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Inactivation of Human Aldehyde Oxidase by Small Sulfhydryl-containing Reducing Agents

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Abbreviations

2-ME, 2-mercaptoethanol; 4-VP, 4-vinyl pyridine; AOX, aldehyde oxidase; DCPIP, 2,6-dichlorophenolindophenol; DDI, drug–drug interactions; DTE, dithioerythritol; DTT: dithiothreitol; FAD, flavin adenine dinucleotide; GSH, glutathione; hAOX1, human aldehyde oxidase; IAM, iodoacetamide; Moco, molybdopterin cofactor; NEM, N-ethylmaleimide; ROS, reactive oxygen species; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

Abstract

Human aldehyde oxidase (hAOX1) is a molybdoflavoenzyme that belongs to the xanthine oxidase (XO) family. hAOX1 is involved in phase I drug metabolism, but its physiological role is not fully understood to date and preclinical studies consistently underestimated hAOX1 clearance. In the present work, we report an unexpected effect of the common sulfhydryl-containing reducing agents, *e.g.* dithiothreitol (DTT), on the activity of hAOX1 and mouse

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aldehyde oxidases. We demonstrate that this effect is due to the reactivity of the sulfido ligand bound at the molybdenum cofactor (Moco) with the sulfhydryl groups. The sulfido ligand coordinated to the Mo atom in the XO family of enzymes plays a crucial role in the catalytic cycle and its removal results in the total inactivation of these enzymes. Since liver cytosols, S9 fractions and hepatocytes are commonly used to screen the drug candidates for hAOX1, our study suggests that DTT treatment of these samples should be avoided, as otherwise false negative results by an inactivated hAOX1 might be obtained.

Significance statement

This work characterizes the inactivation of human aldehyde oxidase (hAOX1) by sulfhydryl-containing agents and identifies the site of inactivation. The role of DTT in the inhibition of hAOX1 should be considered for the preparation of hAOX1-containing fractions for pharmacological studies on drug metabolism and drug clearance.

Introduction

Human aldehyde oxidase (hAOX1) is a molybdoflavoenzyme that together with other aldehyde oxidases (AOXs) and xanthine oxidoreductases (XOR), including xanthine oxidase (XO) and xanthine dehydrogenase (XDH), belong to the XO family of molybdoenzymes (Hille et al., 2014). hAOX1 plays an important role in drug metabolism (Mota et al., 2018) and it has been suggested to be involved in potential drug–drug interactions (DDI) (Takaoka et al., 2018; Beedham, 2020). However, the physiological endogenous substrate of this enzyme is not known to date and allometric scaling from preclinical data mostly underestimates hAOX1 clearance (Manevski et al., 2019). As a result, promising drug candidates are frequently dropped from consideration, mainly due to inadequate studies on hAOX1 metabolism in preclinical systems.

hAOX1 is a 150 kDa cytosolic enzyme that is active as a homodimer (Hille et al., 2014). Each monomer contains four cofactors including two [2Fe–2S] clusters, one flavin adenine dinucleotide (FAD), and one molybdenum cofactor (Moco) that contribute to the redox-cycling catalytic mechanism of this family of enzymes. In each catalytic cycle, the substrate is oxidized at the Moco site, the electrons generated are internally transferred via the FeS clusters to the FAD cofactor and an electron acceptor at FAD receives the electrons. Molecular oxygen is the physiological electron acceptor of AOXs. The Moco center in the XO family is formed by a Mo atom coordinated to one oxo, one sulfido, one hydroxyl and a bidentate pterin ligand in a five-coordinate square pyramid arrangement. One typical feature of enzymes of the XO family is the so-called cyanolyzable sulfur: an essential sulfur atom (Massey and Edmondson, 1970; Nishino

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et al., 1975; Wahl and Rajagopalan, 1982) present as a sulfido ligand in the equatorial position of the Mo coordination sphere (Okamoto et al., 2004). It was observed that in bovine milk XO upon treatment with cyanide (CN^-), a sulfur group was released as thiocyanate (SCN^-) resulting in the formation of an inactive form of the enzyme that occurs naturally in high amounts at least in eukaryotes (Massey and Edmondson, 1970; Nishino et al., 1975). The cyanolyzable sulfur atom was shown to be a ligand bound at Mo, later identified as being present as a Mo=S group by electron paramagnetic resonance (EPR) studies (Gutteridge et al., 1978). The active form of bovine milk XOR that contains the sulfido ligand can be reproducibly isolated using a folate affinity chromatography procedure coupled with allopurinol (Nishino et al., 1981; Nishino and Tsushima, 1986). However, when heterologous expression systems, *i.e. E. coli*, are applied to produce the mammalian AOXs (including hAOX1 and mouse isoenzymes), due to different enzymatic machinery for sulfuration, the sulfido ligand is not efficiently inserted. Therefore, an *in vitro* chemical reconstitution of the sulfido ligand is required to obtain the more sulfurated active form of the mammalian AOXs (Foti et al., 2016; Kücükgöze et al., 2017).

The related enzymes, *i.e.* the mammalian XORs, are redox regulated and can interconvert between the XDH and XO states (the so-called dehydrogenase to oxidase (D/O) conversion) upon formation of a disulfide bond (*e.g.* between Cys992 and Cys535 in bovine XDH), or irreversibly by mild proteolysis (Rajagopalan and Handler, 1967; Nishino et al., 2008). Dithiothreitol (DTT) was the reducing agent that was used to convert the oxidase form of XO back into the dehydrogenase form by reducing the disulfide bond formed between two cysteines (Turner et al., 1995; Nishino et al., 2008). In contrast, there is no reported redox regulation of AOX with studies on rabbit liver AOX (Turner et al., 1995) showing that this enzyme is stable in the oxidase form, and it cannot switch to the dehydrogenase form. This arises as the two cysteines identified to be crucial for the D/O conversion in XORs, are not present in the AOXs (Turner et al., 1995; Coelho et al., 2015). Since AOXs are cytoplasmic enzymes containing free thiol groups and have no reported thiol-based redox regulation, the use of DTT during their production is expected to improve the yield.

In the present work, we identify a previously unreported and unexpected negative effect of DTT and other small sulfhydryl-containing reducing agents on the activity of hAOX1. In addition, we demonstrate that the negative effect is due to the reactivity of the sulfhydryl group towards the sulfido ligand bound at Moco. hAOX1 is involved in phase I drug metabolism (Mota et al., 2018) and liver cytosol and S9 fractions (De Sousa Mendes et al., 2020), or cryopreserved primary hepatocytes (Hutzler et al., 2012) are among the systems frequently employed to screen

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drug candidates for hAOX1 activity. DTT treatment of these fractions consequently will have a negative effect on hAOX1 activity and will give false negative results in the related studies.

Materials and Methods

Enzyme production

hAOX1 and mouse AOXs were expressed and purified as previously described (Foti et al., 2016; Kücükgöze et al., 2017), except that a Superose 6 column (self-packed) was applied for the size exclusion chromatography (SEC) of hAOX1 purification using 50 mM TrisHCl, 200 mM NaCl, 1 mM EDTA, pH 8.0 as SEC buffer. The enzyme metal saturation was estimated using inductively coupled plasma-optical emission spectroscopy (ICP-OES) method (Optima 2100 DV, PerkinElmer Life and Analytical Sciences) using a multi-element solution (Solution XVI, Merk) as external standard (Neumann and Leimkühler, 2008). Protein concentration was calculated using the absorbance at 450 nm using a Shimadzu UV-2401PC spectrophotometer. A molar extinction coefficient of 21100 $M^{-1}cm^{-1}$ and molecular weight of 150 kDa (MW) were considered for both hAOX1 and mouse AOXs as previously reported (Foti et al., 2016; Kücükgöze et al., 2017). See **SI-Table 3** for the details of metal saturation and purification index ratios of the enzymes used in this study.

