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ORIGINAL ARTICLE

# Involvement of the Cys-Tyr cofactor on iron binding in the active site of human cysteine dioxygenase

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**Abstract** Sulfur metabolism has gained increasing medical interest over the last years. In particular, cysteine dioxygenase (CDO) has been recognized as a potential marker in oncology due to its altered gene expression in various cancer types. Human CDO is a non-heme iron-dependent enzyme, which catalyzes the irreversible oxidation of cysteine to cysteine sulfinic acid, which is further metabolized to taurine or pyruvate and sulfate. Several studies have reported a unique post-translational modification of human CDO consisting of a cross-link between cysteine 93 and tyrosine 157 (Cys-Tyr), which increases catalytic efficiency in a substrate-dependent manner. However, the reaction mechanism by which the Cys-Tyr cofactor increases catalytic efficiency remains unclear. In this study, steady-state kinetics were determined for wild type CDO and two different variants being either impaired or saturated with the Cys-Tyr cofactor. Cofactor formation in CDO resulted in an approximately fivefold increase in $k_{cat}$ and tenfold increase in $k_{cat}/K_m$ over the cofactor-free CDO variant. Furthermore, iron titration experiments revealed an 18-fold decrease in $K_d$ of iron upon cross-link formation. This finding suggests a structural role of the Cys-Tyr cofactor in coordinating the ferrous iron in the active site of CDO in accordance with the previously postulated reaction mechanism of human CDO. Finally, we identified product-based inhibition and α-ketoglutarate and glutarate as CDO inhibitors using a simplified well plate-based activity assay. This assay can be used for high-throughput identification of additional inhibitors, which may contribute to understand the functional importance of CDO in sulfur amino acid metabolism and related diseases.

**Keywords** Cysteine · Cysteine dioxygenase · Cross-linked cofactor · Cysteine sulfinic acid · Ferrous iron · Sulfur metabolism

**Abbreviations**

| | |
|---|---|
| CDO | Cysteine dioxygenase |
| Cys-Tyr cofactor | Cysteine-tyrosine cofactor |
| CSA | Cysteine sulfinic acid |
| GA | Glutarate |
| α-KG | α-Ketoglutarate |

## Introduction

Cysteine dioxygenase (CDO, EC: 1.13.11.20) is a cytosolic non-heme mononuclear iron-dependent enzyme that catalyzes the irreversible addition of molecular oxygen to the sulfhydryl group of cysteine yielding cysteine sulfinic acid (CSA) (Stipanuk 1986). The CDO-catalyzed reaction represents a branch point in mammalian cysteine

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catabolism and CSA is further metabolized via two different pathways producing either taurine or pyruvate and sulfate as final products. CDO is expressed at detectable levels in the brain, kidney, lung, and adipose tissue, and at very high levels in liver (Stipanuk et al. 2002, 2009), where the enzyme plays a critical role in regulating the hepatic concentration of intracellular cysteine (Cresenzi et al. 2003). CDO is mainly regulated at the level of protein turn-over via ubiquitination and degradation by the 26S proteasome system (Dominy et al. 2006). In vivo experiments in rat liver showed that CDO undergoes an up to 45-fold change in concentration in response to a change in protein intake (Bella et al. 1999a, b; Lee et al. 2004). However, the precise mechanism by which cysteine regulates CDO ubiquitination remains unknown.

Regulation of CDO activity is crucial for maintaining a balance between cellular needs for cysteine while keeping its concentration below toxic levels (Andine et al. 1991; Lehmann et al. 1993). In fact, cysteine is essential for glutathione synthesis, which is the most abundant low molecular thiol in animals (Wu et al. 2004). Increase in cysteine supply is directly correlated with an enhanced glutathione synthesis. Thus, cysteine is considered to be the limiting amino acid for glutathione synthesis in mammals (Chung et al. 1990; Jahoor et al. 1999; Lyons et al. 2000). In contrast, elevated intracellular levels of free cysteine have been shown to be both cytotoxic and neurotoxic due to the oxidative damage via formation of free radicals in the presence of iron (Andine et al. 1991; Lehmann et al. 1993; Montine et al. 1997). Furthermore, cysteine accumulation was correlated to various autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Emery et al. 1992; Gordon et al. 1992) as well as neurodegenerative diseases such as Parkinson's and Alzheimer's (Bradley et al. 1994; Heafield et al. 1990; Heafield and Williams 1992). In recent years, CDO has gained increased attention as potential target for the development of therapies for different types of cancer as an increased CDO methylation in cancer cell lines and patients were reported in various studies (Brait et al. 2012; Jeschke et al. 2013). Consequently, those studies implicate a close and important relationship between the sulfur metabolism—in general and CDO in particular—and oxidative stress and neurodegenerative disorders.

