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The inactivation of human aldehyde oxidase 1 by hydrogen peroxide and superoxide

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Running Title: The substrate-dependent inactivation of hAOX1

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Abbreviations: hAOX1: human aldehyde oxidase 1; AOX: aldehyde oxidase; XO: xanthine oxidase; XDH: xanthine dehydrogenase; Moco: molybdopterin cofactor; *E. coli*: *Escherichia coli*. DCPIP: 2,6-dichlorophenolindophenol, superoxide dismutase: SOD.

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ABSTRACT

Mammalian aldehyde oxidases (AOX) are molybdoflavo-enzymes of pharmacological and patho-physiological relevance, being involved in phase-I drug metabolism, and also in the generation of reactive oxygen species (ROS) as a product of their enzymatic activity. So far, the physiological role of aldehyde oxidase 1 in the human body remains unknown. The human enzyme hAOX1 is characterized by a broad substrate specificity, oxidizing aromatic/aliphatic aldehydes into their corresponding carboxylic acids and hydroxylating various heteroaromatic rings. The enzyme uses oxygen as terminal electron acceptor to produce hydrogen peroxide and superoxide during turnover. Since hAOX1 and in particular some natural variants not only produce H_2O_2, but also high amounts of superoxide, we investigated the effect of both ROS molecules on the enzymatic activity of hAOX1 in more detail. We compared hAOX1 to the high $O_2^{\cdot-}$ producing natural variant L438V for their time-dependent inactivation with $H_2O_2/O_2^{\cdot-}$ during substrate turnover. We show that the inactivation of the hAOX1 wild type enzyme is mainly based on the production of hydrogen peroxide, while for the variant L438V both hydrogen peroxide and superoxide contribute to the time-dependent inactivation of the enzyme during turnover. Further, the level of inactivation was revealed to be substrate-dependent, using substrates with higher turnover-numbers resulted in a faster inactivation of the enzymes. Analysis of the inactivation site of the enzyme identified a loss of the terminal sulfido-ligand at the molybdenum active site by the produced ROS during turnover.

SIGNIFICANCE STATEMENT

This work characterizes the substrate-dependent inactivation of human aldehyde oxidase 1 under turnover by reactive oxygen species and identifies the site of inactivation. The role of ROS in the inhibition of hAOX1 will have a high impact on future studies.

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INTRODUCTION

Human aldehyde oxidase (hAOX1) is a cytosolic homodimeric molybdoflavoenzyme that binds the molybdenum cofactor (Moco), 2x [2Fe-2S] clusters and FAD as prosthetic groups (Terao, Garattini et al. 2020). The enzyme belongs to the xanthine oxidase (XO) family of molybdoenzymes (Hille, Hall et al. 2014). So far, no clear physiological function has been described for hAOX1 (Garattini, Fratelli et al. 2007, Terao, Romao et al. 2016). hAOX1 is a phase-I drug metabolizing enzyme, and its broad substrate specificity, and its unpredictable interindividual variability still presents a challenge to fully understand the role of this enzyme in the human body (Beedham 2020). hAOX1-mediated metabolism has led to several failures in clinical trials and to the termination of drug discovery programs, raising the awareness for the importance of this metalloenzyme (Manevski, King et al. 2019).

Like XO, an enzyme involved in the catabolism of purines (Coughlan 1980), eukaryotic AOX enzymes use oxygen as the terminal electron acceptor and produce reactive oxygen species (ROS) during turnover (Coelho, Foti et al. 2015, Foti, Dorendorf et al. 2017). Mammalian XO, however, exists in two interconvertible forms, the oxidase form (XO) that uses O_2 as electron acceptor and the dehydrogenase form (XDH) that uses NAD^+ as electron acceptor (Enroth, Eger et al. 2000, Kuwabara, Nishino et al. 2003). In contrast, mammalian AOX enzymes solely exist in the oxidase form and cannot be converted to the dehydrogenase form using NAD^+ as electron acceptor (Kurosaki, Bolis et al. 2013). ROS represent prominent key molecules in physiological and pathological conditions in the cell (Oberley 2002). When ROS achieve higher and unbalanced concentrations, they are a dangerous source of damage to several molecules within the cell. ROS can cause damage to the DNA, leading to an oncogenic effect, damage to lipids with consequent peroxidation and damage to residues or cofactors of proteins, e.g. iron-sulfur clusters (Holmstrom and Finkel 2014). So far, mainly XO had been implicated to generate significant amounts of $O_2^{\cdot-}$ during the course of its

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catalytic activity with purine substrates (Terada, Rubinstein et al. 1991, Harris and Massey 1997, Kundu, Hille et al. 2007, Lee, Velayutham et al. 2014).