Activity measurements

All activity measurements were carried out at room temperature and the average of at least three independent measurements with the standard deviation indicated as ±SD was reported as the final values. Activity of hAOX1 and mouse AOXs were measured in a 500 µL reaction containing air-saturated SEC buffer as mentioned above, 40 µM phenanthridine and 150-200 nM of the enzyme with molecular oxygen as the final electron acceptor. Appearance of phenanthridinone (ε_{321}: 6400 $M^{-1}cm^{-1}$) was followed at 321 nm for 30 seconds (Shimadzu UV-2401PC spectrometer). When mentioned, 100 µM of 2,6-dichlorophenolindophenol (DCPIP) (ε_{600}: 16100 $M^{-1}cm^{-1}$) was used as an alternative electron acceptor and the reaction tracked at 600 nm for 60 seconds.

E. coli aldehyde oxidase PaoABC was purified as described previously (Otrelo-Cardoso et al., 2014). 0.7 µM enzyme (ε_{450}: 23686 $M^{-1}cm^{-1}$, MW: 136 kDa) and 500 µM benzaldehyde were used in a 500 µL total volume and formation of the oxidized product (ε_{295}: 1321 $M^{-1}cm^{-1}$) was followed in air-saturated buffer (50 mM TrisHCl, 200 mM NaCl, 1 mM EDTA, pH 6.0). *Rhodobacter capsulatus* XDH (*Rc* XDH) was obtained according to previous reports (Leimkuhler et al., 2003). 10 nM of *Rc* XDH (ε_{450}: 31600 $M^{-1}cm^{-1}$, MW: 135 kDa and 80% Mo saturated) was used in a 500 µL reaction containing 1 mM xanthine and 1 mM nicotinamide

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adenine dinucleotide (NAD^+) in the activity buffer (50 mM TrisHCl, 200 mM NaCl, 1 mM EDTA, pH 6.0) and the formation of NADH (ε_{340}: 6220 $M^{-1}cm^{-1}$) was monitored at 340 nm. Bovine milk xanthine oxidase (bXO) was purchased from Roche (ε_{450}: 35800 $M^{-1}cm^{-1}$, MW: 150 kDa, 100% Mo) and the enzymatic reaction was followed at 295 nm using 1 mM xanthine (ε_{295}: 8400 $M^{-1}cm^{-1}$) and 10 nM bXO in air-saturated buffer (50 mM TrisHCl, 200 mM NaCl, 1 mM EDTA, pH 8.0).

To study the effect of different chemicals including DTT, dithioerythritol (DTE), 2-mercaptoethanol (2-ME), glutathione (GSH), 4-vinyl pyridine (4-VP), iodoacetamide (IAM) or N-ethylmaleimide (NEM) on the activity of hAOX1 or the XO family enzymes, the enzyme was incubated with the chemical at a 100x molar ratio (*i.e.* 1 mM final concentration for the samples of about 10 µM enzyme) for 30 minutes at RT, the enzyme was desalted using PD10 columns (GE Health Care) to remove the chemical and the residual activity was measured as explained above.

Circular Dichroism (CD) Spectroscopy

CD measurements were carried out at 22 ± 0.1 °C using a JASCO 815 CD spectropolarimeter equipped with a Peltier PTC-423S device to maintain the temperature. The instrument was calibrated using 1S-(+)-10-camphorsulphonic acid (Kelly et al., 2005). hAOX1 samples contained 400 µL of about 5 µM protein in SEC buffer as mentioned above. CD spectra were recorded in three accumulations using a quartz cuvette (1 mm path length, Hellma, Germany) over a wavelength ranging from 300 to 600 nm. Buffer was used to correcting the baseline and ellipticities were converted to mean residue ellipticities Θ_{MRW} after being normalized to the protein concentration using the absorbance at 280 nm (peptide backbone) and at 450 nm (FAD cofactor absorption). All measurements were done at least three times and the final spectra were reported as the average of all acquisitions.

Electrospray Ionization Mass Spectrometry (ESI)

hAOX1 was incubated with 100x molar ratio IAM or NEM (*i.e.* 1 mM final concentration for the protein samples around 10 µM) for 30 minutes at RT and resolved on a reducing SDS-PAGE. Bands were cut and dried using a SpeedVac (Christ RVC 2-18). They were then incubated with DTT and subsequently with a different second label, (*i.e.* .methyl methanethiosulfate (MMTS) or IAM, respectively for the IAM and NEM treated samples) to protect the cysteines residues which became exposed after DTT treatment. In-gel tryptic digestion was carried out at 37 °C overnight. Peptides were extracted from the gel, separated by HPLC (Evosep, Odense) and measured on an ESI-TIMSTOF mass spectrometer (Bruker, Bremen). Peptides were identified and quantified using the Peaks Software (BSI, Waterloo, Ontario, Canada). For the analysis the percentage of

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each modification was calculated in a semi-quantitative method (similar to the method applied by Shetty and colleagues (Shetty et al., 2007)), *i.e.* the intensity of the peptides carrying the desired modification was considered against all the peptides containing a cysteine of interest and the percentage of the modification was obtained for each cysteine.

Results:

The Effect of Dithiothreitol (DTT) on Enzyme Activity

DTT is often added during the purification of cytoplasmic enzymes to improve the yield of active and more homogenous enzyme, *e.g.* as previously reported for XO enzymes (Leimkuhler et al., 2003). We, therefore, examined if the addition of DTT would improve the yield of the dimeric active form of hAOX1. The cell pellet was split into two halves and hAOX1 was purified in parallel, with 2.5 mM DTT being added to the SEC buffer in one part but not to the other. The SEC profile presented a slightly sharper dimer and slightly smaller aggregation peak in the hAOX1 sample purified with the SEC buffer supplemented with DTT (**Figure 1, A**). DTT was then removed and the UV-visible spectra were obtained for both samples. Comparing the UV-Vis absorption of the two samples showed nearly identical spectra including the typical peaks at 280, 450 and 550 nm, respectively, for the peptide backbone, FAD and FeS clusters (**Figure 1, A** inset). In addition, the metal content of the samples purified in the presence or absence of DTT was measured using ICP-OES, but no difference was observed in the metal saturation (**SI-Table 3**). Together these results implied that DTT improves the amount of dimeric form of hAOX1, while does not affect the UV-Vis absorption or the metal saturation of the enzyme.

Unexpectedly, the hAOX1 purified in the presence of DTT showed a complete loss of activity (40 μM phenanthridine was used as the reducing substrate and oxygen as the electron acceptor; **Figure 1, B**). To confirm this effect, the active enzyme was incubated with 1 mM DTT (100x molar ratio to enzyme) and a similar effect was observed. DTT was removed, but the lack of reactivation of the enzyme after DTT removal suggests that DTT irreversibly inactivated hAOX1.

