Biocatalysis

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The Oxidation of Thiols by Flavoprotein Oxidases: a Biocatalytic Route to Reactive Thiocarbonyls**

*Tom A. Ewing, Willem P. Dijkman, Jacques M. Vervoort, Marco W. Fraaije, and Willem J. H. van Berkel**

Abstract: *Flavoprotein oxidases are a diverse class of biocatalysts, most of which catalyze the oxidation of C–O, C–N, or C–C bonds. Flavoprotein oxidases that are known to catalyze the oxidation of C–S bonds are rare, being limited to enzymes that catalyze the oxidative cleavage of thioethers. Herein, we report that various flavoprotein oxidases, previously thought to solely act on alcohols, also catalyze the oxidation of thiols to thiocarbonyls. These results highlight the versatility of enzymatic catalysis and provide a potential biocatalytic route to reactive thiocarbonyl compounds, which have a variety of applications in synthetic organic chemistry.*

Flavoprotein oxidases constitute a diverse class of oxidoreductases, which contain a flavin cofactor in the form of flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). They catalyze the oxidation of a wide range of substrates, with molecular oxygen functioning as the final oxidant. Their catalytic cycle consists of two half reactions. During the reductive half reaction two electrons are transferred from the substrate to the flavin cofactor, a process which is generally believed to occur through the direct transfer of a hydride anion to the N5 atom of the flavin.[1,2] In the oxidative half reaction the flavin cofactor is reoxidized by molecular oxygen, which is reduced to hydrogen peroxide. Unlike many other oxidative enzymes, for example dehydrogenases, oxidases do not require stoichiometric amounts of expensive organic cofactors, making them interesting candidates for biocatalytic exploitation. Most of the characterized flavoprotein oxidases catalyze the oxidation of C–O, C–N, or C–C bonds to the corresponding double bonds.[3] A small number of flavoprotein oxidases are known to oxidize sulfur-containing compounds. Prenylcysteine lyase (Enzyme Commission (EC) number 1.8.3.5) and farnesylcysteine lyase (EC 1.8.3.6) catalyze the oxidative cleavage of thioethers into aldehydes and thiols, whereas sulfhydryl oxidases (EC 1.8.3.2 and 1.8.3.3) catalyze the formation of disulfide bonds from thiols. So far, no flavoprotein oxidases have been identified that catalyze the oxidation of thiols to thiocarbonyl compounds. Herein, we present the finding that a number of flavin-dependent oxidases, that hitherto had been thought to act solely on alcohols, also oxidize thiols that are structurally similar to their alcohol substrates, yielding the corresponding thiocarbonyls.

Alditol oxidase from *Streptomyces coelicolor* (AldO; EC 1.1.3.41) is a flavin-dependent oxidase that catalyzes the regioselective oxidation of the primary hydroxy groups of a broad range of polyols, with the best substrates being alditols, such as xylitol, D-sorbitol, and L-threitol.[4,5] AldO displays stereoselectivity: for example, D-sorbitol is converted into its oxidized form significantly more efficiently than its C2 epimer D-mannitol. AldO belongs to the vanillyl alcohol oxidase (VAO) family of structurally homologous flavoproteins. These flavoproteins contain a conserved FAD-binding domain as well as a variable substrate-binding domain, which determines their substrate specificity.[6] The enzyme contains an FAD cofactor which is covalently linked to a histidine residue.[7] To investigate whether AldO reacts with thiols, the enzyme was first expressed and purified as a fusion protein with an N-terminal maltose-binding protein tag. This fusion protein (m-AldO) displayed identical catalytic properties to the wild-type enzyme.[4] Oxygen consumption measurements showed that m-AldO catalyzes the oxidation of DL-dithiothreitol (DL-DTT), which is structurally identical to the known AldO substrate threitol ((2*R*,3*R*/2*S*,3*S*)-butane-1,2,3,4-tetrol), except for the presence of thiol rather than hydroxy groups at the terminal carbon atoms. Steady-state kinetic analysis revealed that m-AldO oxidizes racemic DTT with a turnover number $k_{cat} = 0.79\ s^{-1}$ and a Michaelis constant $K_M = 3.7$ mM (Table 1 and Figure S1 in the Supporting Information). The L-enantiomer of DTT was preferentially converted into its oxidized form, displaying a similar k_{cat} value ($0.71\ s^{-1}$), but a lower K_M (2.4 mM) than the racemic mixture (Table 1 and Figure S2). m-AldO also oxidizes dithioerythritol, but the reaction with this epimer of DTT is less efficient, primarily because of a higher K_M value, and displayed substrate inhibition with an inhibition constant $K_i = 353 \pm 41$ mM (Table 1 and Figure S3). The tested thiols are remarkably good substrates for m-AldO. The catalytic efficiency (k_{cat}/K_M) for the oxidation of L-DTT is very similar to that for the oxidation of its alcohol analogue L-threitol. In fact, of all known m-AldO substrates, only xylitol and D-sorbitol display higher catalytic efficiencies than L-DTT.[4,5]