The crystal structure of human CDO revealed high structural similarities to mouse and rat CDO and confirmed a core monomeric structure (McCoy et al. 2006; Simmons et al. 2006b; Ye et al. 2007). The active site of human CDO is buried inside a deep cavity under the protein surface, and harbors a catalytically important ferrous iron, which is coordinated by N atoms of His86, His88 and His140 (Ye et al. 2007). The crystal structures of mouse, rat and human CDO also revealed the presence of a unique intramolecular thioether bond between the active site residues Cys93 and Tyr157, a cross-linked cofactor (McCoy et al. 2006; Simmons et al. 2006b; Ye et al. 2007), which was named Cys-Tyr cofactor. Further biochemical studies identified that the Cys-Tyr cofactor is responsible for the characteristic two-band migration pattern of CDO on SDS-PAGE (Dominy et al. 2008). Accordingly, the formation of the Cys-Tyr cofactor in CDO results in a slightly faster migration on SDS-PAGE than for the protein without the cofactor thus providing an easy tool to evaluate the ratio of both species.

Cross-link formation has been identified and well characterized in other enzymes such as galactose oxidase (Whittaker and Whittaker 2003). However, the Cys-Tyr cofactor in CDO has two unusual characteristics, which are different from galactose oxidase. First, cross-link formation in CDO does not occur spontaneously and strictly depends on substrate availability in addition to iron and molecular oxygen (Dominy et al. 2008). Second, cofactor formation is not essential for catalytic activity as several CDO variants, which are impaired in cross-link formation remain partially active (Dominy et al. 2008; Ye et al. 2007). Moreover, biochemical studies reported that cross-link formation increases catalytic efficiency of CDO up to tenfold in comparison to the Cys-Tyr cofactor-free CDO (Dominy et al. 2008). However, cross-link formation in CDO remains enigmatic with respect to the underlying mechanism that enhances the catalytic activity of CDO. A clear separation and characterization of the two CDO forms, with and without cofactor, may contribute to a better understanding of the role of this particular type of post-translational modification.

Despite the high sequence and structural similarity among mammalian CDOs, the determined kinetic parameters vary considerably in the literature (McCoy et al. 2006; Simmons et al. 2006a; Ye et al. 2007). Those variations are mostly due to different assay techniques and different enzyme preparations. A possible source of variation might be caused by the iron saturation of purified recombinant CDO, which has been shown to vary considerably (10–70 %) (Chai et al. 2005; McCoy et al. 2006; Simmons et al. 2006a, b). Thus, it became common practice to add ferrous iron to the activity assay in order to ensure high iron saturation of the enzyme (Dominy et al. 2008; Simmons et al. 2006a). In contrast, other research groups do not include iron (Ye et al. 2007), while a recent study suggests the use of reductants instead of iron in the CDO activity assay (Imsand et al. 2012). However, iron is not only important for catalysis, but it is also essential for Cys-Tyr cofactor formation in CDO. Thus, differences in cross-link saturation of recombinantly expressed CDO will also impact on the kinetic properties of the enzyme. In addition, in some studies an inhibition of CDO at high substrate concentrations was reported (Dominy et al. 2008; Ye et al. 2007). Therefore, in this study we aimed to characterize the

kinetic properties of recombinant human CDO taking into account the requirement for iron, oxygen and the presence of the Cys-Tyr cofactor. Furthermore, we determined the impact of cross-link formation on catalytic efficiency by investigating a cofactor-free and a cofactor-saturated CDO. Subsequently, iron binding to CDO was investigated using both CDO variants to understand the contribution of the Cys-Tyr cofactor in coordinating iron in the active center of CDO. Finally, a well plate-based activity assay for CDO was used to identify CDO inhibitors.

## Materials and methods

### Molecular biology

Cysteine dioxygenase (CDO; wildtype) expression construct was generated by cloning the coding sequence for the human CDO1 (accession no. BG708901.1) into pQE80L (QIAGEN) using *Sal*I and *Hin*dIII restriction sites. CDO C93A variant was generated from the human CDO wt construct by fusion PCR and cloned into pQE80L expression construct using the same restriction sites. Identities of all generated constructs were confirmed by sequencing (GATC).

### Protein expression and purification

All CDO proteins were expressed in the *E. coli* BL21 (DE3) strain. Expression was induced with 0.1 mM isopropyl β-thiogalactoside at $OD_{600} = 0.5$ and continued for 15 h at 30 °C. Knowing that CDO harbors a catalytically important iron in its active site, expression media as well as all purification buffers were supplemented with 1 mM ammonium iron sulfate to ensure proper iron saturation. All expressed CDO proteins carried an N-terminal His-tag and were purified by nickel nitrilotriacetic acid (Ni-NTA) affinity as recommended (QIAGEN) followed by size exclusion chromatography using a preparative superdex 200 column (GE healthcare) and the resulting proteins corresponding to monomeric CDO were exchanged into the same buffer (20 mM Tris/HCl pH 8.0, 50 mM NaCl) and stored at −80 °C.