Inactivation of hAOX1 with Other Sulfhydryl-containing Agents

DTT contains two sulfhydryl groups (see supplementary **SI-Table 1** for the structures of the chemicals used in this study) and we hypothesized that these reactive groups were responsible for the inactivation of hAOX1. To test this, hAOX1 was incubated with 100x molar ratio (*i.e.* 1 mM final concentration for the protein samples around 10 μM) of 2-ME containing a 2-carbon backbone and one sulfhydryl, the reduced form of glutathione (GSH), a tri-peptide containing a

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sulfhydryl on a cysteine residue, DTE, an epimer of DTT with less reactivity, and erythritol, a structural homologue of DTE containing two hydroxyl groups instead of the sulfhydryls. In addition, the effect of three widely used cysteine alkylating agents, *i.e.* IAM, NEM and 4-VP was studied. In each condition after the removal of the chemical, the activity was measured in parallel with a control hAOX1 sample incubated under the same conditions (**Figure 1, B**).

It was observed that GSH only decreased the enzyme activity less than 30 percent, while 2-ME inactivated hAOX1 completely in a similar manner to DTT (**Figure 1, B**). DTE caused more than 90 percent loss of hAOX1 activity, but erythritol, the epimer without sulfhydryl groups, only reduced the activity of hAOX1 less than 20 percent. Among the thiol alkylating agents NEM and 4-VP completely inactivated hAOX1, but IAM treatment only decreased the activity of hAOX1 less than 30 percent. The IAM treated sample was then treated with DTT, which resulted in a complete loss of activity.

These results imply that a sulfhydryl group is the cause of the inactivation of hAOX1 and that at least one reactive thiol group is present in hAOX1 which is essential for activity. Furthermore, the size and/or charge of the thiol-containing agents is important, as the bulkier and charged GSH showed only less than 30 percent negative effect on the activity, while the smaller and charged 2-ME, DTT and DTE completely inactivated the enzyme.

Investigating the hAOX1 Potential Sites that React with Sulfhydryl-containing Agents

The sulfhydryl group on small reducing agents could potentially react with three sulfur-containing groups in hAOX1, any of which may alter the biological activity: **a)** any solvent exposed disulfide bonds, **b)** inorganic sulfur ligands coordinated to the surface-exposed FeS clusters and **c)** the terminal sulfido ligand bound at Moco. Since hAOX1 is a cytosolic enzyme and no structural disulfide bond has been identified in this enzyme or any other XO family enzymes structures (Hille et al., 2014; Coelho et al., 2015), nor has any transient disulfide bond been reported during the catalytic cycle (Huber et al., 1996), it seemed very unlikely that inactivation occurs via reduction of a disulfide bond.

ESI-MS to Identify the Sites Affected by NEM

To uncover potential binding sites and to map the cysteines in hAOX1, the effect of NEM (that strongly inactivated the enzyme) and IAM (that had a much lower effect on enzyme activity) were studied using ESI-MS.

The ESI-MS labeling experiments demonstrated that 40 out of 42 cysteines present in hAOX1 were fully or partially modified by NEM (see **SI-Table 2**), indicating that at least 40 cysteine residues in hAOX1 do not form a disulfide bond. Out of the two remaining cysteines, Cys1192

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was not detected in any of the peptides, while Cys562 was not alkylated by NEM in any of the peptides identified. The crystal structure of hAOX1 (Coelho et al., 2015) shows that the thiol side chain of Cys562 and Cys1192 locate at 28 Å distance from each other and hence these residues cannot be in a disulfide bond.

Comparing NEM (inactivating) and IAM (much lower effect on activity) showed that 32 cysteine residues in hAOX1 were modified by both IAM and NEM, implying their alkylation was not responsible for inactivation. In contrast, 3 cysteine residues, *i.e.* Cys170, Cys798 and Cys943, were predominantly reactive towards NEM (see **SI-Table 2**).

Cys170 is part of linker I located at the C-terminus of the FeS domain. This residue is 40 percent alkylated when treated with NEM, while is not alkylated by IAM. Specific NEM alkylation of Cys170 could potentially affect the FeS clusters and internal electron transfer, eventually resulting in a loss of activity. However, this is unlikely to be the underlying cause of the difference between NEM and IAM as both NEM and IAM similarly alkylated the FeS cluster binding sites, *i.e.* Cys49, 51, 74, 79 and Cys149, 151, respectively, bound to FeSII and FeSI, indicating that both FeS clusters may be impacted by these alkylating agents. Since IAM decreased hAOX1 activity only less than 30 percent, and since the alkylation of the cysteines bound at the FeS clusters would impair the internal electron transfer, it seems that the modification of FeS binding sites might have occurred only on inactive enzyme fractions that were devoid of FeS clusters, and hence allowed exposure of the cysteines to the alkylating agents.

Cys943 and 798 were about 30 percent accessible for IAM, but more than 95 percent reactive towards NEM, values which correlate with the loss of activity of the enzyme by these two reagents. These cysteine residues have not been mentioned to be involved in substrate or cofactor binding or orienting, however, the Moco domain might be affected if either of these cysteines reacts with an alkylating agent.

Overall, it seems that while alkylation of Cys943 and 798 by NEM most likely results in the inactivation of hAOX1, it is unlikely that a direct reaction of these residues is involved in the inactivating effect of small sulfhydryl-containing agents including DTT.

Sulfur Ligands Bound to Iron-Sulfur Clusters

Small sulfhydryl-containing agents could possibly react with the inorganic sulfur ligands coordinated at the FeS clusters. The IAM vs NEM mapped peptides indicated that the FeS clusters binding cysteines were partially modified (see **SI-Table 2** in the supplementary information) suggesting that FeS clusters might potentially be affected when hAOX1 is treated

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with DTT. To examine this, circular dichroism (CD) was employed to investigate the possible effect of DTT treatment on the FeS clusters.

CD has been previously reported as a method to distinguish the two FeS clusters in *Rhodobacter capsulatus* xanthine dehydrogenase (*Rc* XDH) (Schumann et al., 2008). CD can also be used to examine the FAD and the pterin ligand of Moco. The CD traces of hAOX1 treated and untreated with DTT were studied in the region from 300 to 600 nm to track any alteration in the absorption of FeS clusters, based on the maximum CD signals at 475 nm and 430 nm, respectively, for FeSI and FeSII (**Figure 2, A** and **Table 1**). The CD profile of hAOX1 pre-incubated with DTT (**Figure 2**, grey curves) exhibited a slightly higher absorption than the sample which was not treated with DTT (**Figure 2**, black curve). This can be due to a slightly higher concentration of hAOX1. However, the maximum peak ratios for the FeSI to FeSII in the samples treated and untreated with DTT were 1.2 and 1.3, respectively, at 475 and 430 nm. Since the peak ratios did not show any major differences, it implies that the FeS clusters were not disturbed by DTT and therefore are unlikely to be the sites of DTT inactivation. Similarly, the FAD peaks, with a negative minimum at 377 nm and a positive maximum at 342 nm (Kelly and Price, 2000), showed a similar ratio, again implying that the FAD site was unaffected by DTT. In contrast, in the region around the peaks related to the pterin ligand of Moco around 550 to 580 nm (Ryan et al., 1995) the spectra became more divergent (**Figure 2, A**). The CD trace in DTT treated enzyme (**Figure 2, A**, grey curve) in the range of 500 to 600 nm did not increase to the positive values, while for the sample treated with DTT (**Figure 2, A**, black curve) it increased to the positive values at 580 nm. This resulted in a 1.9 peak ratio at 555 and 580 nm for DTT untreated to DTT treated hAOX1 samples (**Table 1**), which is higher than the peak ratios related to FeS (1.2 and 1.3) and FAD (1.2 and 1.5). Altogether, while CD studies did not suggest that FeS clusters or FAD were affected upon treatment with DTT, they implied that Moco might get disturbed by DTT and that this may result in hAOX1 inactivation.

