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Influence of Cysteine 164 on Active Site Structure in Rat Cysteine Dioxygenase

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Abbreviations: CDO, cysteine dioxygenase; CSA, cysteine sulfinic acid.

Abstract

Cysteine dioxygenase (CDO) is a non-heme mononuclear iron enzyme with unique structural features, namely an intramolecular thioether crosslink between cysteine 93 and tyrosine 157, and a disulfide between substrate L-cysteine and cysteine 164 in the entrance channel to the active site. We investigated how these posttranslational modifications affect catalysis through a kinetic, crystallographic and computational study. The enzyme kinetics of a C164S variant are identical to WT indicating that disulfide formation at C164 does not significantly impair access to the active site at physiological pH. However, at high pH the cysteine-tyrosine crosslink formation is enhanced in C164S. This supports the view that disulfide formation at position 164 can limit access to the active site. The C164S yielded crystal structures of unusual clarity in both resting state and with cysteine bound. Both show that the iron in the cysteine bound complex is a mixture of penta- and hexa-coordinate with a water molecule taking up the final site (60% occupancy), which is where dioxygen is believed to coordinate during turnover. The serine also displays stronger hydrogen bond interactions to a water bound to the amine of the substrate cysteine. However, the interactions between cysteine and iron appear unchanged. DFT calculations support this and show that WT and C164S have similar binding energies for the water molecule in the final site. This variant therefore provides evidence that WT also exists in this equilibrium between penta- and hexa-coordinate forms and presence of the sixth ligand does not strongly affect dioxygen binding.

Key Words: Cysteine dioxygenase, post-translational modification, *ES* coordination, kinetics, DFT.

Introduction

The mononuclear non-heme iron enzyme cysteine dioxygenase (CDO, EC 1.13.11.20) converts L-cysteine and molecular oxygen to cysteine sulfinic acid. CDO is part of a larger class of enzymes, the thiol dioxygenases, that catalyze formation of the corresponding sulfinate from a range of different thiols. This group includes cysteamine dioxygenase, [1] 3-mercaptopropionate dioxygenase[2-5] and mercaptosuccinate dioxygenase, [6] which have been found to have a range of substrate specificities. CDO catalyzes the first step in oxidative cysteine breakdown in mammals.[7] Tight regulation of cysteine levels has been shown to be important for health and linked to many diseases with recent studies also linking CDO activity to human cancer. [8, 9]

The active site ferrous iron in mammalian CDO is chelated by a 3-histidine motif in close proximity to an unusual posttranslational modification, a cysteine-tyrosine crosslink. Although the cysteine and tyrosine residues that form the crosslink are completely conserved in eukaryotes, a glycine replaces the cysteine in most bacterial sequences where CDO activity remains. [10] Interestingly, other thiol dioxygenases appear to also lack the crosslink, demonstrating that the crosslink is not required for activity. Nonetheless, we and others have shown that mammalian CDO activity is dependent upon the amount of crosslink.[11, 12] Indeed, the kinetic parameters of the two isoforms were shown to be quite different.[13]

A second posttranslational modification forms in mammalian CDO; a disulfide between cysteine 164 and exogenous cysteine. This was initially observed crystallographically[14] and subsequently characterized by us using mass spectrometry.[15] Indeed, the first cysteine bound structure for CDO (PDB ID 2ICI) [16] has been reanalyzed and interpreted as containing such a disulfide.[17] Furthermore, sequence alignment and structural comparison shows that although cysteine is conserved at this position in CDO of multicellular eukaryotes, it is replaced by a

catalytically important arginine residue in 3-mercaptopropionate dioxygenases.[3] This disulfide is believed to be important because cysteine 164 lies at the entrance to the channel to the active site.

However, removal of cysteine 164 from CDO has been reported to lead to reduction in activity and not an increase as one might expect if only steric factors are considered. This reduction in activity has been shown in both human[16] and rat CDO,[18] but the size of the decrease reported is quite different, depending on substrate concentration. To resolve seemingly conflicting statements about the role of cysteine 164, we re-investigated the C164S variant to explore what changes in activity could be observed and to understand if these could be explained structurally.