In our previous studies, the hAOX1 enzyme was shown to produce superoxide radicals with a rate of around 10% as compared to the amount of hydrogen peroxide produced during the overall reaction (Foti, Dorendorf et al. 2017). This value, however, is lower than the reported rates of 16-20% for bovine XO (Nishino and Tamura 1991). Further, it was reported that significant alterations in the rate of superoxide anion production are present in human natural variants of hAOX1 based on single nucleotide polymorphisms (SNPs) (Foti, Dorendorf et al. 2017). In particular the SNP-based amino acid exchange L438V of hAOX1in proximity to the isoalloxanzine ring of the FAD cofactor resulted in an increased rate of superoxide radical production to 75%. This variant is therefore considered to be an overproducer of $O_2^{\cdot -}$. Considering the high toxicity of superoxide in the cell, the hAOX1-L438V SNP variant is a considerable candidate for pathological roles within the human population. The fact that also wildtype hAOX1 produces a significant amount of H_2O_2 and $O_2^{\cdot -}$ radicals might therefore also be of patho-physiological interest (Kundu, Hille et al. 2007). Particularly, it is noticeable that hepatic hAOX1 is calculated to generate 24-fold larger amounts of $O_2^{\cdot -}$ than XO. These calculations are based on the relative levels of XO and AOX enzymatic activity in human liver (Krenitsky, Neil et al. 1972, Kundu, Hille et al. 2007). Thus, hAOX1 and other mammalian AOXs may represent significant sources of ROS in the cytosol of liver cells and other tissues and may play a critical role in ROS-mediated tissue-injury under specific conditions (Hunt and Massey 1992, Kundu, Velayutham et al. 2012). Oxidative damage has long been implicated in both the malignant phenotype and carcinogenesis (Oberley 2002). A role of hAOX1 and XO in cancer have been assigned (Qiao, Maiti et al. 2020). In a recent study on XO, it was shown that the block of ROS production slowed down tumor growth (Kusano, Ehirchiou et al. 2019). Therefore, the

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contribution and role of hAOX1 in ROS production that leads to cancer development needs further investigation in the future.

More than 95 years ago Dixon (Dixon 1925) showed that incubation of bovine milk XO with hydrogen peroxide resulted in an inactivation of the enzyme. This observation has been confirmed several times with XO and with the related XDH (Bergel and Bray 1959, Betcher-Lange, Coughlan et al. 1979). Apart from nonspecific modes of inactivation, the various prosthetic groups in these enzymes have been suggested to be the targets for attack by hydrogen peroxide (Lynch and Fridovich 1979, Terada, Leff et al. 1991). However, the nature of the reaction involved has not been investigated in detail nor has the relation between the sites of inactivation and of catalysis been clearly established. It has been concluded, however, that H_2O_2 treatment of the enzyme results in the desulfo-form at the molybdenum active site (Betcher-Lange, Coughlan et al. 1979).

Since hAOX1 and in particular some variants not only produce H_2O_2, but also high amounts of superoxide, we investigated the effect of both ROS molecules on the enzymatic activity of hAOX1 in more detail. We show that the enzyme is inactivated by hydrogen peroxide, or when produced, also by superoxide during turnover, when the enzyme is in its reduced state. We identified the sulfido-ligand at the active site as target of inactivation and the enzyme activity was restored by a chemical sulfuration procedure, which restores the sulfido-ligand at the Moco active site, being essential for the activity of the enzyme.