Sulfido Ligand Coordinated to molybdenum in Moco

Another sulfur-containing site of hAOX1 that could be affected by DTT is the sulfido ligand coordinated at the molybdenum atom. The presence of the sulfido ligand is essential for the activity of the enzyme. During the heterologous expression of hAOX1 in *E. coli* this ligand is not efficiently inserted by the *E. coli* sulfuration system and therefore a chemical reconstitution step in the presence of sodium sulfide and sodium dithionite is mandatory to obtain an active enzyme (Foti et al., 2016; Kücükgöze et al., 2017). If the sulfhydryl group of small reducing agents removes the sulfido ligand, then a chemical reconstitution of this ligand should, at least partially, restore the enzyme activity.

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To test this, protein samples treated with DTT and 2-ME were subjected to a chemical resulfuration step. In parallel, a sample treated with cyanide, a suicide inhibitor that is able to remove the sulfido ligand from Moco (Massey and Edmondson, 1970) was resulfurated as a positive control. After chemical resulfuration, it was observed that the samples treated with DTT or 2-ME regained activity to about 20 to 30 percent, similar to that obtained for the cyanide-treated hAOX1 (**Figure 2, B**). This suggested that the sulfido ligand bound at the molybdenum ion was removed by the sulfhydryl-containing agents resulting in the inactivation of hAOX1 in a similar manner that cyanide affected the enzyme.

The effect of DTT on the Activity of Other Xanthine Oxidase Family Enzymes

To examine whether small sulfhydryl-containing agents have a similar effect on other XO family enzymes, the activities of *E. coli* periplasmic aldehyde oxidoreductase PaoABC*, Rc* XDH, bXO and the four mouse aldehyde oxidase isoenzymes *i.e.* mAOX1, mAOX2, mAOX3 and mAOX4, was recorded with and without prior DTT treatment. In the enzyme assays, molecular oxygen was used as the terminal electron acceptor, except for *Rc* XDH where NAD^+ was the final oxidizing substrate. In addition, DCPIP was employed as an alternative electron acceptor for human and mouse enzymes.

The results obtained showed that *E. coli* PaoABC and bXO did not lose activity upon pre-incubation with DTT, while for *Rc* XDH, and in consistence with previous studies (Leimkuhler et al., 2003), the activity was slightly increased (**Figure 3, B**). Similar to hAOX1, mAOX1, mAOX2 and mAOX3 presented more than 90 percent loss of activity after DTT treatment when using oxygen or DCPIP as electron acceptors (**Figure 3, A**). However, mAOX4 only showed 10 and 30 percent loss of activity, respectively, when molecular oxygen or DCPIP were used as the electron acceptor. Note that mAOX4 among the four mouse isoenzymes shows the least broad substrate specificity and phenanthridine is not among the most active substrates for this enzyme, which is the reason for the low activity obtained in the enzyme assays (Kücükgöze et al., 2017).

Discussion

It was discovered in the 1970s that the XO family enzymes can be inactivated by reagents such as cyanide, which are known as sulfhydryl inhibitors (Massey and Edmondson, 1970). It has been shown that this class of inhibitors is able to remove the sulfido ligand bound at Moco and replace it by an oxo ligand, a substitution that leads to a total abolishment of the enzyme activity (Massey and Edmondson, 1970; Branzoli and Massey, 1974). The sulfido ligand coordinated at Moco can behave as a sulfhydryl group. It can react with a sulfhydryl-containing agent, a reaction through which the activity of the enzymes in this study might have been affected. When the samples treated with 2-ME and DTT were subjected to a chemical resulfuration, the activity

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of hAOX1 was regained up to 20 - 30 percent, which is similar to the amount observed for the sample treated with cyanide (**Figure 2, B**). Hence, hAOX1 inactivation upon treatment with small sulfhydryl-containing agents involves the sulfido ligand bound at Moco. The sulfido ligand may be attacked nucleophilically by one of the sulfhydryl groups on DTT, forming a mixed-disulfide species (**Figure 4,** step 1). Recruitment of a water molecule then results in an oxo group substituting the sulfido ligand, while the mixed-disulfide species generates a persulfide-DTT species (**Figure 4,** step 2).

The redox potential of the species present in the equilibrium might thereby determine the generation of the persulfide-DTT, mixed-disulfide-Moco, and oxo-Moco species. Since hAOX1 crystals for the structural studies were only formed when the enzyme was pre-incubated with DTT (Coelho et al., 2015), and hence, this potentially allows for observation of the mixed disulfide or oxo-Moco species in the X-ray crystal structures of hAOX1. Unfortunately, the resolution of the hAOX1 structures currently available is insufficient to directly see the sulfido ligand; hence, more detailed investigations of the oxo-Moco or mixed-disulfide-Moco species possibly formed after DTT treatment are not possible at the moment.

DCPIP and molecular oxygen were used as two different oxidizing substrates to investigate how DTT affected the activity of mAOXs and hAOX1 (**Figure 3, B**). Oxygen is the physiological electron acceptor that takes electrons from the FAD cofactor, while DCPIP is a redox-active dye often used as an electron acceptor in *in vitro* assays (VanderJagt et al., 1986) which receives electrons directly from Moco (Hartmann et al., 2012). Here, the activities with molecular oxygen for mAOXs were 2 - 10 times higher than the values obtained using DCPIP as electron acceptor. This suggested that the inhibitory effect of DCPIP previously reported for hAOX1 (Foti et al., 2016) is also observed for mouse isoenzymes. More importantly, the loss of activity observed with DCPIP that directly takes electrons from Moco implies that Moco is likely the site that is affected upon treatment with DTT.

In the experiments where other AOXs, e.g. rabbit liver AOX, were investigated for an *in vitro* interconversion of the oxidase form to the dehydrogenase form, the use of DTT as the reducing agent has been mentioned, but no loss of activity was observed (Turner et al., 1995). This implies that the negative effect of DTT on the enzyme activity was specific to hAOX1. To further investigate the effect of small sulfhydryl-containing agents on other XO family enzymes, *Rc* XDH and bXO in parallel with the four mouse isoenzymes and *E. coli* PaoABC, were treated with DTT. Consistent with previous work, DTT did not show negative effects on the activity of bXO (Nishino et al., 2008) and *Rc* XDH (Leimkuhler et al., 2003). In contrast, in hAOX1,

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mAOX1, mAOX2 and mAOX3 DTT treatment caused more than 90 percent loss of activity, while mAOX4 seemed to be only less than 30 percent influenced (**Figure 3**).

