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Exploring the catalase activity of unspecific peroxygenases and the mechanism of peroxide-dependent heme destruction

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ABSTRACT

The catalase activity of three unspecific peroxygenases (UPOs) from the agaric basidiomycetes *Agrocybe aegerita*, *Coprinopsis cinerea* and *Marasmius rotula* was investigated. The study included analysis of pH dependency of the catalase reaction and H_2O_2 mediated enzyme inactivation as well as experiments on the influence of a second substrate on the course of catalase reaction. Apparent kinetic parameters (K_m, k_{cat}) for the catalase activity of UPOs were determined. Inactivation of UPOs by H_2O_2 is discussed with regard to O_2 production and remaining UPO activity. Furthermore formation of biliverdin as heme destruction product was demonstrated along with the formation of UPO compound III as a possible intermediate that forces the destruction process. Radical trapping experiments with methyl benzoate gave indication for the formation of hydroxyl radicals in the presence of excess H_2O_2. Eventually, a plausible pathway of heme destruction has been proposed, proceeding via UPO compound III and subsequent hydroxyl radical formation, which in turn may cause heme bleaching and verdoheme and biliverdin formation.

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1. Introduction

Unspecific peroxygenases (UPOs, EC 1.11.2.1) are ubiquitous biocatalysts in the kingdom of fungi and are (next to cytochrome P450 monooxygenases) the most versatile oxyfunctionalizing enzymes (with regard to substrate spectrum and reaction variety). UPOs catalyze diverse reactions such as hydroxylation, epoxidation, sulfoxidation, heterocyclic *N*-and *S*-oxidation, *N*-dealkylation, as well as ether cleavages [1–4]. The so far mostly studied UPO is secreted by the agaric basidiomycete *Agrocybe aegerita* (*Aae*UPO) [5]. *Aae*UPO has been shown to convert over 100 substrates via peroxygenation and beyond that, it catalyzes one-electron oxidations of a broad spectrum of phenolic compounds [1,6–11]. Despite the broad substrate spectrum, reactions catalyzed by UPOs can be highly regio- and enatio-selective [9,10]. This versatility combined with specificity for certain substrates has drawn attention of biotechnologists and organic chemists [12].

Besides utilizing hydrogen peroxide (H_2O_2) as electron acceptor and oxygen donor for hydroxylation reactions, UPOs catalyze H_2O_2 dismutation via a catalase-like reaction [11] that produces one molecule of dioxygen (O_2) and two molecules of water (H_2O) from two molecules of H_2O_2. Catalase activity may be a drawback of potential UPO applications because the unproductive consumption of the co-substrate will increase the costs for any industrial synthesis and beyond that, this reaction is accompanied by enzyme inactivation. The latter is a common problem of heme-containing oxidoreductases and particularly pronounced when the enzymes are confronted with H_2O_2 in excess [13–15]. Thus, loss of enzyme activity and concomitant heme-bleaching caused by H_2O_2 has been described for diverse peroxidases. Cytochrome-P450 monooxygenases (P450s) undergo even faster peroxide-mediated inactivation via reactive intermediates, particularly when they act in the peroxide-shunt mode ("peroxygenase" side activity of P450s) [16–20].

A competition of peroxidase reactive species (compound I) for the target substrate and another molecule of the co-substrate H_2O_2 was proposed in several studies [17,21]. Thus, the second substrate may protect a peroxidase from inactivation by H_2O_2. Oxidation of amino acids of the peroxidase apoprotein protein was reported for different peroxidases upon treatment with excess H_2O_2, e.g. for ascorbate peroxidase, lignin peroxidase and versatile peroxidase [22–24]. On the other hand, it was shown that heme destruction is the main mechanism leading to the inactivation of chloroperoxidase (CPO, EC 1.11.1.10), a heme-thiolate protein secreted by the ascomycete *Leptoxyphium fumago* (also known as *Caldariomyces fumago*) [25]. CPO has about 30% sequence homology to *Aae*UPO,

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and hence is related to fungal UPOs; in fact, CPO can be regarded as special UPO preferably oxygenating halides [11]. Thus, the inactivation mechanism caused by excess H_2O_2 may be similar in CPO and UPOs [15,26]. However, the products resulting from heme destruction in CPO have not yet been identified.

Reactive intermediates formed in the catalytic cycle of *Aae*UPO have been recently studied using stopped-flow techniques [27,28]. Unsurprisingly, the spectroscopic properties of compound I (cpd-I) and compound II (cpd-II) of *Aae*UPO and CPO are rather similar (though cpd-II of *Aae*UPO is more basic and reactive), underlining their molecular relation [29–31]. However, compound-III (cpd-3) of *Aae*UPO has not been studied so far but was characterized for CPO [29].

Cpd-III of CPO and classic heme peroxidases is formed upon treatment of cpd-II with additional H_2O_2 [14,29]. In the case of horseradish peroxidase, a so-called verdoheme compound (P-670; absorption band at 670 nm) was observed [17], when cpd-III further reacted with H_2O_2. Greenish P-670 was shown to arise from auto-hydroxylation of the porphyrin ring and hence may be an intermediate on the way to heme destruction [32]. Verdoheme is also known as an intermediate in the natural destruction of hemoglobin by heme oxygenases (HOs, EC 1.14.99.3) [33]. HOs are membrane-bound, heme-containing dioxygenases that catalyze the oxidation of hemoglobin to form biliverdin [34]. The formation of biliverdin can be divided in three phases: (i) the heme α-*meso*-hydroxylation, (ii) rearrangement to verdoheme and (iii) biliverdin formation [35–37]. Whereas the first two steps are certainly enzyme-dependent and require NADPH/O_2 and O_2, respectively [35], the third step is thought to be enzyme-independent (in form of a chemical hydrolysis reaction) [38].