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MATERIALS AND METHODS

Expression and Purification of hAOX1 and variants

The hAOX1 wild-type protein and the L438V variant were expressed and purified as described previously, with minor modifications (Foti, Hartmann et al. 2016). The constructs pTHcohAOX1 (hAOX1 wild-type) (Foti, Hartmann et al. 2016), pTHcohAOX1-L438V (Foti, Dorendorf et al. 2017) were transformed into *E. coli* TP1000 (Δ*mobAB*) cells (Palmer, Santini et al. 1996). For protein expression, *E. coli* cell cultures were grown at 30°C in LB medium supplemented with 150 µg/ml ampicillin, 1 mM sodium molybdate and 20 µM IPTG. Cells were harvested by centrifugation after 24 h of cell growth and resuspended in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl. After cell lysis the crude extract containing hAOX1 was first purified using a nickel-nitrilotriacetic acid resin (QIAGEN GmbH, Hilden, Germany) and then subjected to chemical sulfuration before the final purification by a size exclusion chromatography step using a Superdex 200 10/300 GL Column (GE Healthcare) as described previously (Foti, Hartmann et al. 2016).

Metal quantification

Inductively coupled plasma optical emission spectroscopy with an Optima 2100 DV (PerkinElmer Life and Analytical Sciences, Waltham, MA) was used to measure the metal content (Neumann and Leimkühler 2008). 500 µL of purified hAOX1 (about 10 µM) and an equal volume of 65% nitric acid were mixed to wet ash the protein over night at 100°C overnight. The samples were diluted with 4 ml of water. The buffer used as reference was 50 mM Tris HCl, 200 mM NaCl, EDTA 1 mM pH 8.0. The detection was at wavelengths of 203.845 nm, 202.031 nm and 204.597 nm for molybdenum and 238.204 nm, 239.562 nm and 259.939 nm for iron. A standard was used for calibration and quantification of the detected metals (Standard Solution XVI, Merck). The resulting mass concentrations were calculated

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and related as percent of protein saturated with Moco and iron corresponding to the two [2Fe-2S] clusters.

Steady-state Kinetics

Steady state enzyme kinetics were performed with purified 200 nM hAOX1 or 200 nM L438V in 50 mM Tris and 1 mM EDTA buffer, pH 8.0, at 25°C in a final volume of 500 µL. Phthalazine and benzaldehyde were used in a range of 5-200 µM, and for zoniporide the concentrations used were from 75µM to 600µM. The electron acceptor was molecular oxygen, in air-saturated EDTA-Tris buffer pH 8.0. The oxidized product (benzoic acid, phenanthridone or 2-oxo-zoniporide) were detected by UV absorbance (295 nm, 304nm or 315nm, respectively). Reactions were monitored over a range of 60 seconds. Activities were calculated using the molar extinction coefficients of 1166 $M^{-1}cm^{-1}$ (at 304 nm) for phthalazine, 1321 $M^{-1}cm^{-1}$ (at 290 nm) for benzaldehyde and 5775 $M^{-1}cm^{-1}$ (at 315 nm) for zoniporide. Mean values with standard deviations were obtained from at least 3 independent measurements. k_{cat} and K_M values were normalized to 100% molybdenum content. The kinetic constants were obtained using the Michaelis-Menten equation by non-linear regression with the software Origin Pro 8.1G (Waltham, MA). The enzyme kinetics assays were performed on a Shimadzu UV-2401PC photometer at room temperature.

Inactivation of hAOX1 under turnover conditions

The inactivation of hAOX1 WT and the L438V variant by substrates was analyzed with 20 µM of each enzyme that were incubated in air-saturated 50 mM Tris-HCl, 1mM EDTA, pH 8.0 in the presence of 1.5 mM of benzaldehyde, 1.5 mM of zoniporide, or 1.5 mM of phthalazine. A pH of 8.0 was used in our study, since the pH optimum of hAOX1 is at pH8.0 (Foti, Hartmann et al. 2016) The enzyme activity was tested every 15 min by taking a 5 µL

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aliquot diluted into 500 µL of fresh in air-saturated 50 mM Tris-HCl, 1mM EDTA, pH 8.0 containing either 150 µM of benzaldehyde, 150 µM of phthalazine and 150 µM of zoniporide. 2000 U/mL of catalase and/or SOD were included when required.

Activity assays using DCPIP as electron acceptor

Inactivation of hAOX1 WT and the L438V variant by substrates was monitored using 100 µM DCPIP as electron acceptor. The specific activities were measured by monitoring the product formation of $DCPIPH_2$ at 600 nm using an extinction coefficient of 16100 $M^{-1}cm^{-1}$. The measurements were performed under aerobic conditions using air saturated buffer and under anaerobic conditions, using degassed buffer in an anaerobic chamber (Coy).