### CDO standard activity assay

Activity assay for recombinant expressed human CDO was similar to the protocol used for rat CDO (Stipanuk et al. 2008). Briefly, the activity assay was conducted in a total volume of 200 µl containing the following components: 50 mM MES buffer pH 6.1, 0.3 mM ammonium iron sulfate, 62.5 µM bathocuproine disulfonate, 0.15 µM purified CDO and varying concentrations of L-cysteine (0–20 mM). The reaction was started by addition of cysteine and incubated at 37 °C with vigorous shaking to ensure proper oxygenation using a thermomixer (Eppendorf). The CDO-catalyzed reaction was terminated by the addition of 200 µl 5 % sulfosalicylic acid and centrifugation for removal of precipitated proteins followed by quantification of the reaction product CSA.

### Quantification of cysteine sulfinic acid

Cysteine sulfinic acid (CSA) formation in the CDO standard assay was quantified using high-performance liquid chromatography (HPLC) and pre-column derivatization with *o*-phthalaldehyde (OPA) as previously described (Belaidi et al. 2012). Briefly, HPLC analyses were carried out using an Agilent 1200 SL system controlled by Agilent ChemStation software (Agilent technologies). Pre-column derivatization was achieved using an automated autosampler, which was programmed to mix 20 µl of the sample with 5 µl of derivatization reagent and after an incubation time of 0.5 min, the derivatization mixture was separated using isocratic elution on a reversed-phase C18 XBridge column (75 × 4.6 mm, 2.5 µm, Waters). Detection was carried out using a fluorescence detector (excitation 240 nm, emission 450 nm) and compound identification was achieved by comparing the retention time with that obtained for CSA standard (Sigma).

### CDO well plate-based activity assay

The well plate-based assay was developed for identification of potential CDO inhibitors. The assay is based on the quantification of the CDO substrate cysteine using the Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The DTNB reagent reacts quantitatively with thiol groups, forming mixed disulfides and releasing the anion 5-sulfido-(2-nitrobenzoic acid), which absorbs light at 412 nm, thus permitting to determine the concentration of free cysteine in the sample. The assay was conducted in 96-well plate and contained following components: 70 µl 100 mM MES buffer pH 6.1, 5 µl 6 mM ammonium iron sulfate (freshly prepared), 10 µl 0–20 mM inhibitor, 10 µl 30 µM CDO and the reaction was started by addition of 5 µl 4 mM cysteine. The reaction mixture was incubated at room temperature for 10 min and terminated by the addition of 100 µl of DTNB reagent mixture containing: 10 µl 2 mM DTNB reagent (prepared in 50 mM NaAc), 20 µl 1 M Tris/HCl pH 8.0 and 70 µl $H_2O$. The concentration of the free remaining cysteine was determined by measuring the absorption at 412 nm using a well plate reader (Bio-Tek Instruments EL808) and calibration of the method was performed by measuring the absorption of defined cysteine concentrations.

In vitro formation of cross-link in purified CDO

Cross-link saturated CDO protein was generated according to the protocol of Dominy et al. (2008). Briefly, a preparative reaction mixture containing 0.3 mM wt CDO; 3 mM ammonium iron sulfate and 10 mM cysteine was incubated at 37 °C for 20 min under vigorous shaking to ensure proper oxygenation. Following, the reaction mixture was buffer-exchanged to 20 mM Tris/HCl pH 8.0, 50 mM NaCl to eliminate excess cysteine and iron and stored at −80 °C. Evaluation of cross-link formation was carried out on a 15 % SDS-PAGE and the enrichment of the CDO band corresponding to cross-linked CDO could be visualized.

## Results

Kinetic characterization of human CDO

CDO catalyzes the formation of cysteine sulfinic acid by adding two oxygens to the cysteine thiol. The reported loose binding of iron in the active center of CDO suggests an additional requirement of exogenous iron for the accurate determination of CDO activity. Given that different kinetic parameters were reported for CDO by using different assay conditions (Dominy et al. 2008; Ye et al. 2007), we asked the question to what extent iron saturation did impact on the activity of CDO. Indeed, the $K_m$ values reported for human and rat CDO were 3.1 and 0.45 mM, respectively and therefore quite different. Furthermore, CDO inhibition at high substrate concentrations was only reported for rat CDO and not for the human enzyme, despite a sequence identity of 92 % between both proteins. In this study, we first investigated the kinetic properties and inhibition of human CDO, which was recombinantly expressed in *E. coli* and purified to homogeneity (Fig. 2a).

CDO activity was determined based on the protocol of Stipanuk and colleagues (Stipanuk et al. 2008). All activity measurements were conducted at low enzyme concentrations (0.15 μM), in order to keep oxygen levels under saturating conditions, thus allowing the determination of cysteine-dependent CDO activity under pseudo-first order conditions. We first measured CDO activity using a broad substrate concentration of 0–20 mM and found an increased activity at cysteine concentrations up to 3 mM (Fig. 1a). At cysteine concentrations above 3 mM, a clear inhibition was observed and CDO activity continuously decreased (Fig. 1a). The data were therefore fitted using a kinetic substrate inhibition model. Due to the strong inhibition at high substrate concentrations, CDO activities were further measured using a substrate range of 0–3 mM, which could be fitted well by applying the Michaelis–Menten type of equation resulting in a $K_m$ for cysteine of 0.77 ± 0.14 mM and a $k_{cat}$ of 10.35 ± 0.69 $s^{-1}$ (Fig. 1b).