Materials and Methods

Expression, purification and kinetic assays of the WT and C164S CDO variant. Anaerobic expression and purification of C164S CDO was performed as described previously.[12] As-purified protein was enriched with ferrous iron as and excess iron removed by Chelex, already described in greater detail elsewhere. [12, 13, 19] The crosslink fraction was ~50% as previously demonstrated for WT. [12] Activity assays were performed by HPLC-ELSD [20] at 37°C in phosphate buffer between pH 5.7 and 7.5.

All cysteine solutions, including dilutions from a stock, were freshly prepared in buffer at the beginning of each experiment and adjusted to the desired pH. The final protein concentration in all assays was 1.5-3.0 μ M. CDO activity and crosslink formation was typically assessed over a period up to 3 hours. The initial linear proportion of the product formation curves was used to analyse Michaelis-Menten type kinetics. All kinetic parameters were calculated relative to active iron containing protein. The fraction of crosslinked CDO in relation to non-crosslinked CDO was

determined densitometrically from SDS-PAGE gel images using the program ImageQuant™ TL (GE Healthcare, UK). Data were analyzed using Microsoft Excel 2007/2010 and GraphPad Prism version 5.00 for Windows.

Crystallization and Structural Determination of the resting state and cysteine bound C164S CDO variant. Crystals were grown and processed using the same conditions described previously. [13, 21] Hanging drops of 2 μ L of approximately 50 mg/mL C164S CDO (10 mM sodium phosphate and 20 mM NaCl, pH 7.5) that had been reconstituted with iron and 1.5 μ L of reservoir buffer containing crushed WT CDO seeds were allowed to equilibrate above the reservoir buffer (26% (w/v) polyethylene glycol 4000, 200 mM ammonium acetate, and 100 mM sodium citrate, pH 6.3). Crystals grew within two days as thick needles up to 1 mm in length. For the resting state, data were collected at the home source at the University of Otago Department of Biochemistry at a rotating anode (Rigaku MSC 007HF copper anode with Osmic VariMax optics) using an image plate detector (Raxis IV++). With the crystal at 93 K and X-rays of wavelength of 1.542 Å, 300 images were collected at an exposure time of two minutes, using 0.5° oscillation and a detector distance of 100 mm. For the cysteine bound structure, a crystal was soaked for six minutes in a drop containing the reservoir solution mentioned above and 100 mM cysteine, with an adjusted pH of 8.1. This approach was based on a report by Driggers et al. [17] Data were collected in a similar manner to the resting state; 400 images were collected, using 0.5° oscillation and a detector distance of 110 mm. Data collection for both structures was done by CrystalClear-d*TREK. [22] Data were indexed in space group $P4_32_12$, with unit cell dimensions of ~58, 58, and 122 Å (Table 1), and integrated in iMosflm. [23] Scaling was done using Aimless,[24, 25] 5% of reflections were reserved for the calculation of R_{free} using the MTZ from 4KWJ as a reference. The resolution (Table 1) was cut back based $I/\sigma I > 2$, which resulted

in CC1/2 values of >0.8 (all values reported by Aimless). [24] PDB entry 4KWJ [21] with important sidechains substituted by alanines was used for molecular replacement in Phenix Phaser [26] with one monomer in the symmetric unit. The active site iron atom was placed using Phaser-EP [26] (MR-SAD). Phenix.Refine, [26] Phenix.Readyset, [26] and COOT [27] were used for refinement. The cysteine model and constraints were created using Phenix.eLBOW.[28] MolProbity [29] was used to validate the structures. Coordinates and structure factors were deposited in the Protein Data Base, for the resting state as entry 4YYO, for the cysteine bound as entry 4Z82.