Reactivation of the enzymes by resulfuration

First, an aliquot of 1 mL of 20 µM of enzyme with 150 µM benzaldehyde was prepared. The enzyme activity of the aliquot was measured at time point 0 and after 60 minutes of incubation with benzaldehyde and/or 2000 U/mL catalase. After 60 min, the assay mixture was exchanged into 50 mM KH2PO4 • KOH, 0.1 mM EDTA pH 7.4 by using a PD-10 column.

Further, the enzyme concentration was measured by using the absorbance at 450nm and the activity of half of the reaction was measured and used as a non-sulfurated control. The remaining enzyme was used for chemical sulfuration and transferred to an anaerobic chamber (Coy). An adapted procedure to the chemical sulfuration protocol originally reported by Wahl et al. (Wahl and Rajagopalan 1982) was used. To 10 µM of enzyme 200 µL of 5 mM sodium dithionite, 1 mM sodium sulfite and 12 µM methylviologen were added and the mixture was incubated for 30 min on ice. Afterwards, the buffer was exchanged into 50 mM Tris-HCl,

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1mM EDTA, pH 8.0 using a PD-10 column. The activity was determined with benzaldehyde as substrate and compared to the activity of the non-sulfurated control.

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RESULTS

Steady state kinetics of hAOX1 and the superoxide overproducing variant L438V with different substrates

A substrate-dependent inactivation has been reported previously for hAOX1 (Abbasi, Paragas et al. 2019) and the XO/XDH enzymes (Dixon 1925, Betcher-Lange, Coughlan et al. 1979). For chicken liver XDH it had been suggested that the enzyme is inactivated by hydrogen peroxide that is produced during turnover (Betcher-Lange, Coughlan et al. 1979). Therefore, we wanted to investigate the nature of the inactivation of hAOX1, by comparison of two enzyme variants, the purified hAOX1 wildtype enzyme and the natural variant L438V, that was shown to produce 75% superoxide, while the wildtype only produces 10% superoxide during substrate turnover with oxygen as electron acceptor (Foti, Dorendorf et al. 2017) Both enzymes were expressed in a heterologous system in *Escherichia coli* (Foti, Hartmann et al. 2016). The goal was to analyze whether hydrogen peroxide or superoxide produced by the enzyme influence the substrate-dependent inactivation of the enzyme that has been observed under turnover conditions (Abbasi, Paragas et al. 2019). First, we determined the steady state kinetic constants of the two enzymes with three selected substrates for comparison: phthalazine as fast substrate, benzaldehyde as intermediate substrate, and zoniporide as slow substrate (Table 1). The kinetic constants of these substrates with oxygen as electron acceptor were not reported before for hAOX1 wildtype and the L438V variant. The results show that both hAOX1 proteins have similar kinetic constants with the three substrates, which is consistent with previously published data (Foti, Dorendorf et al. 2017) using a different substrate and therefore both proteins can be directly compared in our study.

Wildtype hAOX1 is inactivated by hydrogen peroxide while the L438V variant is inactivated by both hydrogen peroxide and superoxide under turnover conditions

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To analyze the substrate and time-dependent inactivation of hAOX1 wildtype and the variant L438V, we monitored product formation over a time of 60 min with three different substrates (phthalazine, benzaldehyde and zoniporide) using oxygen as terminal electron acceptor. Since oxygen is converted to hydrogen peroxide and superoxide at different rates by the two enzyme variants, we also included superoxide dismutase and catalase in the reaction mixtures, to analyze whether the enzyme inactivation can be slowed down by ROS scavengers. Aliquots were withdrawn from the incubation mixtures every 15 min and assayed for activity with fresh substrate in oxygen-saturated buffer to avoid substrate-limitation caused by the consumption of substrate during the incubation time. Also, interferences of the products by product inhibition are avoided this way.

