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Enzymatic one-pot conversion of cyclohexane into cyclohexanone: Comparison of four fungal peroxygenases

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ABSTRACT

Unspecific peroxygenases (UPO; EC 1.11.2.1) represent a group of secreted heme-thiolate proteins that are capable of catalyzing the mono-oxygenation of diverse organic compounds, using only H_2O_2 as a co-substrate. Here we show that the four peroxygenases *Aae*UPO, *Mro*UPO, r*Cci*UPO and rNOVO catalyze the stepwise hydroxylation of cyclohexane to cyclohexanol and cyclohexanone. The catalytic efficiencies (k_{cat}/K_m) for the initial hydroxylation were in the same order of magnitude for all four peroxygenases ($\sim 10^4\ M^{-1}\ s^{-1}$), whereas they differed in the second step. The conversion of cyclohexanol by *Aae*UPO and r*Cci*UPO was 1–2 orders of magnitude less efficient ($\sim 10^2\ M^{-1}\ s^{-1}$) than by *Mro*UPO and rNOVO ($\sim 10^4\ M^{-1}\ s^{-1}$). The highest conversion rate in terms of H_2O_2 utilization was accomplished by *Mro*UPO under repeated addition of the peroxide (87% in relation to the total products formed). Using the latter UPO, we successfully established a micro-mixing reaction device (SIMM-V2) for the oxidation of cyclohexane. As cyclohexanone is a chemical of high relevance, for example, as starting material for polymer syntheses or as organic solvent, new enzymatic production pathways for this compound are of interest to complement existing chemical and biotechnological approaches. Stable and versatile peroxygenases, as those presented here, may form a promising biocatalytic platform for the development of such enzyme-based processes.

1. Introduction

Cyclohexanone (CHone) is an important bulk chemical used for the synthesis of Nylon and Perlon via ε-caprolactam as well as a widely used organic solvent. The industrial production of CHone today is typically accomplished either by oxidation of cyclohexane (CH) under pressure and at elevated temperature in air using cobalt catalysts, or by hydrogenation of phenol with noble metal catalysts under similar conditions. Apart from that, CHone can be obtained by hydrogenation of cyclohexene to cyclohexanol (CHol) and subsequent dehydrogenation to CHone at high temperature or with metal catalysts [1]. All these chemical methods consume a lot of energy and are based on the use of toxic and/or expensive metal catalysts.

An alternative approach would be to use non-toxic biocatalysts under mild conditions. There are several reports on monooxygenases, such as cytochrome P450 enzymes (P450s, EC 1.14.1.4.1) or methane monooxygenase (EC 1.14.18.3), catalyzing the most challenging first step, the hydroxylation of non-activated CH [2–5]. However, these systems suffer, in most cases, from low enzyme stability and the need of complex protein purification methods and expensive cofactors. Apart from that, efforts have been made to mimic the activities of oxygenating biocatalysts by "artificial enzymes". Thus Ni^{II} and non-heme iron complexes were shown to catalyze the hydroxylation of CH [6–8], but their efficiency is still lower than that of natural systems, in particular regarding the catalysts vs. substrate ratio.

For several years, a group of glycosylated, extracellular, H_2O_2-dependent monooxygenases has been known, which are subsumed as unspecific peroxygenase (EC 1.11.2.1) [9]. The best-characterized fungal peroxygenase, from the basidiomycetous fungus *Agrocybe aegerita*, was shown to be stable in organic solvents[1] and catalyze

Abbreviations: CH, cyclohexane; CHol, cyclohexanol; CHone, cyclohexanone; UPO, unspecific peroxygenases.

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[1] Tests on the temperature stability of *Aae*UPO and calculations being based on these data have indicated half-live times of several years (storage at 4 °C under sterile conditions; unpublished result).

a variety of oxyfunctionalization reactions [10–17] including the hydroxylation of CH to CHol as well as the further oxidation to CHone [18]. Here we report on the screening of four fungal peroxygenases, *Aae*UPO (*A. aegerita* unspecific peroxygenase, [19]), *Mro*UPO (*Marasmius rotula* unspecific peroxygenase, [20]), r*Cci*UPO (recombinant unspecific peroxygenase from *Coprinopsis cinerea*, [21]) and rNOVO (recombinant peroxygenase from a not further characterized soil mold) with regard to the stepwise oxidation of CH via CHol to CHone in a one-pot reaction system.