Computational chemistry. All structures were optimized using the *Gaussian-09* software package using models based on the crystal structure coordinates.[30] A minimum level of constraints was included in the model to enable fidelity in the structural representation of the active region. Our original model[31] consisted of the iron bound to the cysteine substrate with imidazole groups representing histidine residues 86, 88, 140 and 155. However, here we expanded the histidine groups to methylimidazole. As before, also included were the methylguanidinium group to represent arginine 60, a phenolate group in place of tyrosine 157 and methylsulfide for cysteine 93. We further expanded the model with the methylsulfide group of cysteine 164, a bridging water molecule towards the carboxylate of the substrate and the phenolate group of tyrosine 58. We calculated the resting state structure with a water molecule bound to the sixth ligand position for WT as well as the C164S mutant. These systems have an overall charge of +1 and were calculated in the quintet spin state only.

Geometry optimisations were carried out for each structure using the unrestricted hybrid density functional method B3LYP,[32, 33] with a basis set containing 6-31G+ double- ζ basis set on all atoms except for the iron where a double- ζ quality LACVP basis set that includes an

effective core potential was used (basis set BS1).[34] Additional calculations were done with the triple- ζ quality LACV3P+ basis set on iron (with core potential) and 6-311+G* on the rest of the atoms (basis set BS2). Vibrational frequencies were calculated on the optimised structures using the *Gaussian-09* software package and free energies were calculated at 298.15 K and 1 atm, which resulted in local minima with real frequencies only.

Results

C164S CDO activity is the same as WT. The activities of C164S and WT were compared in phosphate buffer at pH 5.7, 6.5 and 7.5 at 37°C (Figure 1). For both, activity increases with increasing pH as observed previously for WT.[19] Kinetic parameters are indistinguishable at pH 5.7 ($k_{\text{cat}} = 0.04 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 3 \pm 2 \text{ mM}$) and pH 6.5 ($k_{\text{cat}} = 0.40 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 11 \pm 2 \text{ mM}$). At pH 7.5 k_{cat} is not significantly different (C164S $k_{\text{cat}} = 0.48 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 5 \pm 2 \text{ mM}$; WT $k_{\text{cat}} = 0.40 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 9 \pm 2 \text{ mM}$). However, differences in K_{m} do suggest that C164S is more catalytically efficient than WT at this higher pH.

Overall reaction profile and crosslink formation. Over longer time periods, cysteine depletion and CSA formation were seen to be sigmoidal (Figure 2). An initial linear phase (0-30 min) fulfilling the Michaelis-Menten requirements described above was observed. This was followed by a second faster phase (~30-90 min) and eventually a slower phase (~90-180 min) as substrate concentrations were depleted. The HPLC-ELSD method[20] allows both substrate depletion and product formation to be monitored and the sum of substrate consumed and product produced was constant throughout the time-course within the error of the method for each set of data, indicating that non-enzymatic oxidation of cysteine was insignificant during the experiment, suggesting a CSA:cysteine coupling of ~100%.

During assays the overall crosslink formation as a function of time could be followed by SDS-PAGE and densitometry. As described previously for WT CDO,[12] crosslink formation occurs almost simultaneously with CSA production (Figure 2). Crosslink formation was pH dependent with an almost linear increase in the final amount of crosslink formed with pH (Figure 2). Slightly more crosslink formation was observed using C164S than WT and nearly 100 % crosslink was formed at high pH, something we have not observed under our conditions with WT CDO.

Comparison of C164S resting state structure with WT. The crystal structure of C164S CDO variant shows a similar overall structure compared to WT CDO. The active site also appears to be largely unchanged with only subtle changes. The cysteine 93 tyrosine 157 crosslink is present and as in WT appears to be the only isoform that crystallizes. Analysis of the diffraction data was aided by anomalous scattering by sulfur atoms, e.g. cysteines 76, 93 and 130 and methionines 73, 117 and 179 show significant anomalous peaks (Figure 3A). Although not optimized for exploitation of the anomalous signal from these atoms, the Cu K_{α} radiation used for data collection gives both iron and sulfur have sufficiently large f'' (3.15 and 0.56, respectively) to provide interpretable maps. In fact a strong anomalous signal was observed, most likely because of a combination of the wavelength (1.542 Å), the very large crystal (~1 mm) and the relatively high overall resolution (1.77 Å). As expected no anomalous peak was observed for serine 164.