When benzaldehyde was used as intermediate substrate (Figure 1A), the hAOX1 wildtype enzyme was 80% inactivated to 20% of its initial activity during the incubation time of 60 min. For comparison, the control reaction without substrate also showed a loss of activity over 60 min, but only of 10% maximum. The presence of SOD in the incubation mixture had no positive effect on the inactivation rate of hAOX1 WT with benzaldehyde as substrate (Figure 1A). However, when catalase was included in the incubation mixture with benzaldehyde, the enzyme showed a slower rate of inactivation of 20% maximum (Figure 1A). The same activities were obtained when both catalase and superoxide dismutase were present (Figure 1A). In comparison, the L438V variant was more rapidly inactivated with benzaldehyde as substrate with a complete inactivation after 30 min of incubation (Figure 1B). In contrast to the hAOX1 wild type enzyme, the inactivation of the variant was slowed down by the inclusion of either SOD or catalase in the reaction mixture (Figure 1B), showing that the inactivation is likely caused by both superoxide and hydrogen peroxide, since this variant produces a higher amount of superoxide as the wild type enzyme. Similar results were obtained for both enzymes when zoniporide as slow substrate was used (Figures 1 C+D). The wildtype enzyme was inactivated to 50% of its initial activity after an incubation time of 60

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min. While SOD had no effect on the inactivation rate, the inclusion of SOD and catalase had a minor positive effect on the inactivation rate, which was only reduced to 40% of its initial activity after 60 min. With zoniporide as slow substrate, the L438V variant was inactivated to 60% of its initial activity, showing that with slower substrates, the rate of inactiviation is reduced (Figure 1D). In consistency with the reaction of L348V with benzaldehyde as substrate, the rate of inactivation was only affected by the inclusion of SOD as ROS scavenger and not by catalase (Figure 1D).When we analyzed the inactivation rate of both enzymes with phthalazine as fast substrate, a fast inactivation of the wildtype enzyme was obtained with a loss of almost 60% of its activity during the first 15 min (Figure 1E). The inclusion of catalase or superoxide dismutase in the incubation mixture of the wildtype enzyme had mainly no effect on the rapid inactivation rate (Figure 1E). Only when both catalase and superoxide dismutase were included in the incubation mixture, the inactivation rate was slower and the enzyme showed a remaining 40% activity after 60 min of substrate turnover (Figure 1E). Similarly, the L438V variant was rapidly inactivated during the incubation time of 60 min and the rate of inactivation was only little reduced when SOD included in the incubation mixture. For the L438V variant, the inclusion of SOD or catalase and SOD resulted in a slower rate of inactivation, with a remaining activity of 40% after 60 min of incubation (Figure 1F).

Substrate-dependent inactivation of hAOX1 is largely reduced under anaerobic conditions

To get more insights into the site of inactivation of hAOX1 (Figure 2A+B) and the L438V variant (Figure 2C+D), we performed the assays using DCPIP instead of oxygen as terminal electron acceptor. DCPIP directly accepts the electrons from the Moco active site and therefore the electron transfer via the 2x[2Fe-2S] clusters and FAD is avoided (Foti, Dorendorf et al. 2017). When using DCPIP as electron acceptor under aerobic conditions, still

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an inactivation of both enzymes under turnover conditions with benzaldehyde as intermediate substrate was obtained (Figure 2A+C). Since the inactivation was completely diminished by the inclusion of catalase during the reaction, this shows that the oxygen present in the incubation mixture likely reacted with the enzyme as preferred electron acceptor to produce hydrogen peroxide that inactivated the enzymes (Figure 2A+C). Consequently, we performed the assay under anaerobic conditions to avoid a possible electron transfer to oxygen when using DCPIP as terminal electron acceptor (Figure 2B+D). The results show that the inactivation of both enzymes was almost completely diminished (Figure 2B+D). Likely still residual amounts of oxygen (10 ppm) were present in the reaction mixture, since catalase was still able to slightly reduce the rate of inactivation. The results conclusively show that oxygen is reacting with the enzyme very fast as preferred electron acceptor and that the site of inactivation is the Moco active site.

Inactivated hAOX1 during turnover can be reactivated by chemical sulfuration

To analyze the nature of the inactivation of the Moco active site, one possible target is the terminal sulfido-ligand at the molybdenum atom that is characteristic for enzymes of the XO family and essential for their catalytic activity. Since the sulfido-ligand can be ligated to AOX1 by using a chemical sulfuration reaction, we analyzed whether the enzyme activity can be restored by this procedure. As control, we chemically sulfurated the hAOX1 and L438V enzymes that were treated with cyanide, since cyanide treatment was shown to release the sulfido-ligand as thiocyanide, resulting in an inactive oxo-enzyme. Afterwards the enzyme can be resulfurated by a chemical sulfuration procedure using dithionite and sulfide under anaerobic conditions which restores the sulfido-ligand and enzyme activity is regained to 60% of its initial activity (Figure 3A+B, yellow bars). For comparison, we chemically sulfurated the hAOX1 wildtype enzymes that were incubated with benzaldehyde (grey bars) and benzaldehyde and catalase (green bars) under turnover conditions for 60 min (Figure 3A). As