The different size and shape of the substrate funnels in hAOX1, mAOX1, mAOX2 and mAOX3 in comparison with the rabbit liver AOX, mAOX4, PaoABC, bXO and *Rc* XDH can be used to explain why these enzymes behaved differently when they were treated with DTT. The substrate funnel of bXO (Cao et al., 2011), *Rc* XDH (Truglio et al., 2002) and mAOX4 (Terao et al., 2020) in the available X-ray crystal or modeled structures of these enzymes was shown to be narrow, while hAOX1, mAOX1, mAOX2 and mAOX3 have a broader substrate specificity with wider substrate funnel (Terao et al., 2020). In the latter enzymes, the wider substrate funnel potentially allows agents such as DTT access to Moco and provides space for formation of a mixed-disulfide species. However, PaoABC deviates from this pattern as it has a broad substrate funnel (Otrelo-Cardoso et al., 2014) and yet it was not inactivated upon treatment with DTT. It is conceivable that the broad substrate funnel of PaoABC makes it easier for substrate and/or DTT to access Moco so that the mixed-disulfide species formed by DTT at Moco is displaced by the substrate and/or another molecule of DTT. Differences between the Moco redox potential of the different XO family enzymes could also explain the different behavior of these enzymes in reaction with DTT. DTT has a redox potential of -366 mV at pH 8.0 (Cleland, 1964), which is in the range of the values measured for Moco redox center in the XO family enzymes (*e.g.* bXO Mo(IV/V): -355 mV (Barber et al., 1982), mAOX3 Mo(IV/V): -468 mV (Mahro et al., 2013) and hAOX1: -476 mV (Mahro PhD thesis, 2013)). This pattern suggests that the enzyme is susceptible to inactivation by DTT when the redox potential of Mo(IV/V) is more negative than that of DTT. Hence, it is probable that the reactivity of the sulfido ligand to sulfhydryl groups is dictated by variations in Moco redox potential. Unfortunately, the redox potential of PaoABC is not available to fully support our hypothesis.

Only with high resolution structural data can the precise mechanism by which DTT inactivates hAOX1 or other XO family enzymes be completely elucidated. It seems that in AOXs accessibility of the sulfido ligand, which is governed by the size and shape of the substrate binding funnel, affects the sulfido ligand's sensitivity to sulfhydryl groups. It also seems that the reactivity of the Moco towards a sulfhydryl group is determined by the redox potential of Mo redox active center.

In conclusion, DTT is a common reagent used to keep the thiol side chains of cysteines of cytoplasmic enzymes in their reduced state. In particular, for XOR enzymes the usage of DTT is mandatory to keep the enzyme in the dehydrogenase form, while for AOXs as the highly related enzymes to XORs, the use of DTT is not thought to have an effect on the activity of the enzyme.

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However, here we show that some members of the aldehyde oxidases including hAOX1 get inactivated upon treatment with DTT and other small sulfhydryl-containing agents. This implies that in pharmacological studies, the usage of DTT (*e.g.* during cell lysis) should be avoided, as otherwise misleading results due to the inactivation of hAOX1 will be obtained. Hence, this report is highly important to consider in pharmacological studies and phase I drug metabolism.

Author Contributions

Participated in research design: Silke Leimkühler, Mariam Esmaeeli.

Conducted experiments: Mariam Esmaeeli, Manfred Nimtz.

Contributed new reagents or analytic tools: Lothar Jänsch, Lloyd Ruddock.

Wrote or contributed to the writing of the manuscript: Silke Leimkühler, Mariam Esmaeeli, Lloyd Ruddock.

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Conflict of interests: No author has an actual or perceived conflict of interest with the contents of this article.
Data availability statement:
"The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Figure Legends:

Figure 1: A: Effect of DTT on hAOX1: Size exclusion chromatography (SEC) profile of hAOX1 in the absence and presence of DTT: Purification was performed using a Superose 6 column using SEC buffer (50 mM TrisHCl, 200 mM NaCl, 1 mM EDTA, pH 8.0) supplemented with 2.5 mM DTT when DTT was present. **Inset:** UV-visible spectra of hAOX1 in the absence and presence of DTT in the range of 250 to 800 nm. **B: Nature of the inactivation of hAOX1 by DTT:** hAOX1 (stored in SEC buffer) was treated with 100x molar ratio of DTT, DTE, 2-ME, reduced glutathione (GSH), Erythritol, NEM, IAM and 4-VP for 30 min at RT. Chemicals were removed after treatment and the specific activity of hAOX1 was measured using oxygen as electron acceptor and 40 µM phenanthridine in an air-saturated SEC buffer as mentioned above at 321 nm. Control bar shows the activity of hAOX1 when incubated at RT for 30 minutes.

Figure 2: Potential effect of DTT on hAOX1 cofactors. A: CD of hAOX1 of about 5 µM native and DTT treated samples in the range from300 to 600 nm in SEC buffer: FeS clusters show two distinct peaks at around 475 and 430 nm, respectively, for FeSI and FeSII, FAD gives CD signal at 342 and 377 nm and Moco shows absorption at 555 and 580 nm. **B:** Resulfuration of about 10 µM protein samples treated with 1 mM cyanide, DTT or 2-ME was performed under anaerobic condition (98% N_2, 2% H_2) using 500 µM of sodium dithionite, 2 mM sodium sulfide and 25 µM methylviologen in the sulfuration buffer (50 mM KH_2PO_4, 0.1 mM EDTA, pH 7.4). The initial activity was about 1300 nmol/min.mg as presented in **Figure 1, A**, control bar.

Figure 3.: Effect of DTT on hAOX1 in comparison with other XO family enzymes: A: Specific activities of *E. coli* aldehyde oxidase PaoABC, bXO and *Rc* XDH for the samples pre-incubated with 100x molar ratio of DTT to enzyme (1 mM final concentration for about 10 µM protein samples). 500 µM benzaldehyde, 1 mM xanthine and 1 mM NAD^+ were used to measure the activities of *E. coli* PaoABC, bXO and *Rc* XDH, respectively, by tracking the reaction at 295, 295 and 340 nm. **B:** Specific activities of hAOX1 and four mouse isoenzymes, native condition

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compared with incubated samples with 100x molar ratio of DTT to enzyme using two different electron acceptors: Specific activities were measured in the SEC buffer using 40 μM of phenanthridine and molecular oxygen in air-saturated buffer and 100 μM DCPIP as electron acceptors. **Inset:** Zoom-in mAOX4 specific activity for better visualization.

Figure 4: The proposed mechanism for inactivation of Moco in the reaction with small sulfhydryl-containing agents: In step 1, the nucleophilic attack of a thiol group present on the reducing agent to the sulfido ligand bound at Moco forms a mixed disulfide species. In step 2, the mixed disulfide species is resolved by an electron transfer from a water molecule that results in an inactive oxo-Moco and a persulfide on the reducing agent. **R** represents the carbon backbone including the hydroxyl groups of DTT, DTE or 2-ME.

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Tables:

Table 1: The CD signal peak values of hAOX1 cofactor in the samples treated and untreated with DTT. CD spectra were recorded in the oxidized state of 5 µM hAOX1 untreated (-DTT) and treated with DTT (+DTT) in a buffer containing 50 mM TrisHCl, 200 mM NaCl, 1 mM EDTA, pH 8.0.