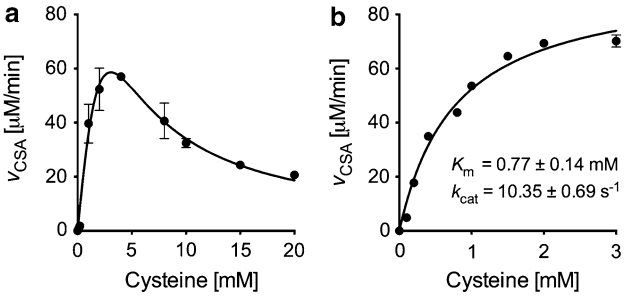


**Fig. 1** Kinetic characterization of recombinant human CDO. Activity of recombinant human wt CDO (0.15 μM) was investigated using a wide substrate range (0–20 mM), which revealed a markedly inhibition of the enzyme at cysteine concentration above 3 mM (**a**). Due to the strong inhibition at high substrate concentrations, kinetic parameters were determined at cysteine concentrations below 3 mM (**b**). Hyperbolic regression analysis according to Michaelis–Menten resulted in a $K_m = 0.77 \pm 0.14$ mM and $k_{cat}$ of 10.35 ± 0.69 $s^{-1}$

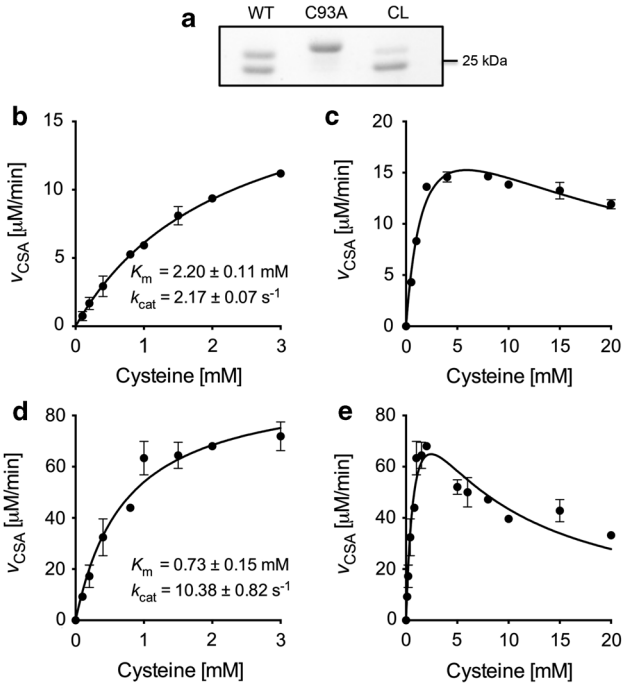


**Fig. 2** Impact of cross-linked cofactor on catalytic properties of human CDO. SDS-PAGE analysis of wt CDO, a cross-link-free CDO variant (C93A) and a cross-link-saturated CDO variant (CL) (**a**). Activity of the cross-link free variant was markedly reduced and hyperbolic regression analysis according to Michaelis–Menten resulted in a $K_m = 2.20 \pm 0.11$ mM and $k_{cat}$ of 2.17 ± 0.07 $s^{-1}$ (**b**). The C93A variant showed also inhibition at high substrate concentrations but to a lesser extent than wt CDO (**c**). Cross-link-saturated CDO showed similar kinetic properties as wt CDO resulting in a $K_m$ of 0.73 ± 0.15 mM and $k_{cat}$ of 10.38 ± 0.82 $s^{-1}$ (**d**) and showed a strong inhibition at high substrate concentrations (**e**)

## Impact of the Cys-Tyr cofactor on kinetic properties

The different kinetic parameters reported for CDO (Dominy et al. 2008; Ye et al. 2007) may also result from differences in enzyme preparations, which could impact the ratio of cofactor-containing versus cofactor-free fraction of the protein. In fact, Cys-Tyr cofactor formation between cysteine 93 and tyrosine 157 in CDO has been reported to significantly enhance catalytic activity (Dominy et al. 2008; Ye et al. 2007). To gain a better understanding of the impact of the Cys-Tyr cofactor on catalytic activity, we followed two different approaches. First, we measured the kinetic parameters of a CDO variant being impaired in cross-link formation by replacing cysteine 93 to an alanine. As result, the produced C93A variant showed only a single band on the SDS-PAGE while wildtype (wt) CDO showed the typical two-band pattern corresponding to the two forms of the enzyme with and without cross-link (Fig. 2a). Impairment in cross-link formation did not result in a complete loss of activity (Fig. 2b). However, we found a three- and five-fold decrease in $K_m$ and $k_{cat}$ in comparison to wt CDO, respectively (Fig. 2b), while inhibition at high cysteine concentration remained persistent although to lower extent (Fig. 2b, c).