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the CN inactivated control reaction, the activity of the inactivated enzyme incubated with benzaldehyde under turnover conditions was regained to 55% of its initial activity. The chemical sulfuration had mainly no effect on the hAOX1 wild type enzyme that contained catalase under turnover conditions, since the enzyme already showed 70% of the initial activity. For comparison, we chemically sufurated the L438V variant. This direct comparison is intended to differentiate the inactivation site of the L438V enzyme with superoxide from the one of the wildtype enzyme with hydrogen peroxide, and will reveal whether both ROS molecules target the sulfido-ligand at the molybdenum atom. The results in Figure 3B show, that after sulfuration of the CN and benzaldehyde inactivated L438Vvariant, the activity of both enzymes was restored to 60% and 40%, respectively, revealing that the sulfido-ligand was also the target of inactivation of the L438V variant (Figure 3B). No higher reactivation rate was obtained for the enzyme which was incubated in the presence of catalase under turnover conditions , which shows that the sulfido-ligand is the main target for enzyme inactivation of both hydrogen peroxide and superoxide.

DISCUSSION

In this report we show a substrate-dependent inactivation of hAOX1 and the L438V variant under turn-over conditions in the presence of oxygen over time. We show that in dependence of the substrate, the enzyme inactivation of the WT enzyme can be reduced by the inclusion of catalase in the incubation mixture. In particular with slower substrates, catalase could prevent the damaging effect of hydrogen peroxide on the enzyme, while using substrates with a high turnover rate like phthalazine, catalase had mainly no effect. We conclude that with fast substrates hydrogen peroxide is produced at high rates and the inactivation of the enzyme is faster than the scavenging reaction of catalase to convert hydrogen peroxide to water and oxygen. That would provide an explanation why with fast substrates, no effect of the

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inclusion of catalase in of incubation mixture has been observed in the past (Abbasi, Paragas et al. 2019).

When the enzyme variant L438V was used that produces high amounts of superoxide, also superoxide was damaging the enzyme, an effect that could be prevented by the inclusion of SOD in the assay. Also in this case, SOD had a better effect to prevent enzyme inactivation with substrates with a slower turnover number in comparison to fast substrates with a high turnover number. Our results clearly show that the time-dependent inactivation under turnover conditions is based on the presence of oxygen as electron acceptor, since with other electron acceptors like DCPIP (that takes the electrons directly from the molybdenum center (Foti, Dorendorf et al. 2017)) and additionally under anaerobic conditions, the AOX1 enzymes were not inactivated. When both DCPIP and oxygen were present as electron acceptor, the enzyme still reacted with oxygen as preferred electron acceptor, since the electron transfer to oxygen is faster than to DCPIP (which also is a weak inhibitor of the enzyme) (Foti, Dorendorf et al. 2017). Further, we were able to show that the site of inactivation is the terminal sulfido-ligand at the molybdenum ion, since we were able to reactivate the enzyme by a chemical sulfuration reaction.

A similar inactivation has been reported for XDH and XO (Dixon 1925, Betcher-Lange, Coughlan et al. 1979). While the inactivation of XO by hydrogen peroxide has been reported already in 1925 by Dixon (Dixon 1925), the nature of the reaction was mainly revealed by a study on chicken liver XDH in 1979 (Betcher-Lange, Coughlan et al. 1979), an enzyme that cannot be converted to the oxidase form (Rajagopalan and Handler 1967, Nishino, Schopfer et al. 1989). However, XDH is nevertheless able to react with oxygen as electron acceptor even though the turnover rate is less than 2% of that obtained with the physiological electron acceptor NAD^+ (Nishino, Schopfer et al. 1989). In the study by Betcher-Lange and coworkers (Betcher-Lange, Coughlan et al. 1979), it was shown that hydrogen peroxide reacts more rapidly with the reduced enzyme than with the oxidized form of the enzyme and that the site

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of inactivation is the molybdenum active site and not at the FAD site. It was assumed that inactivation results from modification of the cyanolysable sulfur (Wahl and Rajagopalan 1982) present at the molybdenum center, the nature of the terminal sulfido-ligand at the molybdenum ion had not been identified at the time of the publication in 1979. Our results on hAOX1 are fully consistent with the results of the inactivation of chicken liver XDH and show the common sensibility of these enzymes of the XO family to their reaction products hydrogen peroxide and superoxide in the reduced form. Consequently, the cyanolyzable sulfur ligand is oxidized and exchanged by an oxygen more easily in the reduced form than in the oxidized-form.