Cofactor	Wavelength (nm)	CD signal (molar ellipticity) - DTT	CD signal (molar ellipticity) +DTT	Ratio (-DTT/+DTT)
FeSI	475	33580	41662	1.2
FeSII	430	50847	65593	1.3
FAD	342	7334	10968	1.5
	377	-27120	-32405	1.2
Moco	555	-19849	-37108	1.9
	580	7132	-13412	1.9

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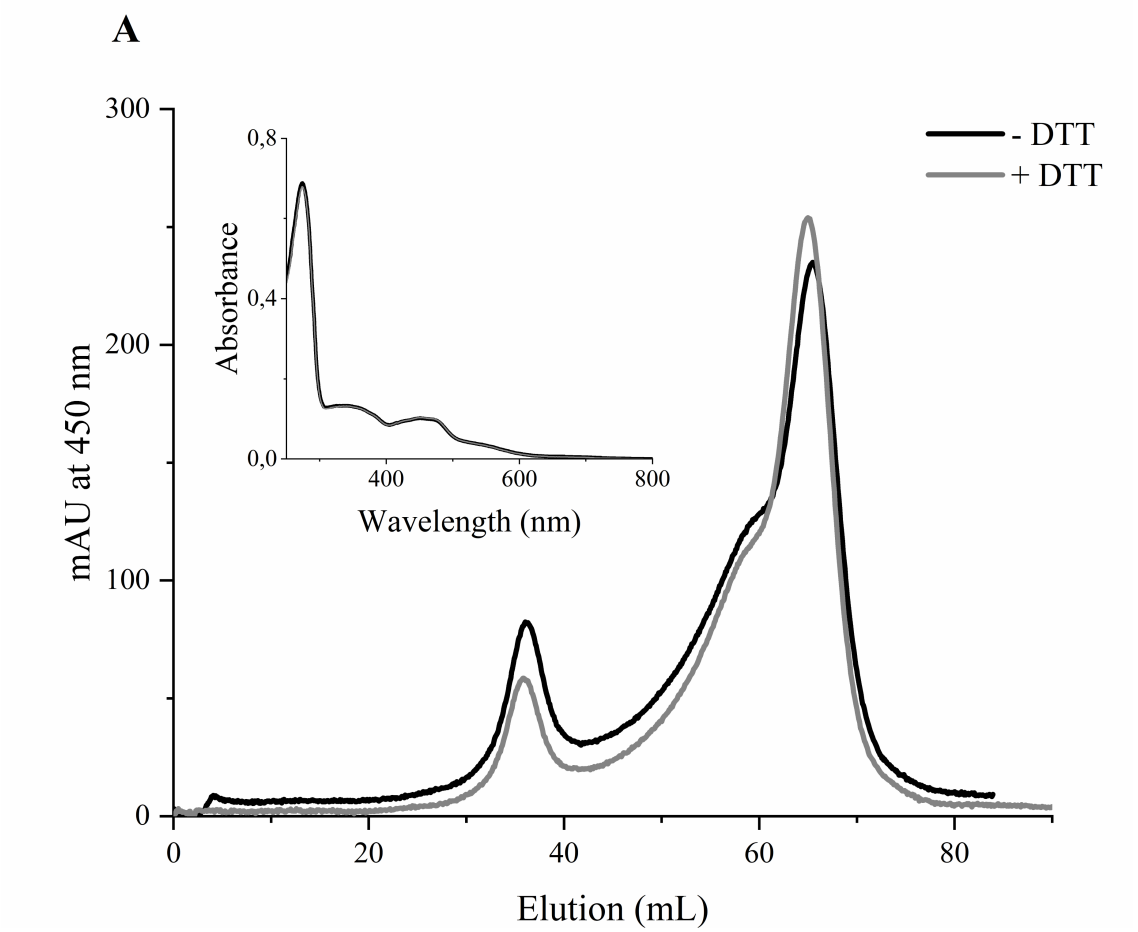