In the second approach, we produced a cross-link-enriched fraction of CDO, which should represent maximal CDO activity. Based on previous results on rat CDO, which showed that cross-link formation in CDO is dependent on cysteine, iron and oxygen, we incubated wt CDO with cysteine and iron under vigorous shaking to ensure proper oxygenation for 30 min and subsequently exchanged the buffer to remove all remaining components. Under those conditions, the production of a Cys-Tyr cofactor-saturated enzyme (CL-CDO) was clearly visible by a nearly complete shift of the CDO band to a single band, representing the cofactor-containing fraction (Fig. 2a). The subsequent kinetic measurement with the cross-link saturated CDO did not reveal any difference to wt CDO as documented by comparable $K_m$ or $k_{cat}$ values (Fig. 2d). Furthermore, inhibition at high substrate concentration was again observed thus excluding any influence of the cross-link (Fig. 2e). Overall, these data demonstrate that under our assay conditions, CDO was converted to a Cys-Tyr cofactor-enzyme and thus the kinetic parameters measured here correspond to a fully active human CDO.

## Influence of the Cys-Tyr cofactor on iron binding

Ye and colleagues proposed a structural role of the Cys-Tyr cofactor in iron coordination in the active site of CDO (Ye et al. 2007). In order to explore the potential relationship between cross-link formation and iron binding, we investigated enzyme inactivation upon iron chelation by EDTA for wt CDO and the C93A variant (Fig. 3a). Wt CDO was

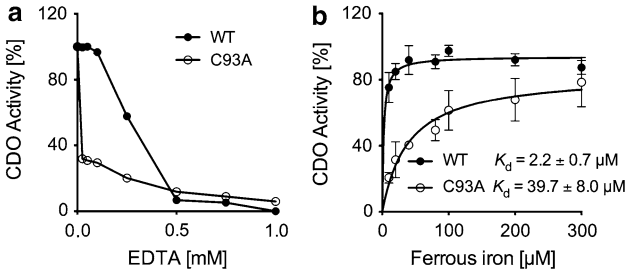


**Fig. 3** Formation of the cross-link cofactor results in stronger binding of iron in the active center of human CDO. Activity of wt CDO and C93A was assayed using increasing concentration of the metal chelator EDTA and converted to % CDO activity as a function of the activity measured for each variant in the absence of EDTA, which was converted to 100 % (**a**). The titration experiments with EDTA showed a progressive inactivation of wt CDO, while the cross-link-free C93A variant was readily inactivated at low EDTA concentrations (**a**). Activity of wt CDO and C93A variant was assayed using increasing iron concentrations and hyperbolic regression analysis according to one site-specific binding fitting resulted in a $K_d$ of 2.2 ± 0.7 and 39.7 ± 8.0 µM for wt CDO and C93A variant, respectively (**b**). An enzyme concentration of 3 µM was used in all experiments

progressively inactivated with increasing EDTA concentration and the activity was completely abolished at EDTA concentrations above 0.5 mM, which is in line with the importance of iron in the catalytic mechanism of cysteine oxygenation by CDO. In contrast, the C93A variant was inactivated at much lower EDTA concentration with 70 % decrease in activity already at 0.05 mM EDTA while wt CDO was not affected at the same EDTA concentration (Fig. 3a). This result suggests that iron binding is stronger in wt CDO as compared to the cofactor-free C93A variant.

Next, we performed iron titration experiments using wt CDO and C93A variant and investigated the iron dependence of CSA formation. In case of wt CDO, maximal activity was reached using very low iron concentrations (10 µM, Fig. 3b). In contrast, CDO activity of the C93A variant increased with a much lower rate of iron supplementation and consequently a much higher concentration was required to approximate saturation (over 100 µM, Fig. 3b), however, the full activity of wt CDO was not reached at any iron concentration. Fitting the data using the one site-specific binding equation resulted in an apparent $K_d$ of 2.2 ± 0.7 µM for wt CDO while an 18-fold higher $K_d$ of 39.7 ± 8.0 µM was determined for the C93A variant (Fig. 3b). Our results demonstrate that the Cys-Tyr cofactor strongly contributes to efficient iron coordination in the active center of CDO.

## Development of a well plate-based CDO activity assay

Cysteine is the only known substrate for cysteine dioxygenase (Stipanuk et al. 2009). However, to our knowledge no