[*] T. A. Ewing,[+] Prof. Dr. J. M. Vervoort, Prof. Dr. W. J. H. van Berkel
Laboratory of Biochemistry, Wageningen University
Dreijenlaan 3 (The Netherlands)
E-mail: willem.vanberkel@wur.nl
Homepage: http://www.bic.wur.nl
W. P. Dijkman,[+] M. W. Fraaije
Molecular Enzymology Group
Groningen Biomolecular Sciences and Biotechnology Institute
University of Groningen, Nijenborgh 4 (The Netherlands)
[+] These authors contributed equally to this work.

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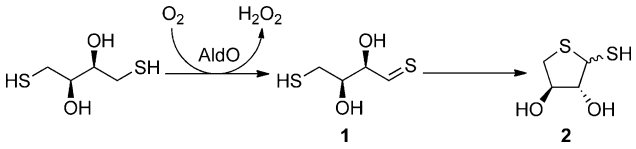
Table 1: Steady-state kinetic parameters for the oxidation of thiols and their alcohol analogues by flavoprotein oxidases.

Enzyme[a]	Substrate	$k_{cat(app)}$ [s^{-1}]	$K_{M(app)}$ [mM]	$k_{cat(app)}/K_{M(app)}$ [s^{-1} mM^{-1}]
m-AldO	DL-dithiothreitol	0.79 ± 0.01	3.7 ± 0.1	0.21
m-AldO	L-dithiothreitol	0.71 ± 0.01	2.4 ± 0.1	0.30
m-AldO	L-threitol	6.3[b]	25[b]	0.25
m-AldO	dithioerythritol	0.45 ± 0.02	29 ± 3	0.016
m-AldO	erythritol	N.D.	>100	N.D.
HMFO	phenylmethanethiol	2.1 ± 0.1	3.5 ± 0.8	0.60
HMFO	benzyl alcohol	13.5 ± 0.3[b]	1.4 ± 0.1[b]	9.6
HMFO	(4-nitrophenyl) methanethiol	3.0 ± 0.09	15 ± 1.4	0.20
HMFO	(4-nitrophenyl) methanol	4.5 ± 0.1	0.078 ± 0.01	58
GO	1-thio-β-D-glucose	50 ± 12	71 ± 21	0.70
GO	D-glucose	223 ± 2	35 ± 1	6.4

Steady state kinetic parameters were determined at 25 °C in the following buffers: m-AldO: KP_i buffer (20 mM, pH 7.2); HMFO: KP_i (50 mM, pH 8.0); GO: citrate buffer (50 mM, pH 5.5). [a] m-AldO = maltose-binding-protein alditol oxidase fusion protein, HMFO = HMF oxidase, GO = glucose oxidase. [b] The kinetic parameters have been described elsewhere.[4,14] N.D. = not determined.

HPLC analysis revealed that three main products are formed during the oxidation of DL-DTT by m-AldO (Figure S4). Addition of the hydrogen peroxide scavenger sodium pyruvate to the reaction mixture demonstrated that two of the products are formed by side reactions involving hydrogen peroxide. The third compound, which is the product of the enzymatic reaction, was collected and identified by NMR spectroscopy as the dithiohemiacetal **2** (Scheme 1, showing



Scheme 1. The oxidation of L-DTT catalyzed by alditol oxidase (AldO).

the L-enantiomers, and Figure S5). Formation of this product presumably occurs via the thioaldehyde intermediate **1**, which is formed upon oxidation of DL-DTT by m-AldO and subsequently undergoes an intramolecular cyclization to yield **2**. This dithiohemiacetal is relatively unstable, being entirely oxidized to the corresponding sulfenic acid after 6 days of storage at −20 °C dissolved in acetonitrile. By comparison to a reference compound, one of the two side products was identified as the disulfide *trans*-4,5-dihydroxy-1,2-dithiane, which was shown to be formed upon reaction of DL-DTT with hydrogen peroxide. The second unidentified compound is likely the disulfide formed after reaction of two molecules of **2** upon its oxidation by hydrogen peroxide. Alternatively, it may be the mixed disulfide formed between one molecule of **2** and one molecule of DTT.

