 mutants appeared to be exactly proportional to the measured iron content relative to the wild type (both to 50% of the wild type), indicating that reduction of the iron content is the only reason for the decreased activity of Cys-93 mutants.

In the case of Arg-60, both the iron and zinc content decreased. It is necessary to point out that reduction of the iron content is only part of the reason for the diminished activity of Arg-60 mutants. For example, R60Q lost ~55% coordinated iron but lost ~70% activity.

DISCUSSION

Comparison with Other Cysteine Dioxygenases—To understand how the active site confers its function, it is necessary to compare human CDO with other cysteine dioxygenases reported previously, especially at the active center (Fig. 6A).

The source of the first crystal structure of CDO was mouse, which was reported by McCoy and co-workers (18). In addition to His-86, His-88, and His-140, three water molecules (Wat160 through Wat162) were found around the nickel ion, forming a distorted tetragonal bipyramidal geometry (Fig. 6C). Nevertheless, two of the three water molecules (Wat161 and Wat162) are thought to be hydrogen-bonded because of their long distances from the metal center (19).

The structure of rat CDO was then reported by Simmons and colleagues (19). An iron center was observed, coordinated by only one water molecule (Wat4) in addition to the three absolutely conserved histidine residues (Fig. 6D).

Superposition of the two above CDO structures and our structure of human CDO show very small r.m.s.d. values for the three conserved histidine Ne atoms and the metal ion (0.18 Å between human and mouse CDOs; 0.10 Å between human and rat CDOs). An unanticipated similar pattern is shared by these four atoms irrespective of the four-, five-, and six-coordination state. This is an example of the entactic state of proteins and enzymes whereby the rigidity of the protein structure imposes unusual coordination environments on a metal center for optimal activity.

It is interesting that the water molecule (Wat-160) closest to the nickel ion in mouse CDO (Fig. 6C) occupies nearly the same position as the unique coordinated water (Wat-4) in rat CDO (Fig. 6D). These waters do not appear at the formal coordination site of the nickel ion in mouse CDO (Fig. 6C) occupies nearly the same position as the unique coordinated water (Wat-4) in rat CDO (Fig. 6D).

A distance between the distal oxygen atom and Tyr-157 hydroxyl group is measured as 2.3 Å with the bond distance of O-Fe designated as ~2 Å and the bond angle Fe-O-O as 106° (37, 38). The strong hydrogen bond to the distal oxygen should enhance π* back-donation from the Fe2+ to the dioxygen π* orbital to favor the ferric-superoxide structure.

Recently, a new crystal structure of Cupriavidus necator CDO was deposited in the PDB but remains unpublished (PDB entry 2GM6). Primary sequence alignment shows an identity of 18.1% and a similarity of 35.2% with human CDO. Superposition of these two structures shows greater differences than between human and mouse or human and rat, with an overall r.m.s.d. of 4.96 Å (Fig. 6F). The iron center adopts a near-octahedral coordination involving an SO42- ion hydrogen-bonded to the hydroxyl group of Tyr-164, the counterpart of Tyr-157 in human CDO (Fig. 6E). However, the C. necator CDO does not have the Tyr-Cys cofactor in human CDO due to the substitution of the cysteine by a glycine (Fig. 1). Therefore, this structure provides powerful evidence to support the hypothesis that the thioether-bonded Tyr-Cys cofactor leads to the distorted coordination in Eukaryotic CDOs.
Key Roles of the Tyr-Cys Cofactor—In the first known protein structure involving a Tyr-Cys cofactor, galactose oxidase, the cooper center is directly coordinated by the tyrosyl oxygen of the Tyr-Cys cofactor. This thioether bond leads to decreased bond dissociation energy of the O–H bond, thus facilitating the oxidation of Cu(II)-tyrosinate to a Cu(II)-tyrosyl radical in catalysis (39, 40).

Based on our work on CDO and previous work on cross-linked protein-derived cofactors involving tyrosine, we suggest two key roles for the CDO Tyr-Cys cofactor, which differ from the role played by the cofactor in galactose oxidase. The first role is structural. The strong hydrogen bond to the iron-bound water stabilizes the near-tetrahedral coordination of the ferrous ion in the resting enzyme even though the d^6 Fe^{2+} prefers octahedral geometry and protects the ferrous ion from oxidation. As a strictly Fe^{2+}-dependent oxygenase, the ferric active site of CDO leads to catalytic inactivity and loss of the metal, as reported in the other 2His-1-carboxylate non-heme iron enzymes (41). Loss of the Tyr-Cys cofactor in Tyr-157 or Cys-93 mutants will weaken Fe^{2+} binding by allowing its oxidation, but the 4-fold coordination would also allow the three histidines to coordinate a zinc ion (Fig. 5B). The second role of the Tyr-Cys cofactor is to prevent the production of highly damaging free hydroxyl radicals. During the cleavage of the dioxygen bond and the coupling of oxygen-sulfur radicals, the Tyr-Cys cofactor firmly holds the oxygen radical via the hydroxyl hydrogen. Free radicals are highly damaging to organisms (42–44), and free hydroxyl radicals are one of the most dangerous species (45). Higher organisms have a complicated set of mechanisms to prevent the production of free hydroxyl radicals (46), of which a small component may be the Tyr-Cys cofactor of CDO.

Catalytic Mechanism—Summarizing the results and discussion above, we propose a new plausible mechanism for cysteine thiol oxidation catalyzed by cysteine dioxygenase (Fig. 7).