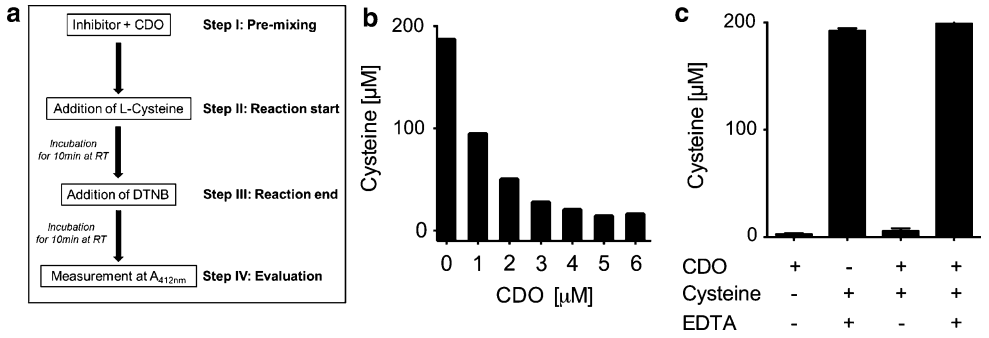


**Fig. 4** Development of a well plate-based activity assay for CDO using substrate detection with Ellman's reagent. Schematic representation of the experimental set-up of the well plate-based assay for activity measurement of CDO (**a**). The activity assay was conducted by using a cysteine concentration of 0.2 mM and different protein concentrations (0–4 µM) (**b**). The final set-up of the well plate-based assay included 3 µM CDO and a cysteine concentration of 0.2 mM and a systemic variation of substrate, protein and EDTA (1 mM) as a negative control was conducted for assay validation (**c**)

extensive inhibition study of CDO has been conducted so far, probably due to the commonly used complex and time-intensive HPLC detection method of its product cysteine sulfinic acid. Therefore, we aimed to establish a simple well plate-based assay, which can be used for identification of CDO inhibitors in a high-throughput screening format. For this purpose, we developed an assay based on the quantification of cysteine as CDO substrate using the Ellman's reagent 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) (Fig. 4a).

We first set a concentration of 0.2 mM cysteine, which is below the apparent $K_m$ of CDO, and probed the decrease in cysteine concentration as a function of CDO concentration (Fig. 4b). Results showed that a CDO concentration of 3 µM was able to reduce 80 % of cysteine within an incubation time of 10 min (Fig. 4b). CDO concentrations higher than 3 µM also reduced cysteine levels but failed to reach complete cysteine conversion, pointing to a limitation of oxygen in this set-up, as this assay was performed without shaking (Fig. 4b). In the following, systematic variation of assay components has been performed, with EDTA addition as negative control, which completely abolished CDO activity due to iron chelation (Fig. 4c).

## Identification of CDO inhibitors

Our initial characterization of wt CDO showed a clear inhibition at high substrate concentrations, which was attenuated when the activity of the C93A CDO variant was probed. Knowing that catalytic activity of the C93A variant was lower than wt CDO, we asked whether this inhibition is attributed to the CDO product CSA rather than cysteine. To test this hypothesis, we used the well plate assay and probed CDO activity in the presence of increasing concentrations of CSA. The results showed that CSA was in fact able to decrease CDO activity and 50 % inhibition was

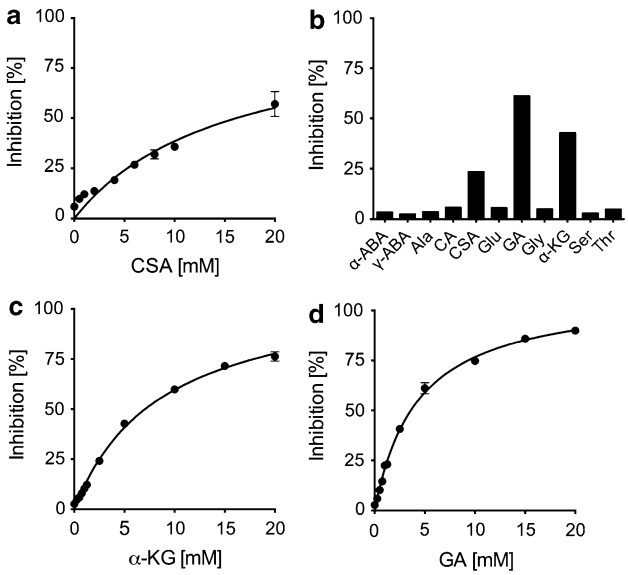


**Fig. 5** Identification of human CDO inhibitors using the well plate-based activity assay. Activity of wt CDO (3 µM) was assayed at a cysteine concentration of 0.2 mM and increasing amounts of CSA using the well plate assay (**a**). Based on the observed product-based inhibition of CDO, activity of CDO was investigated in the presence of 5 mM of various compounds being structurally similar to CSA (**b**). Among all investigated compounds, α-ketoglutarate (α-KG) (**c**) and glutarate (GA) (**d**) were further investigated using a concentration range of 0–20 mM (**c**, **d**). The abbreviations used are: *α-ABA* α-aminobutyric acid, *γ-ABA* γ-aminobutyric acid, *Ala* alanine, *CA* cysteic acid, *CSA* cysteine sulfinic acid, *Glu* glutamic acid, *GA* glutarate, *Gly* glycine, *α-KG* α-ketoglutarate, *Ser* serine, *Thr* threonine

reached with concentrations above 10 mM (Fig. 5a), which is in line with our observed inhibition at cysteine concentrations higher than 3 mM.