Indeed inactivation of UPOs seems to be peroxide-dependent. Loss of *Aae*UPO activity has been observed within minutes, when performing oxyfunctionalization reactions of C—H bonds using stoichiometric amounts of H_2O_2. However, when H_2O_2 was added slowly, enzyme activity remained stable over hours [12]. Considering the diversity of total turnover numbers for miscellaneous UPO substrates, it becomes evident that their inactivation must be additionally dependent on the particular substrate [10]. The phenomenon of heme bleaching in the case of *Aae*UPO was already observed by the authors in previous tests, but it has not been studied in detail so far. Therefore, in the present work, we studied the catalase reaction of three different UPOs from basidiomycetes *Marasmius rotula* (*Mro*UPO), *Coprinopsis cinerea* (r*Cci*UPO) and *A. aegerita* (*Aae*UPO) and propose an inactivation pathway initiated by excess H_2O_2.

2. Material and methods

2.1. Chemicals

All chemicals used were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) with the highest purity available.

2.2. Enzyme preparations

*Aae*UPO and *Mro*UPO were produced and purified as described previously [5,39]. Recombinant UPO from *C. cinerea* (expressed in *Aspergillus oryzae*) was kindly provided by Novozymes A/S (Bagsvaerd, Denmark) and was prepared according to Babot et al. [40].

2.3. Measurement of H_2O_2 consumption

To determine the catalase activity of UPOs, the conversion of H_2O_2 (as sole substrate) was directly measured (online) in 1-cm quartz cuvettes with a Carry 50 Varian spectrophotometer (Darmstadt, Germany) at 240 nm (ε_{H2O2} = 40 M^{-1} cm^{-1}).

The reaction setup for the determination of pH optima and residual UPO activity was as follows: *Aae*UPO – 2 U_{VA}[1] (0.6 μM), r*Cci*UPO – 0.3 U_{VA} (0.05 μM), *Mro*UPO –3.3 U_{VA} (0.73 μM) and 50 mM potassium phosphate buffer (KP_i) varying between pH 2 and 9. It had been intended that the amount of H_2O_2 added caused significant changes in the residual activity; therefore, in dependence on the actual catalase activity of UPOs, different H_2O_2 concentrations were used. Thus, the reactions were started by the addition of 2 mM and 5 mM H_2O_2 in the case of *Aae*UPO/*Mro*UPO and r*Cci*UPO, respectively, and instant mixing in a quartz cuvette. The consumption of H_2O_2 was followed at 240 nm; the linear slope of the first 25 s after starting the reaction was used for activity calculation. Two examples of such catalase activity measurements are given in the Supplementary material (Fig. S1).

Residual UPO activities were determined with veratryl alcohol (VA, 5 mM) in KP_i 50 mM (pH 7 *Aae*UPO/*Cci*UPO and pH 5.5 *Mro*UPO, respectively). Reaction was started by addition of 1 mM H_2O_2 and rapid mixing. The absorbance increase was measured at 310 nm and the activity was calculated via Beer-Lambert (ε of veratraldehyde: 9300 M^{-1} cm^{-1} at 310 nm).

2.4. Online O_2 monitoring

To study the catalase activity of UPOs more in detail, a method for online monitoring of dioxygen (O_2, formed as the result of H_2O_2 decomposition) was established using an oxygen meter (Fibox 4 trace PreSens – Precision Sensing GmbH, Regensburg, Germany). A catalase (E.C. 1.11.1.6) Terminox Ultra (activity 50,000 U/ml) from Novozymes (Bagsværd, Denmark) was used as standard enzyme. An example of such O_2 measurement in a stirred reaction tube (20 ml total volume) is given in the Supplementary material (Fig. S2). Intrinsic O_2 was partly stripped with helium resulting in a basic O_2 level around 20–45 μM. Then the O_2 concentration was constantly measured (over about 30 s) prior to the addition of H_2O_2. After peroxide addition, the amount of dissolved O_2 steadily increased until a plateau was reached.

2.5. O_2 production and TTN calculation

*Aae*UPO (0.3 U_{VA} ml^{-1}, 0.068 μM when constant; 0.01–0.09 U_{VA} ml^{-1}, 1 U ml^{-1} = 0.227 μM when varied), r*Cci*UPO (0.044 μM) and *Mro*UPO (0.063 μM) was mixed in 50 mM KP_i pH7.0 (*Aae*UPO/r*Cci*UPO) or pH 5.5 (*Mro*UPO), respectively, in a total volume of 20 ml at room temperature. O_2 was measured according to the setup above. The reaction was started by addition of H_2O_2 (0.5 mM when constant; 0.06–2.0 mM [*Aae*UPO], or 0.06–20 mM [r*Cci*/*Mro*UPO], respectively, when varied).

2.6. Inhibition of AaeUPO's catalase activity by different substrates

*Aae*UPO (0.3 U_{VA} ml^{-1}, 0.068 μM) was mixed in 50 mM KP_i pH 7.0 without any additional substrate, or with varying concentrations of VA (1–40 mM) or ethyl benzene (EB; 0.5–2 mM) in a total volume of 20 ml. O_2 was measured according to the setup above. The reaction was started by addition of 1 mM H_2O_2. The samples with additional substrate were analyzed by HPLC and the column used was a Luna C18 (2) (5 μ, 100 Å, 150 × 2 mm; Phenomenex, Aschaffenburg, Germany). Following separation conditions were used: water (eluent A) and acetonitrile (eluent B), flowrate –

[1] U_{VA} – units based on the oxidation of veratryl alcohol (VA) to vertraldehyde.

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