In the resting enzyme, the central ferrous ion is coordinated by His-86, His-88, His-140, and a water oxygen atom, thus forming a distorted tetrahedral geometry as in rat CDO (Fig.
The hydroxyl group of Tyr-157 indirectly stabilizes the ferrous ion by hydrogen bonding to the coordinated water. This water molecule is replaced by the thiol group of cysteine when this substrate enters the active cavity (Fig. 7B). The second docking point of cysteine is the amino group that coordinates trans to His-140-Ne. The iron centers in both this substrate-bound (five coordination) state and the resting (four coordination) state are coordinately unsaturated. The carboxyl group of cysteine is firmly located in a hydrogen bond network involving several highly conserved residues in the second coordination shell, namely Arg-60, Tyr-157, Tyr-58, and His-155 (Fig. 3A). The dioxygen co-substrate is accommodated in a small hydrophobic pocket, and it binds to Fe$^{2+}$ in an “end-on” fashion, trans to His-86, with a hydrogen bond between the distal oxygen atom and Tyr-157 (Fig. 7C). At this point, both substrates are bound to the active center and complete a pseudo-octahedral geometry around the iron. A similar coordination of the intact thiol group and dioxygen molecule together to the heme ferrous ion was first proposed by Perera and colleagues (47).

Strong hydrogen bonding between the distal oxygen and the hydroxyl of Tyr-Cys favors the ferric-superoxyide form of the Fe(O$_2$)$_2$ moiety in CDO (Fig. 7C). Promoted by their close proximity, radical coupling between the distal oxygen and the thiol sulfur forms a peroxo intermediate with a sulfur radical cation (Fig. 7D). Homolytic cleavage of the O–O bond then occurs via abstraction of a hydrogen atom from the Tyr-157 hydroxyl by the distal oxygen atom (48) to form a tyrosyl radical. The other electron in the O–O bond is used to form a bond with the iron center, thus forming the well known oxyferryl (Fe$^{4+}$=O) moiety (Fig. 7E). If there is no adduct between Tyr-157 and Cys-93, the hydroxyl group of Tyr-157 is unable to stabilize the oxygen radical (48) and, consequently, leads to the formation of a highly damaging hydroxyl free radical (Fig. 7I).

The modified phenoxyl radical of Tyr-157 can hydrogen bond to either the S-OH or S-H groups, but ultimately it abstracts a hydrogen atom from the S-H group, returning the sulfur to a formal S(II) state (Fig. 7F). The ferryl species is a powerful oxidizing agent, and a single S–O bond is formed as a likely intermediate (Fig. 7G) that is then reductively eliminated from the iron center to give the S=O group and Fe(II) (Fig. 7H). For the completed CSA molecule, the sulfinic acid group is deprotonated via ionization. Finally, L-CSA is released from the active site, and the enzyme returns to the resting state (Fig. 7A).

Before the present work, several catalytic mechanisms of eukaryotic CDOs were proposed. The proposed mechanism based on the mouse CDO structure with a nickel center shares a similar substrate binding mode with our mechanism. However, the functions of the Tyr-Cys cofactor in catalysis were ignored, such that the S=O group was considered to be formed before the S-OH group (18). Another proposal based on x-ray absorption spectroscopy also suggested an octahedral coordination of the iron center, but the cysteine substrate was thought to be coordinated to the iron center via only one carboxyl oxygen instead of the thiol sulfur or amino nitrogen (26). Significantly different from the above two proposals and our mechanism, the iron center in the proposed mechanism based on the rat CDO structure was thought to be maintained in a tetrahedral coordination geometry during the entire catalytic cycle,
such that only one coordination site is available for substrate binding (19).

Outlook—Our work has revealed the precise binding mode of the cysteine substrate to the CDO active center and a possible coordination site for the co-substrate, a dioxygen molecule. A model of the dioxygen complex indicated key roles for the thioether-bonded Tyr-Cys cofactor. A new mechanism of thiol sulfur dioxygenation catalyzed by CDO was proposed, based on our structural analysis and mutagenesis experiments. Nevertheless, further experiments via chemical methods are necessary to confirm our hypothesis.

As mentioned in the introduction, CDO is degraded when hepatic concentration of free cysteine decreases below proper levels (7, 8). A reasonable explanation for this regulatory step is the presence of another cysteine binding site in CDO besides the active site, leading to a conformational change and subsequent recognition by other ubiquitination-related proteins. In our CDO-cysteine complex structure, a cysteine binding against the bottom of the β-barrel was also well modeled, which, together with the unknown covalent modification of Cys-164, may provide a starting point for further study of this regulatory mechanism.

From enzymatic methodology aspects, we emphasize the substrate role of the dioxygen molecule in the thiol sulfur oxidation reaction and its unsaturated concentration in water solution. The steady-state activity during a long reaction time of the order of minutes is strongly related to the dissolving velocity of oxygen from air. To avoid limited values of $K_m$ or $k_{cat}$, a method to determine the initial velocity before 10% soluble oxygen molecules are consumed should be used.

Homocysteine, a natural competitive inhibitor to CDO, does not exist in our diet and is produced by our body itself. High levels of homocysteine have been linked to cardiovascular diseases, such as heart attacks and strokes (49, 50), and neurodegenerative diseases (51). Unfortunately, our efforts to co-crystallize CDO with homocysteine failed, indicating the low affinity of homocysteine for CDO. This is consistent with our conclusions from the activity curve of human CDO with different affinity of homocysteine for CDO. This is consistent with our

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