Next, we examined the inhibition of CDO by different compounds being structurally similar to CSA and found significant inhibition with 5 mM α-ketoglutarate and

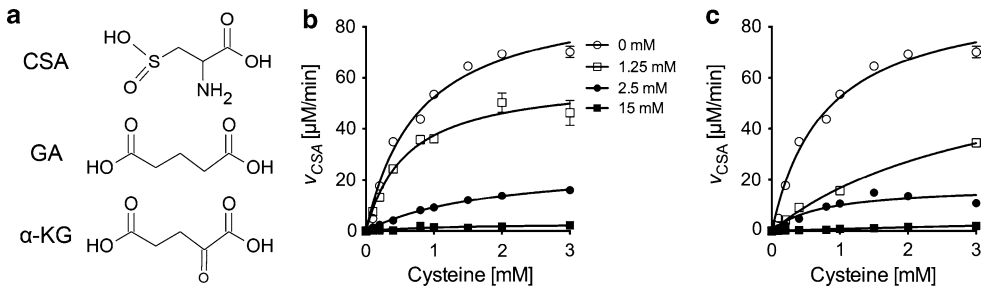


**Fig. 6** Characterization of CDO inhibition through α-ketoglutarate and glutarate. Structure of α-ketoglutarate (α-KG) and glutarate (GA) showing the similarity to CSA (**a**). Investigation of the inhibition pattern of α-ketoglutarate (**b**) and glutarate (**c**) was assayed using the standard kinetic assay with HPLC at different concentrations of each compound (**b**–**c**). Kinetic characterization of the inhibition resulted in a lower $v_{max}$ and $K_m$ as compared to control and complete inhibition of the enzyme was reached using 15 and 10 mM of α-ketoglutarate and glutarate, respectively

glutarate (Fig. 5b). When applying different concentration of α-ketoglutarate and glutarate, which both showed stronger inhibition than that observed for CSA, 50 % CDO inhibition was reached for both compounds at approximately 5 mM (Fig. 5c, d).

Our results using the well plate assay pointed toward an effective inhibition of CDO by α-ketoglutarate and glutarate. In order to better quantify the inhibition of α-ketoglutarate and glutarate (Fig. 6a), we used the standard kinetic protocol and CSA detection via HPLC, which due to high oxygenation levels and sensitivity is more suitable for determination of the kinetic parameters and inhibition pattern. Already a low milimolar range of both compounds was able to effectively inhibit CDO and both $v_{max}$ and $K_m$ were lowered indicating a mixed inhibition pattern (Fig. 6b, c). Complete inhibition of CDO was achieved at concentrations above 15 and 10 mM of α-ketoglutarate and glutarate, respectively.

## Discussion

Mammalian CDO is crucial in controlling intracellular cysteine levels and increase in cysteine dietary intake is directly accompanied by an increase in CDO protein levels due to a decreased proteasomal degradation of the protein in response to substrate availability (Dominy et al. 2006). Crystal structures of various CDO proteins identified the Cys-Tyr cofactor (McCoy et al. 2006; Simmons et al. 2006b; Ye et al. 2007), which was found to increase catalytic efficiency (Dominy et al. 2008). Given that cysteine treatment increases cofactor formation, one can see this cross-link as a post-translational regulatory mechanism in response to substrate availability (Stipanuk et al. 2009). Unlike other cofactor-containing enzymes (Dooley 1999; Whittaker and Whittaker 2003), cross-link formation in CDO requires the substrate cysteine in addition to ferrous iron and oxygen (Dominy et al. 2008). Furthermore, iron plays a crucial role not only in catalysis but also in cofactor formation in CDO and differences in iron saturation (10–70 %) (Chai et al. 2005; McCoy et al. 2006; Simmons et al. 2006a, b) are probably attributed to the loosely bond character of the ferrous iron in the active center of the recombinantly purified enzyme. However, no binding studies between iron and CDO have been conducted so far. Therefore, we investigated in this study the kinetic properties of cofactor-free and cofactor-saturated human CDO and found a strong increase in catalytic efficiency, which correlated with an increased binding of ferrous iron to CDO. Furthermore, by establishing a well plate-based CDO activity assay, we identified product inhibition of CDO and found α-ketoglutarate and glutarate as additional inhibitors.

Several groups reported different kinetic parameters for recombinant CDO, which are in part a result of the different kinetic assays used (Chai et al. 2005; Imsand et al. 2012; McCoy et al. 2006; Simmons et al. 2006a; Ye et al. 2007). Despite a sequence identity of 92 % between rat and human CDO, Ye and colleagues reported a $K_m$ of 3.1 mM for human CDO and no substrate inhibition (Ye et al. 2007), while Simmons and colleagues reported a $K_m$ of 0.45 mM for rat CDO accompanied by an inhibition at high cysteine concentrations (Simmons et al. 2006a). It is important to note that the CDO reaction is a dual-substrate reaction, and the determination of kinetic parameters for one substrate (cysteine) should be performed at saturating conditions for the other substrate (oxygen) to ensure pseudo-first order conditions. However, iron is an additional factor, which may influence the activity of CDO through cofactor formation. In fact, our recombinantly expressed and purified CDO displayed an equal distribution of both CDO forms (with and without cofactor), which is in accordance to previous reports (Dominy et al. 2008; Li et al. 2013). Knowing that cofactor formation is dependent on iron and oxygen, all kinetic assays in this study were performed

under conditions, which favor the formation of the cofactor in CDO as previously reported (Dominy et al. 2008). As a result, our findings ($K_m$ = 0.77 mM) are in line with the reported kinetic parameters of rat CDO ($K_m$ = 0.45 mM), suggesting indeed, that iron supplementation is required for full activity of CDO resulting in the determination of reproducible kinetic parameters.