To further investigate the reaction of m-AldO with L-DTT, changes in the spectral properties of the enzyme's flavin cofactor during the reductive half reaction were followed using a stopped-flow apparatus. Upon mixing m-AldO and L-DTT under anaerobic conditions, a rapid increase in absorbance was observed at $\lambda = 530$ nm (Figure 1 A, see Supporting Information for details). This increase in absorbance at longer wavelengths is indicative of the formation of a charge-transfer complex between the thiolate anion form of L-DTT and the FAD cofactor. Such flavin–thiol charge-transfer complexes have been observed in various members of the sulfhydryl oxidase family, though in these cases it is due to an interaction with an active site cysteine residue.[8,9] Subsequently, the absorbance at $\lambda = 530$ nm decays concomitantly with the absorbance at $\lambda = 450$ nm, suggesting the charge-transfer interaction is broken by transfer of electrons from L-DTT to the flavin cofactor (Figure 1 B). Enzyme-monitored turnover experiments under aerobic conditions revealed that during steady-state catalysis m-AldO is predominantly present as the charge-transfer complex (Figure 1 C).

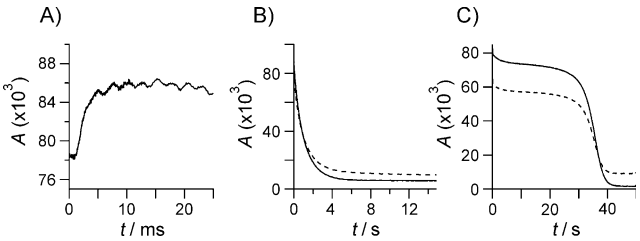


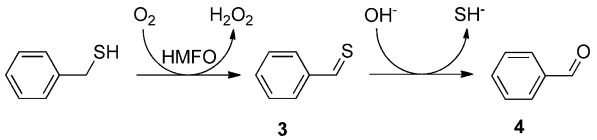
Figure 1. The change in UV/Vis absorption intensity with time measured using a stopped-flow apparatus after mixing m-AldO (10 μM) with L-DTT (25 mM) under anaerobic (A, B) or aerobic (C) conditions. The absorption intensity was monitored at $\lambda = 530$ nm (solid lines) and $\lambda = 450$ nm (dashed line). Time periods for each plot: A) $t =$ 0–25 ms; B) $t = 0$–15 s; C) $t = 0$–50 s. Measurements were performed at least three times, representative traces are shown.

The VAO family, to which AldO belongs, displays a remarkably broad range of reactivities. The oxidation of alcohols and amines, hydroxylation of alkyl groups, C–C bond formation, and the demethylation of ethers have all been described previously.[10–13] The results presented above show that the oxidation of thiols can be added to this list. To determine whether the oxidation of thiols is also performed by more strict alcohol oxidases from the glucose-methanol-choline (GMC) family, we investigated the recently discovered 5-hydroxymethylfurfural oxidase (HMFO) from *Methylovorus* sp. Strain MP688.[14] HMFO catalyzes the oxidation of primary alcohols. Its substrate range includes phenylic, furanic, and linear unsaturated alcohols. In addition, HMFO is active towards several aldehydes. However, this activity is restricted to aldehydes which are spontaneously hydrated to the *gem*-diol form when dissolved in water.[15]

We tested HMFO for activity towards phenylmethanethiol, which is the thiol analogue of its known substrate benzyl alcohol, and the related (4-nitrophenyl)methanethiol. Interestingly, both thiols were readily oxidized by HMFO. The steady-state kinetic parameters of HMFO for these two aromatic thiol substrates are shown in Table 1 and Figures S6

and S8. The k_{cat} values for both aromatic thiols are slightly lower than those for their alcohol counterparts (Table 1, Figures S7 and S9). Also, the K_M values for both thiols are significantly higher. Taken together, the catalytic efficiency of HMFO is higher for alcohols than for thiols. Still, both tested aromatic thiols can be regarded as good substrates with k_{cat} values greater than 2 s^{-1} and K_M values in the millimolar range.

The products formed upon oxidation of the two aromatic thiol substrates by HMFO were identified using GC–MS and LC–MS. At pH 7.0, phenylmethanethiol is oxidized to the corresponding aromatic thioaldehyde, benzothialdehyde (**3**) (Scheme 2 and Figures S10 and S11). No formation of 1,2-dibenzyldisulfane was observed, demonstrating that HMFO



Scheme 2. HMFO-catalyzed oxidation of phenylmethanethiol to benzothialdehyde (**3**) and benzaldehyde (**4**).