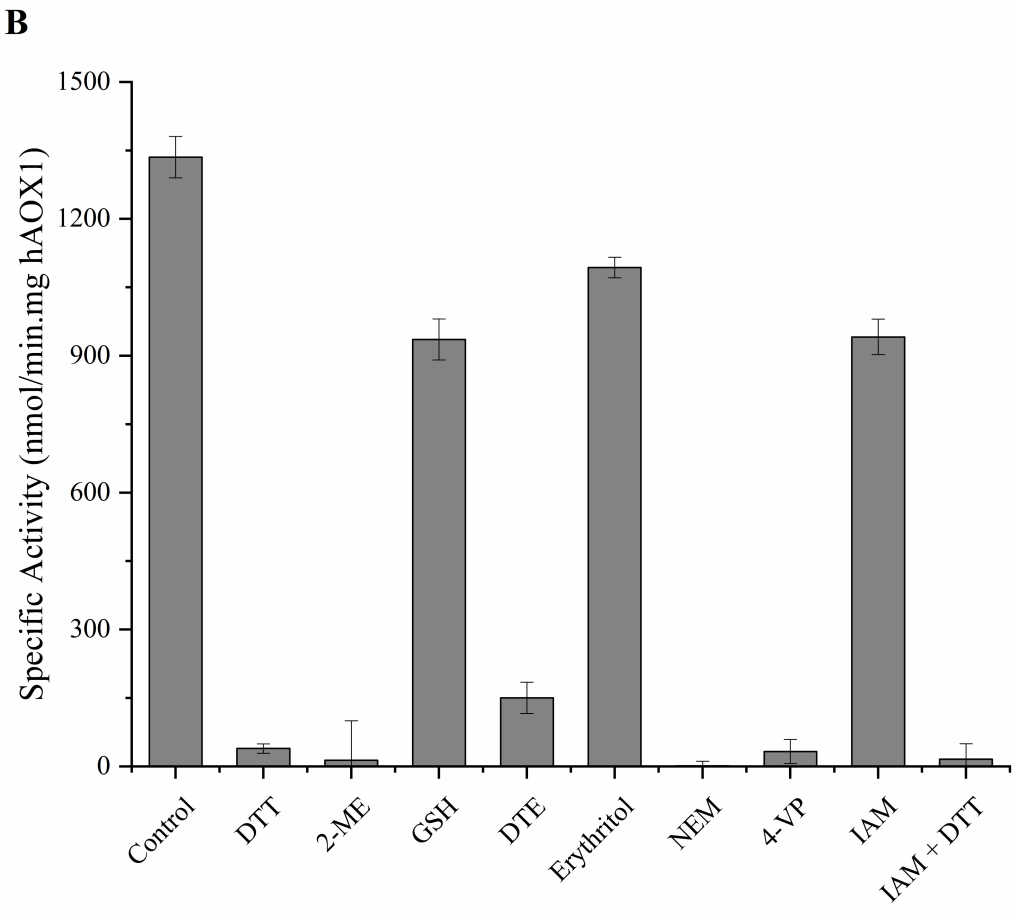

Figure 1

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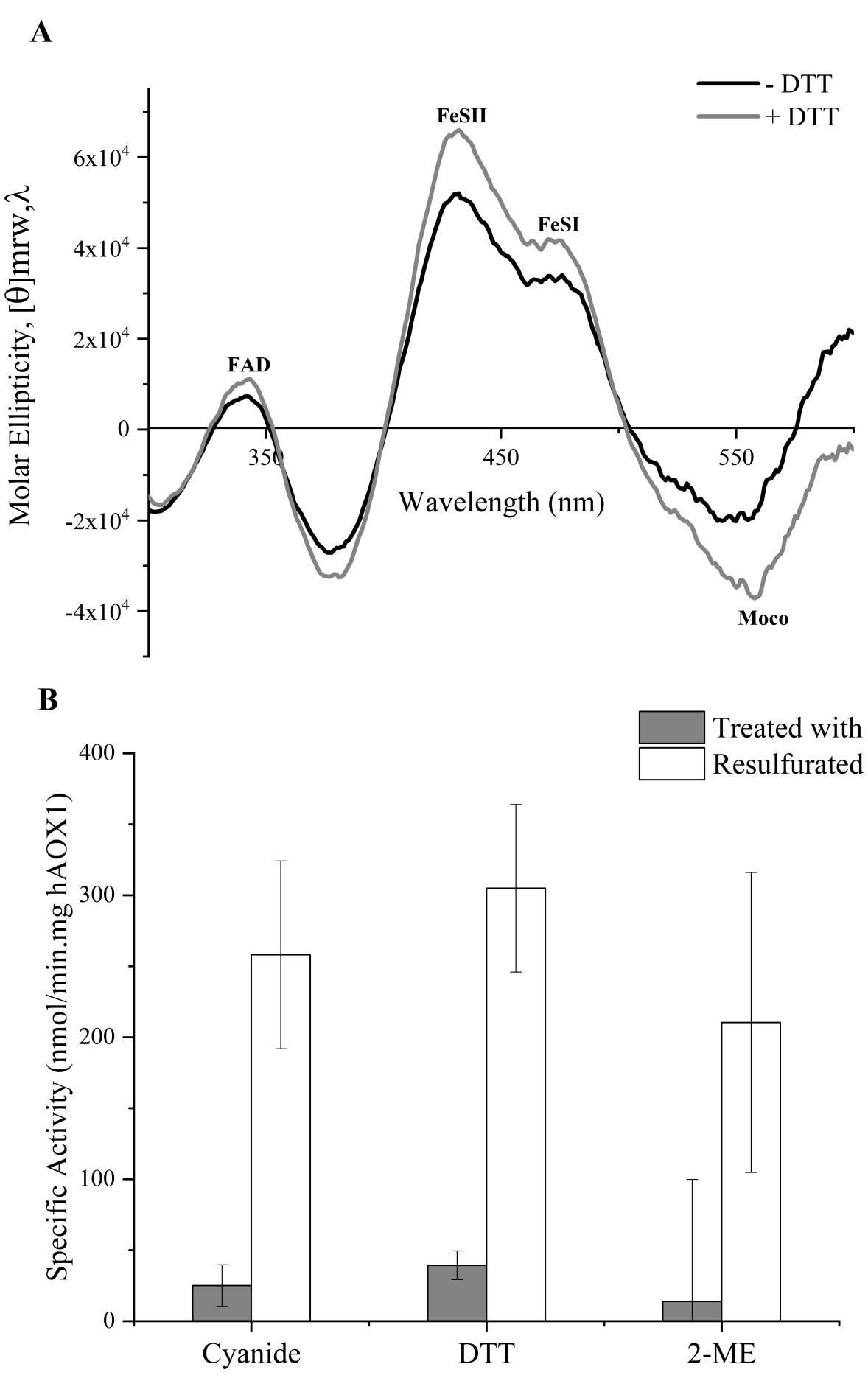

Figure 2

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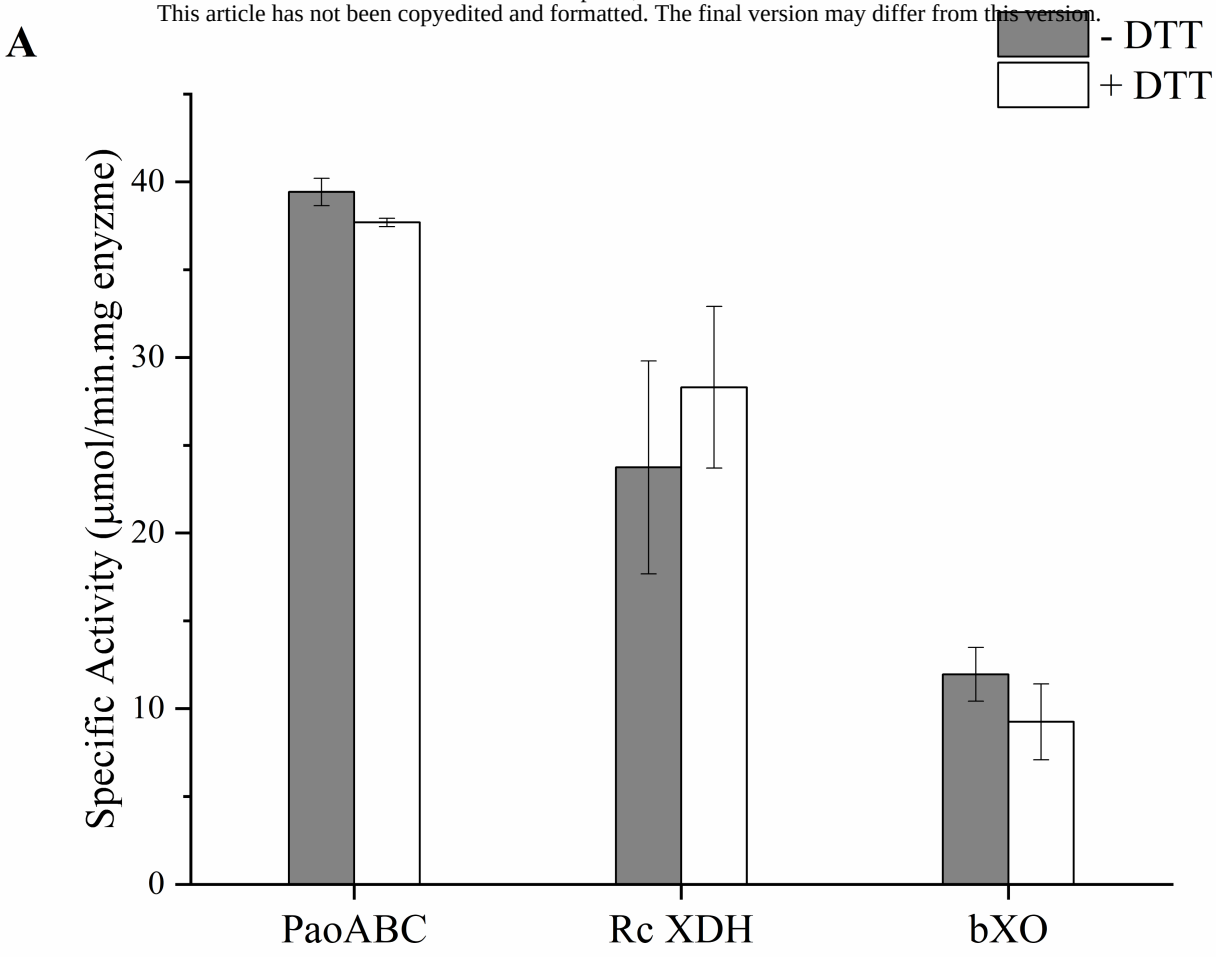