The Cys-Tyr cofactor present in CDO has been reported to increase the catalytic efficiency by a factor of 10, a process that was strictly dependent on the presence of cysteine (Dominy et al. 2008). Also we have identified a decrease in catalytic activity when cross-link formation was impaired resulting in a 13-fold increase in catalytic efficiency of wt CDO (806 $mM^{-1}$ $min^{-1}$) in comparison to the cofactor-free variant (59 $mM^{-1}$ $min^{-1}$). Moreover, in vitro produced cofactor-saturated CDO revealed identical kinetic parameters to wt CDO, attesting that under our assay conditions a complete cross-link saturation of wt CDO was reached.

A recent study suggested that iron is able to promote non-enzymatic CSA formation because a linear dependence of dioxygen consumption with increasing iron concentrations was reported when exogenous iron was added to the activity assay (Imsand et al. 2012). The study also suggested that external reductants such as L-ascorbate instead of iron promote an increase in catalytic efficiency causing a 50-fold increase in $k_{cat}/K_m$ (from 1.7 to 89 $mM^{-1}$ $min^{-1}$) (Imsand et al. 2012). Our own iron titration experiments showed that CDO dependence on iron supplementation does not follow a linear pattern. In fact, very low iron concentrations were sufficient to saturate wt CDO activity, while the cofactor-free C93A CDO variant showed a more linear dependence on iron supplementation similar to a previous study (0–15 μM) (Imsand et al. 2012). However, at higher iron concentration, saturating activity was also reached with the C93A CDO variant. Furthermore, we could not observe non-enzymatic CSA formation using cysteine concentrations up to 3 mM and iron concentrations up to 0.3 mM, which is in contrast to the previous study (Imsand et al. 2012). Also our titration experiments with EDTA showed an efficient inactivation of the cofactor-free CDO variant. Consequently, the loosely bond character of iron and the requirement of higher iron concentrations for cross-link formation resulted probably in a low ratio of cross-linked CDO protein following the assay conditions of Imsand et al. (2012). In fact, the here reported $k_{cat}/K_m$ for wt CDO was one order of magnitude higher than the reported one in their study (from 89 to 806 $mM^{-1}$ $min^{-1}$) (Imsand et al. 2012) and is close to the determined value for the cofactor-free CDO (59 $mM^{-1}$ $min^{-1}$). Most importantly, our determined $K_d$ values of 2.2 ± 0.7 and 39.7 ± 8.0 μM for wt CDO and C93A, respectively, clearly demonstrate that the Cys-Tyr cofactor impacts on iron binding in human CDO. Taken together, the difference in iron dependence between wt CDO and C93A variant suggests a crucial structural role of the Cys-Tyr cofactor in coordinating iron in the active center, which is in accordance to the proposed mechanism of Ye et al. (2007).

Our kinetic characterization confirmed CDO inhibition at high substrate concentrations, as previously reported for rat CDO (Dominy et al. 2008; Simmons et al. 2006a). However, CDO inhibition was markedly attenuated with C93A. Therefore, we investigated product inhibition of CDO using a simple and fast well plate-based assay and found 50 % inhibition with 10 mM CSA. Also, the structurally similar α-ketoglutarate and glutarate showed comparable inhibition of CDO. The latter confirmed a previous study, which identified α-ketoglutarate as inhibitor of rat CDO using an HPLC assay (Chai et al. 2006). Therefore, our well plate-based assay is well suited for inhibition studies with large and complex compound libraries. Furthermore, CDO inhibition by α-ketoglutarate and glutarate was confirmed using the classical activity assay with HPLC detection and a mixed type of inhibition was detected for both compounds. This finding attests that the negatively charged side chain of the carboxylates in α-ketoglutarate and glutarate could substitute the thiolate group of cysteine and bind to the CDO active site. The fact that cysteic acid, which is structurally similar to CSA could not inhibit CDO suggests an important role of the second side chain carboxylate group in the mechanism of CDO inhibition by α-ketoglutarate and glutarate.

In conclusion, our results identified an important function of the Cys-Tyr cofactor in iron binding in the active center of CDO, which would support a tetrahedral coordination geometry along with the three histidines surrounding the iron site in CDO. Thus, we suggest that future studies should take into account the requirement for iron, oxygen and substrate for kinetic determination of CDO. Furthermore, we presented a fast and simple assay for identification of CDO inhibitors that was effective in identifying two CDO inhibitors that structurally mimic the reaction product CSA. This finding could provide the basis for large inhibitor screening studies, which will contribute to understand the functional importance of CDO not only on cysteine homeostasis but also on other diseases given that CDO has been associated with cancer and neurodegenerative disorders (Jeschke et al. 2013; Prabhu et al. 2014).

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**Conflict of interest** The authors do not declare a conflict of interest.

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