does not catalyze the formation of disulfide bonds. At pH 8.0, two products are formed. Although after 10 minutes only benzothialdehyde was formed, after 60 minutes benzaldehyde (**4**) was also detected. This suggests that the thioaldehyde is slowly hydrated, yielding the aldehyde as the final product. This is analogous to the reaction performed by amine oxidases, where the oxidation of amines to imines is followed by their reaction with water, yielding aldehydes or ketones.[16] To confirm the involvement of water, the reaction at pH 8.0 was performed in the presence of ^{18}O-labeled water. In the presence of $H_2{}^{18}O$, benzaldehyde with m/z 108 was observed, whereas in unlabeled water only m/z 106 was detected. This confirms that at pH 8.0, water performs a nucleophilic attack on the initially formed thioaldehyde, yielding benzaldehyde as the final product. Using (4-nitrophenyl)methanethiol as a substrate, the reaction at pH 8.0 again yielded the corresponding aldehyde. Because this aldehyde is easily hydrated to the *gem*-diol,[17] it is oxidized further by HMFO, yielding 4-nitrobenzoic acid as the final product. In addition to these main products, several minor peaks were detected by GC and LC analysis, probably resulting from the reactive nature of the formed thioaldehyde.

To determine whether the ability to oxidize thiols as well as alcohols is a general property of flavin-dependent alcohol oxidases, we investigated a number of commercially available oxidases for their reactivity towards thiols. Glucose oxidase (GO; EC 1.1.3.4) is a fungal glycoprotein, which also belongs to the GMC-family and is widely applied as an oxidative biocatalyst. Comparable to HMFO, GO contains a noncovalently bound FAD cofactor. GO regioselectively oxidizes β-D-glucose to 1,5-D-gluconolactone, which spontaneously hydrolyzes to gluconic acid.[18] Oxygen consumption measurements showed that GO from *Aspergillus niger* indeed oxidizes 1-thio-β-D-glucose with a k_{cat} value of 50 s^{-1}, $K_M = 71$ mM, and with substrate inhibition occurring with $K_i = 42 \pm 13$ mM (Table 1 and Figure S12). However, the oxidation of 1-thio-β-D-glucose by GO is less efficient than that of its alcohol analogue D-glucose, which occurs with a higher k_{cat} value (223 s^{-1}) and a lower K_M value (35 mM) (Table 1 and Figure S13). Methanol oxidase (MO, or alcohol oxidase; EC 1.1.3.13) is another fungal GMC-type oxidase. MO contains a noncovalently bound FAD cofactor and catalyzes the oxidation of linear unbranched primary alcohols of up to 5 carbon atoms in length.[19] MO from *Candida boidinii* was shown to oxidize ethanethiol, displaying a specific activity of 0.32 ± 0.03 U mg^{-1} at a substrate concentration of 20 mM, as compared to a specific activity of 0.51 ± 0.02 U mg^{-1} for the oxidation of 20 mM ethanol.

In summary, all tested flavin-dependent alcohol oxidases are capable of catalyzing the oxidation of thiols, yielding thiocarbonyls. Though various free flavins are also known to react with some thiols, these reactions solely yield disulfides as products, suggesting that they are mechanistically distinct from the flavoenzyme-catalyzed reactions described herein.[20,21] In the reaction of m-AldO with L-DTT, the thiol substrate binds to the enzyme as a thiolate anion, leading to a strong charge-transfer interaction with the oxidized flavin cofactor. Binding of the substrate in its thiolate form is likely stimulated by the presence of two positively charged residues: R322 and K375. These residues are in close proximity to the terminal hydroxy group of the substrate in the crystal structure of AldO with xylitol bound.[7] Subsequently, the flavin is slowly reduced by the transfer of two electrons from the substrate, yielding the thiocarbonyl product. The electron transfer probably occurs through the direct transfer of a hydride anion from the C_α atom of the substrate to the N5 atom of the flavin cofactor, as is believed to be the case for the oxidation of alcohols and amines by flavoprotein oxidases.[1,2] In the case of the oxidation of DTT by m-AldO, the formed thioaldehyde rearranges intramolecularly to yield dithiohemiacetal **2**. For the aromatic thiols oxidized by HMFO, such a rearrangement is not possible because of the absence of a second thiol group and in the case of phenylmethanethiol the formed thioaldehyde **3** is stable enough to be detected. Because HMFO has an exceptionally broad substrate range, this biocatalyst can be used to generate a large variety of reactive, but relatively stable, thioaldehydes. The fusion protein m-AldO also has a broad substrate range and could potentially be used to generate more stable thioaldehydes in cases where no second thiol group is present to enable intramolecular cyclization. As the ability to oxidize thiols appears to be a general property of flavin-dependent oxidases, many other enzymes may be used for similar purposes. Thus, our results not only highlight the promiscuity and versatility of enzymatic catalysis, but also provide a potential biocatalytic route to reactive thiocarbonyl compounds, which have a variety of applications in synthetic organic chemistry.[22]

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