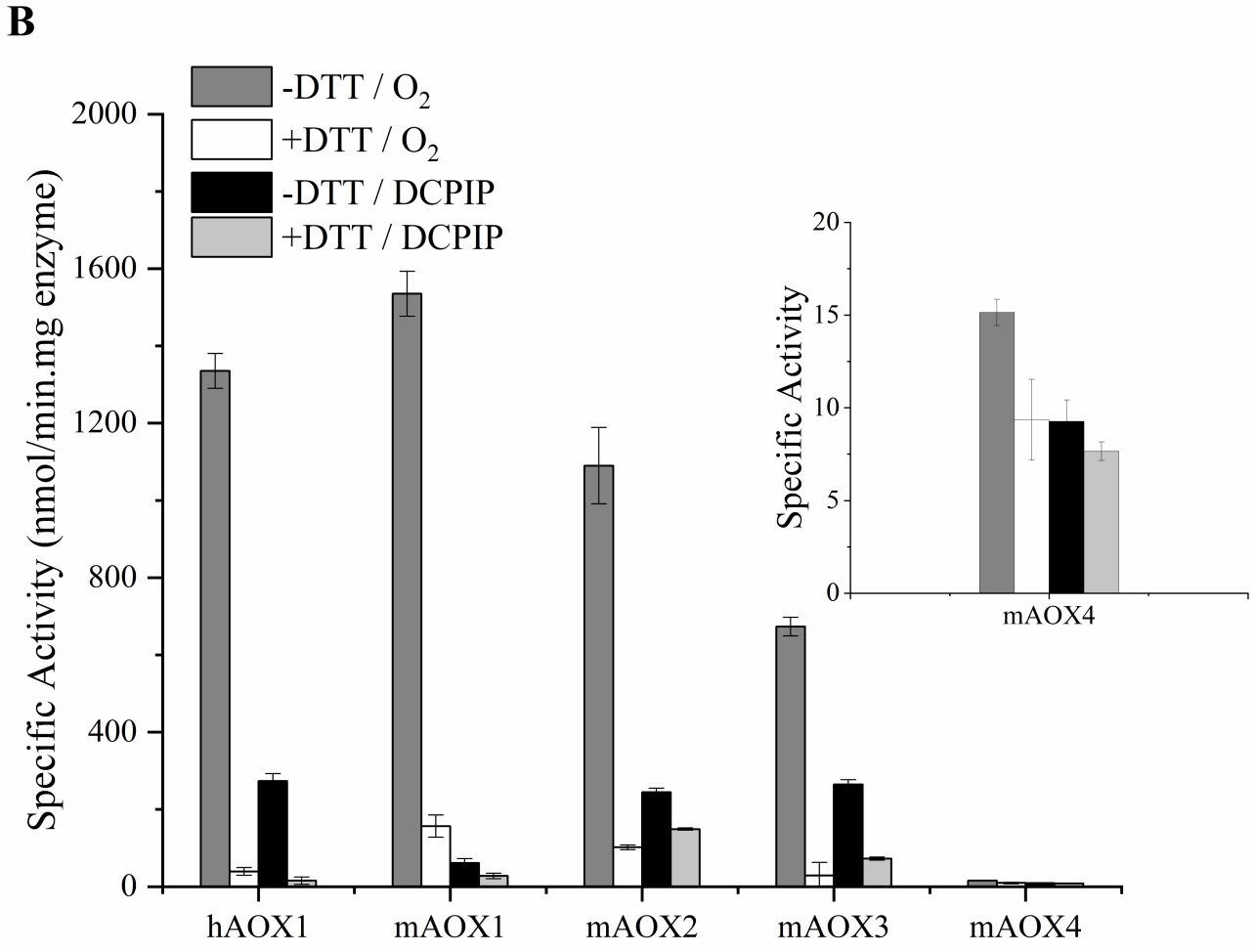

Figure 3

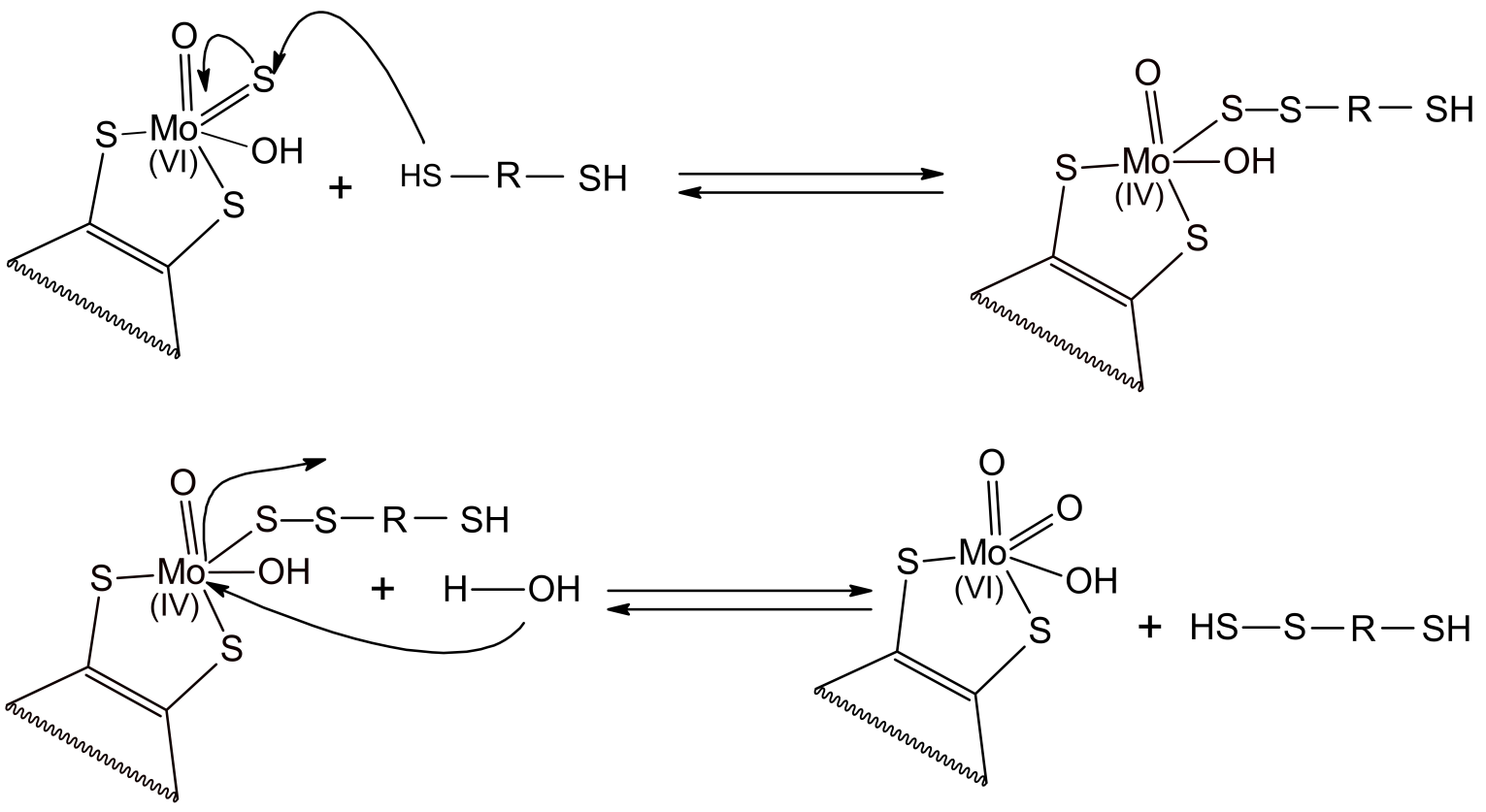
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Figure 4