Superposition with the WT CDO structure PDB 4KWJ[21] (Figure 3B) shows that the sidechain of residue 164 has almost the same orientation in both structures. The active site iron is again chelated by the 3 histidine residues (86, 88 and 140), however a chloride molecule was modeled instead of bound water opposite histidine 86. A chloride molecule accounts better for the observed density, makes chemical sense and is certainly present in the crystallization

conditions used. However, the B factor of the chloride molecule is significantly higher ($44.1 \text{ \AA}^2$) compared to the average protein atom ($22.1 \text{ \AA}^2$) and the iron atom ($14.7 \text{ \AA}^2$), suggesting disorder. We therefore have to state that the modeling of chloride here is speculative and several disordered oxygen atoms could also be possible. We have observed a similar density close to the iron atom for other CDO structures and therefore conclude that the “chloride ion” is not directly related to the C164S substitution.

C164S CDO variant in complex with cysteine. A crystal soaked with 100 mM cysteine at pH 8.1 diffracted to produce data that clearly revealed a cysteine molecule bound to the active site iron. A strong anomalous signal for the iron and the sulfur of the bound cysteine confirms presence of cysteine and aids placement. A $mF_o - DF_c$ electron density map produced from a model with no iron, cysteine or water atoms and no sidechains for arginine 60, cysteine 93 and tyrosine 157 provides an unbiased view of the electron density in the active site (Figure 3C). The fully refined model and electron density are given in Figure 3D.

Overall the cysteine bound structures at pH 8.1 of C164S and WT (4IEV [17]) are very similar with an RMSD of $0.18 \text{ \AA}$ using a $C\alpha$ atom alignment (Figure 3E). [35] Driggers et al. [17] reported that some structural changes occurred at higher pH in the asparagine 76 loop and the same changes are observed with C164S. The only slight differences between the structures are seen in partial occupancy of additional alternate conformations in the C164S structure for residues asparagine 71, glutamic acid 127, histidine 173 (all outside the active site), and threonine 95 (within the vicinity of the active site). Likewise, comparison of the active site shows high similarities. Even residue 164 is very similar with the serine occupying the same rotamer as cysteine in the WT structure. Only methionine 179 occupies a different sidechain orientation in C164S compared to WT consistent with the disorder observed for this residue in

other structures. This view is supported by the fact that no significant anomalous signal was observed for sulfur of residue 179 in comparison with, for example, methionine 73 which appears to be highly ordered (Figure 3D).

The most notable difference between the two structures is that in the C164S cysteine bound structure, unaccounted electron density next to the iron opposite histidine 86 remained after the cysteine ligand was modeled. A water molecule was placed at this position and its occupancy and B-factor were refined to 60 % and 28.11 Å². The cysteine ligand molecule occupancy and B-factor refinement resulted in 91 % and 25.08 Å²; while the iron atom was refined at 100 % occupancy resulting in a B-factor of 21.06 Å² (Table 1).

Closer inspection shows that the hydrogen bonding between cysteine/serine 164 and the bound cysteine differs. The γ -sulfur/oxygen of cysteine/serine 164 is hydrogen bonded to a water molecule that is in turn hydrogen bonded to the amine of the substrate bound to the iron. The serine oxygen to water oxygen bond distance in C164S, is shorter than the cysteine sulfur to water oxygen distance in WT and methionine 179 (although more disordered, see above) moves to take up the space (Figure 4). It is unclear whether the stronger serine-water interaction stabilizes the additional water molecule opposite histidine 86. However, the hydrogen bonding between arginine 60 and the carboxylate of bound cysteine is unchanged.

Computational chemistry. To investigate the hydrogen bonding interactions between cysteine/serine and the bound substrate and water, computational studies were carried out. Optimized geometries are shown in Figure 5. The mutation of cysteine 164 to serine has a significant effect on the local charge distribution nearby the carboxylate group of the cysteine substrate. Thus, the oxygen atom of serine has a charge of -0.70, whereas a sulfur atom in WT only has a charge of -0.10. As a result, a serine moiety in position 164 will pull the bridging

water molecule closer: H₂O---HOSer is 1.74Å, whereas the corresponding bond in WT is 1.93Å (cf. 3.3 Å O---O and 3.6 Å O---S distances in the corresponding crystal structures, respectively). This interaction also affects the bond strength between the bridging water molecule and the amide group of the substrate. In addition, the carboxylate group of the substrate and the crystal water molecule is considerably closer to the substrate in the mutant. Only very small changes in the metal ligand distances are seen.