In a more recent study by Abbasi et al (Abbasi, Paragas et al. 2019), also a non-linear time-course of hAOX1 has been reported over incubation times of 250 min with different substrates. In their study, however, catalase and SOD did not influence the inactivation rate of the enzyme with different substrates. While the authors of this study state that their buffer had an oxygen concentration of 213 µM, it can only be speculated that the actual oxygen concentration was lower at an assay temperature of 37°C. Since the authors also did not aerate their assay over the reaction time of 250 min, the oxygen concentration likely decreased during the long incubation time, resulting in a higher ratio of superoxide formation, that more rapidly inactivates the enzyme, as reported by Lynch and Fridovich (Lynch and Fridovich 1979) and confirmed by our studies using the L438V variant. In addition, in that study the assays were performed at pH 7.4, which is below the pH optimum of hAOX1 of 8.0 (Coelho, Foti et al. 2015). Abbasi et al (Abbasi, Paragas et al. 2019) concluded that inactivation of the enzyme during turnover is based on a slow rate of electron transfer to oxygen, which the authors propose to be the rate limiting step of the reaction. Our results, however, do not confirm their conclusion, since the reaction of the enzyme with oxygen (taking electrons at the FAD site) was faster than the reaction of the enzyme with DCPIP (that accepts the electrons from the Moco active site). Further, the authors did not perform their assay under

anaerobic conditions with an electron acceptor other than oxygen that accepts the electrons from the FAD site (e.g. ferricyanide) to confirm their conclusions. Overall, our study confirms the time-dependent inactivation of hAOX1 under turnover conditions observed by Abbasi et al (Abbasi, Paragas et al. 2019), however, we provide the evidence that the site of inactivation is the terminal sulfido-ligand at the molybdenum ion, as it has been previously reported for chicken liver XDH, another enzyme of the XO family (Betcher-Lange, Coughlan et al. 1979).

Overall, the production of hydrogen peroxide by hAOX1 has long been overlooked as a significant source for cellular ROS production. First reports, however, indicated that inhibition of hAOX1 improves the survival time of cancer patients of certain cancers (Qiao, Maiti et al. 2020). In fatty liver rats treated with the AOX1 inhibitor hydralazine, liver triglyceride levels markedly decreased, indicating that AOX1 inhibitors are capable of ameliorating fatty liver and preventing liver cancer (Takeuchi, Yokouchi et al. 2018). Raloxifene, a potent AOX1 inhibitor, is approved for the prevention of breast cancer in postmenopausal women (Provinciali, Suen et al. 2016). Raloxifene inhibits the matrix metalloproteinase-2 enzyme, which is known to be responsible for tumor invasion and the initiation of angiogenesis during the tumor growth (Agardan, Degim et al. 2016). Inhibition of AOX1 might be another mechanism by which raloxifene reduces breast cancer (Qiao, Maiti et al. 2020).

Oxidative damage has long been implicated in both the malignant phenotype and carcinogenesis (Oberley 2002). Also XO has been implicated to be involved in cancer development. In a recent study on XO knock in mice, it was shown that mice that were expressing the XO-locked form showed strongly enhanced tumor growth when compared to the XDH-locked form of wildtype mice. Further, it was shown that the block of ROS production slowed down tumor growth (Kusano, Ehirchiou et al. 2019). Only mammalian XDH can be converted into the XO form, but the physiological functions still remain elusive.