The binding energy of the water to make the substrate bound complex hexa-coordinate was also calculated for both WT and C164S. The energies were found to be only 0.1 kcal/mol different suggesting that WT is also partially hexa-coordinate.

Discussion

Simmons et al. [36] and Ye et al. [16] independently of each other, noticed unexpected electron density distal to the S γ -atom of residue C164 when analyzing crystal structure data from mammalian CDO crystals that had been either co-crystallised with or soaked in cysteine, but neither group was able to unambiguously identify the origin of this feature by refinement of their crystal structure models. Simmons and colleagues, however, found that this electron density was only observed in the structure of CDO in presence of substrate, but not in its absence. Thus it was proposed that the structural change at C164 was caused by intermolecular disulfide formation with cysteine. This theory has been strengthened recently by a reanalysis of the Ye structure by Driggers et al. [17]. Such reactions on cysteine residues are not uncommon in proteins.[37] It was further suggested that, in consequence, the disulfide would negatively affect CDO activity by obstructing access of substrate to the active site. Ye and co-workers put this hypothesis to the test by designing CDO variants where C164 was replaced by either serine or alanine, both amino acids incapable of forming a disulfide with substrate cysteine. Increased

activity in those variants would have reinforced Simmons' theory, but the results showed an abatement of approximately 20 % with respect to the activity of WT CDO instead. The origin of the unexpected electron density at C164 thus remained unclear. Kleffmann *et al.* [15] investigated covalent post-translational modifications in CDO by MALDI-TOF-MS and found that the modification at C164 after incubation with supraphysiological concentrations of substrate was indeed a mixed disulfide with cysteine.

The Michaelis Menten parameters of C164S and WT CDO that we present are comparable within error, suggesting that only at high cysteine concentrations and higher pH does the disulfide at position 164 interfere with reactivity or modification. Indirect evidence supports this proposal. The reaction profiles measured over longer time scales using C164S are more reproducible than WT but this is difficult to quantify. The final % crosslink formed is, however, accurately quantified using SDS-PAGE densitometry. It is seen that C164S is more readily crosslinked than WT, especially at higher pH (Figure 2B). At higher pH the cysteine thiol is deprotonated and thus more reactive and more likely to form the disulfide. Once formed, the disulfide at position 164 impedes access to the active site and thus abrogates crosslink formation, which has been shown to require cysteine.[15, 18] Conversely, it could be the negative charge on the thiolate itself that might impede access. Either way, the C164S variant is unable to deprotonate in this pH range and/or form this disulfide and therefore is able to be fully crosslinked, in contrast to WT. In contrast to our results with WT, Pierce and coworkers have been able to form fully crosslinked WT mouse CDO,[11] something we have been unable to do using our protein and methods.

The C164S mutation appeared to have little significant effect on the overall structure. The resting state is nearly identical to the WT structure. In X-ray crystallographic models, the iron

environment in resting states of CDO is variously modeled, with one [14], two [13, 17, 21] or three water molecules (when the metal was modeled as a nickel atom [38]) bound. For the resting state of the C164S variant we present a chloride ion as our best model to account for the observed electron density. In general it appears that disordered solvent molecules are exchanged at the iron atom and several interpretations of this disorder can be found in the different crystal structures of CDO. The resting states of CDO structures also show different orientations of arginine 60 and methionine 179, with disulfide formation on residue cysteine 164 influencing their positions. For C164S, only one orientation of arginine 60 is observed in the resting state: pointing towards the active site and a corresponding methionine 179 position.

The orientation of cysteine bound to the C164S structure is unambiguous due to strong anomalous scattering by iron and various sulfur atoms, in particular of the ligand bound sulfur, providing strong independent support for the mode of L-cysteine binding described by Driggers et al.[17] While the cysteine is orientated very similarly to cysteine in the WT structure obtained under similar conditions, the C164S mutation does affect the hydrogen bonding network between residue 164 and the bound substrate. Importantly, there is also extra electron density next to the iron that can be modeled with a water molecule with 60% occupancy to make the iron hexa-coordinate. This is intriguing because previous spectroscopic investigations of CDO have suggested that the cysteine bound complex is penta-coordinate, with the sixth coordination site free to allow dioxygen binding at this position. The exception to this is a recent study of the H155A variant of mouse CDO that showed through magnetic circular dichroism that the cysteine bound complex is 6 coordinate.[39] This difference in coordination number is explained by the alanine substitution breaking the hydrogen bonding network between residue 155 and tyrosine 157 that points towards the substrate coordination site (Figure 5). In our case, the mutation at

position 164 also affects the hydrogen bonding network but from the opposite side of the active site and interacts with the substrate rather than the dioxygen binding site. We further probed its contribution through computational calculations.

The DFT calculations confirm the differences in the hydrogen bonding network and these can be explained by differences in the charge of the oxygen versus sulfur in serine and cysteine. However, no appreciable differences in the iron ligand environment was observed suggesting that, in contrast to the H155A variant, the substitution cannot explain the differences in coordination number. Indeed, the binding energies of water to the penta-coordinate cysteine bound structures of WT and C164S are almost indistinguishable. We therefore believe that the CDO enzyme substrate complex exists as a mixture of 5- and 6-coordinate, but this equilibrium is altered by the H155A variant.

The paradigm for dioxygen binding at non-heme mononuclear iron enzymes is currently based around data collected for enzymes with 2-histidine 1-carboxylate coordination and predominantly those that use α -ketoglutarate as co-substrate.[40] This model involves initial substrate binding that causes the conversion of the iron center from 6- to 5-coordinate. The enzyme is now ready to coordinate dioxygen and it has been suggested that this allows the enzyme to control reactivity and only implement dioxygenation when sufficient substrate is available. However, a weakly coordinating water molecule occupying the sixth coordination site has been observed both crystallographically[41] and spectroscopically[42] in the halogenases SyrB2 and CytC3 respectively. In this case, it has been suggested that 6-coordination is important for ensuring halogenation rather than hydroxylation. It is therefore surprising that CDO allows a mixture of 5- and 6-coordination for the substrate bound complex prior to dioxygen binding. It could be that the difference lies in the neutral 3-histidine coordination,

which allows substitution of water by dioxygen but further study is required to fully understand this process.

Our crystal structure of the cysteine bound C164S variant and related calculations improve our description of the *ES* complex in CDO. Altogether we describe the C164S variant and show that although it does affect cysteine-tyrosine crosslink formation, it does not appear to directly affect reactivity in the lower pH region. Cysteine 164 may act as a gate across the entrance channel, impeding access, particularly at higher pH through either disulfide formation or negative charge on the thiolate.

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Electronic Supplementary Material Available: Coordinates used for computational calculations.

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Table 1: Data collection and refinement statistics for rat CDO 164S structures. Values in parentheses indicate the highest-resolution shell.

	C164S resting state	C164S cysteine bound
Data collection^a		
Resolution range (Å)	21.09 - 1.77 (1.83 - 1.77)	23.88 - 1.70 (1.76 - 1.70)
Space group	P 4 ₃ 2 ₁ 2	P 4 ₃ 2 ₁ 2
Unit cell	57.8 57.8 123.07 90 90 90	58.02 58.02 122.23 90 90 90
Total reflections	165,219 (9,825)	282,218 (13,606)
Unique reflections	21,162 (1,244)	22,847 (1,175)
Multiplicity	7.8 (7.9)	12.4 (11.6)
Anomalous multiplicity	4.2(4.1)	6.6(6.1)
Completeness (%)	99.9 (100)	96.7 (93.1)
Anomalous completeness (%)	100(100)	97.0(92.7)
Mean I/σI	12.2 (2.5)	23.6 (2.3)
R-merge	0.088 (0.321)	0.058 (0.832)
Anomalous R-merge	0.084(0.301)	0.056(0.799)
Half-dataset anomalous difference correlation	-0.171(-0.132)	-0.042(0.037)
CC1/2	0.996 (0.946)	0.999 (0.843)
Refinement^b		
Reflections for refinement	21,073	22,818
R-work	0.175 (0.229)	0.178 (0.244)
R-free	0.208 (0.301)	0.206 (0.271)
RMS bonds (Å)	0.008	0.009
RMS angles (°)	1.08	1.10
Ramachandran favored (%)	98.4	99.5
Ramachandran outliers (%)	0	0
Rotamer outliers (%)	0.6	0.6
Clashscore	5.47	3.28
Average B-factor (Å ²)	24.1	29.9
Macromolecules (Å ²)	22.1	28.5
Iron atom (Å ²)	14.7	21.1
Cysteine (Å ²)	-	25.1
Solvent (Å ²)	34.1	38.9
PDB ID	4YYO	4Z82