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Since the conversion of XDH to the XO form is not completely understood, it might be possible that ROS are also produced by hAOX1 and not only by XO in these cells. Therefore, the contribution and role of hAOX1 in ROS production that leads to cancer development needs further investigation. In summary, our study shows that hAOX1, like XDH or XO, are inhibited by ROS during substrate turnover. The inactivation of hAOX1 is thereby more rapid with substrates with a high turnover number, which cannot be prevented by catalase. When using slower substrates, the inactivation is slower based on lower levels of produced hydrogen peroxide and consequently the inactivation can be prevented by the inclusion of ROS scavengers. Since these enzymes produce a higher ratio of superoxide at lower concentrations of oxygen (Lynch and Fridovich 1979), hAOX1 and XOR might be faster inactivated when cells get oxygen depleted. Further, when substrates are present in too high amounts in the cell, hAOX1 might also get inactivated more rapidly based on a higher rate of hydrogen peroxide production. This might be a regulatory mechanism of the cell to prevent too much ROS production by hAOX1 and XOR, and therefore prevent tumor growth. The role of hAOX1 in cancer and the prevention of cancer by ROS scavengers and hAOX1 inhibitors needs to be investigated in future studies.

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Participated in research design: Leimkühler

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Footnote

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FIGURE LEGENDS

Figure 1: Inactivation of hAOX1 WT and the L438V variant by substrates. 20 µM of hAOX1 and L438V were incubated in air-saturated 50 mM Tris-HCl, 1mM EDTA, pH 8.0 in the presence of 1.5 mM of benzaldehyde (A +B), 1.5 mM of zoniporide (C+D), 1.5 mM of phthalazine (E+F). The enzyme activity was tested every 15 min by taking a 5 µL aliquot diluted into 500 µL of fresh in air-saturated 50 mM Tris-HCl, 1mM EDTA, pH 8.0 containing either 150 µM of benzaldehyde, 150 µM of phthalazine and 150 µM of zoniporide. 2000 U/mL of catalase and SOD were included as indicated. black dotted line: substrate, solid line: no substrate control, red line: SOD + catalase, green line: catalase, and blue line: SOD.

Figure 2: Inactivation of hAOX1 WT and the L438V variant by substrates using DCPIP as electron acceptor. 20 µM of hAOX1 and L438V were incubated in air-saturated 50 mM Tris-HCl, 1mM EDTA, pH 8.0 in the presence of 1.5 mM of benzaldehyde (A +C), or in oxygen-free 50 mM Tris-HCl, 1mM EDTA, pH 8.0. The enzyme activity was tested every 15 min by taking a 10 µL aliquot diluted into 1 mL of fresh in air-saturated 50 mM Tris-HCl, 1mM EDTA, pH 8.0 containing 150 µM of benzaldehyde and 100 µM DCPIP (A + C), or diluted into 1 mL of oxygen-depleted 50 mM Tris-HCl, 1mM EDTA, pH 8.0 containing 150 µM of benzaldehyde and 100 µM DCPIP (B + D). 2000 U/mL of catalase were included as indicated. black dotted line: substrate, solid line: no substrate control, green line: catalase.

Figure 3: Chemical sulfuration of the hAOX1 WT and the L438V variant after substrate-dependent inactivation. The specific activity was measured after 60 min incubation of the enzyme under turnover conditions with benzaldehyde (grey bars) or with benzaldehyde and

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catalase (green bars). AOX1 enzymes that were inactivated with 100 µM KCN were used as control (orange bars). After buffer-exchange using PD-10 columns, the enzymes were chemically sulfurated with sulfide and dithionite and the remaining activity was determined.

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TABLE

Table 1: **Steady state kinetic parameters hAOX1 wildtype and the L438V variant with different substrates.**

Steady-state kinetic parameters were corrected to a molybdenum saturation of 100%. Kinetic parameters were recorded in 50 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA (pH 8.0) in the presence of 250 μM O_2 as electron acceptor using 200-400 nM enzyme. Substrate concentration were varied using 5 to 200 μM for benzaldehyde, 5 to 120 μM for phthalazine and 75 to 600 μM for zoniporide. Data are mean values from three independent measurements (±S.D.).

Enzyme		Benzaldehyde	Phthalazine	Zoniporide
hAOX1-WT	k_{cat} (min^{-1})	14.87±1.42	123.11±1.02	0.43±0.03
	K_M (μM)	104.98±19.96	201.56±31.98	623.82±9.09
hAOX1-L438V	k_{cat} (min^{-1})	10.58±1.07	89.28±1.14	0.42±0.02
	K_M (μM)	101.48±21.01	117.06 ± 28.92	573.41±37.51

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