^aData collection statistics as reported by aimless.[24]

^bRefinement statistics as reported by phenix.refine [26] and Molprobit. [29]

Figure Legends

Figure 1. Plot of k_{obs} of cysteine dioxygenase activity of WT (full circles) and C164S (open circles) as a function of cysteine concentration at pH 5.7, 6.5 and 7.5. The data can be fitted to the Michaelis-Menten equation (solid lines) with parameters: pH 5.7 ($k_{\text{cat}} = 0.04 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 3 \pm 2 \text{ mM}$), pH 6.5 ($k_{\text{cat}} = 0.40 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 11 \pm 2 \text{ mM}$), pH 7.5 (C164S $k_{\text{cat}} = 0.48 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 5 \pm 2 \text{ mM}$; WT $k_{\text{cat}} = 0.40 \pm 0.07 \text{ s}^{-1}$ and $K_{\text{m}} = 9 \pm 2 \text{ mM}$).

Figure 2. (A) Reaction profile of C164S (2.2 μM) at pH 7.5, 37 °C showing cysteine depletion (full squares, left axis), CSA formation (full circles) and % crosslink (open circles, right axis) as a function of time. (B) Final % crosslink formed after reaction as a function of pH. WT (full triangles) and C164S (open circles) show greater differences at higher pH.

Figure 3. Crystal structure of the resting state and cysteine bound C164S CDO variant. Figures and alignments created by UCSF Chimera [35] using coloring-by-element scheme except carbon atoms, iron atoms and iron coordination in black (resting state C164S, PDB entry 4YYO), in cyan (resting state WT, 4KWJ [21]), in purple (cysteine bound C164S, 4Z82) and silver (cysteine bound WT, 4IEV [17]). (A) Active site of the resting state C164S CDO variant. $2mF_o - DF_c$ active site electron density (at 1 RMSD, blue), the anomalous map (at 3 RMSD, yellow) and the refined model. (B) Structural comparison of the resting states of C164S and WT CDO using a C α atom alignment. (C) Unbiased active site density of the cysteine bound C164S CDO variant before model building. Final model compared to a $mF_o - DF_c$ electron density map (green) based on a model with no iron, cysteine or water atoms and no sidechains for arginine 60, cysteine 93 and tyrosine 157 and anomalous map (at 3 RMSD, yellow). (D) Active site of the

cysteine bound C164S CDO variant. $2mF_o - DF_c$ active site electron density (at 1 RMSD, blue), the anomalous map (at 3 RMSD, yellow) and the refined model. (E) Structural comparison of the cysteine bound C164S and WT CDO structure using a C α atom alignment.

Figure 4. Comparison of hydrogen bonding in A) WT (PDB 4IEV) and B) C164S variant of CDO (PDB 4Z82). Figures created and colored as in Figure 3. Proposed hydrogen bonds are shown in red.

Figure 5. B3LYP optimized geometry of the resting state complex (above) and C164S (below) as obtained in Gaussian-09. Bond lengths are in angstroms and the charge (Q) is in au. Details at right include iron, substrate cysteine, residue 164 and associated water molecule, rotated relative to main figures by approximately 90° around a horizontal axis in the plane of the page.

Figures

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.