2. Experimental

2.1. Reagents

Commercially available chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany), TCI Europe (Eschborn, Germany) and Chemos GmbH (Regenstauf, Germany). Extracellular unspecific peroxygenase of *A. aegerita* (*Aae*UPO; wild type, isoform II, 44 kDa) was produced and purified as described previously [19,22]. Wild-type *Mro*UPO was prepared from culture liquid of *M. rotula* as recently reported [20]. Recombinant peroxygenases rNOVO and r*Cci*UPO were a gift from Novozymes A/S (Copenhagen, Denmark) [21]. The specific activities of *Aae*UPO, *Mro*UPO, r*Cci*UPO and rNOVO-preparation were 106 U mg^{-1}, 25 U mg^{-1}, 24 U mg^{-1} and 5.4 U mg^{-1}, respectively, in which 1 U represents the oxidation of 1 μmol of 3,4-dimethoxybenzyl alcohol (veratryl alcohol) into 3,4-dimethoxybenzaldehyde (veratraldehyde) within 1 min at 23 °C [22]. The peroxygenase activities were routinely measured at 310 nm by monitoring the formation of veratraldehyde at 310 nm (ε_{310} = 9.3 mM^{-1} cm^{-1}) in sodium phosphate buffer (50 mM, pH 7). The assay mixture contained 10 mM veratryl alcohol and the reaction was started with 1 mM H_2O_2 [22].

Catalase was purchased from Boehringer Ingelheim Pharma GmbH (Ingelheim am Rhein; Germany).

2.2. Reaction conditions

Typical reaction mixtures (total volume: 0.2 mL) contained purified peroxygenase with specific activity of 2 U mL^{-1} (i.e. 410 nM *Aae*UPO, 2.62 μM *Mro*UPO, 1.98 μM r*Cci*UPO, 15.61 μM rNOVO) dissolved in potassium phosphate buffer (10 mM, pH 7.0), acetone (4%), 50 mM potassium phosphate buffer (pH 7) and CH (40 mM). The reactions were started with manually added H_2O_2 (4-times 5 μL of a 40 mM stock solution with a pipette, in intervals of 1 min, respectively), stirred at room temperature for 4 min, and stopped with 40 μL of 50 mM sodium azide solution. The products were then extracted with 100 μL *n*-hexane by vigorous shaking (separate tests showed that e.g. 90 ± 0.2% of the cyclohexanone can be extracted that way from a sample). All experiments were carried out in triplicate.

2.3. Product identification and quantification

The reaction products were analyzed by GC with a Hewlett Packard 6890 chromatograph equipped with a ZB-Wax plus capillary column (250 μm in diameter, 30 min length, 0.25-μm film thickness; Phenomenex, Torrance, CA, USA) and an autosampler (HP 6890 Series Injector). For analysis, 1 μL of the *n*-hexane extract was injected into the GC system using a splitless injector at 200 °C. GC was performed with the following temperature profile: 90 °C hold 1 min, 20 °C min^{-1} to 110 °C hold 2.5 min. Quantitative analyses of the reaction products were performed by GC/MS as described above, with external standard curves of the respective authentic compounds. All standard curves had linear regression values of $R^2 > 0.98$.

2.4. Enzyme kinetics

The kinetic studies on CH hydroxylation were carried out in stirred reactions as described above using following concentrations of peroxygenases and CH: 6.4 nM *Aae*UPO, 0.1–5 mM CH; 41 nM *Mro*UPO, 0.25–5 mM CH; 31 nM r*Cci*UPO, 0.1–5 mM CH and 122 nM rNOVO, 0.1–1 mM CH. The reactions were initiated with 0.5 mM H_2O_2 and stopped with 40 μL of 50 mM sodium azide solution after 10 s, at which time less than 5% of the substrate had been consumed. The resulting CHol was quantified by GC/MS as described above, and the apparent K_m value for CH was obtained by nonlinear regression using the Michaelis–Menten model in the ANEMONA program [28].

The kinetics of CHol oxidation was studied analogously to that of CH (see above). The reaction mixtures contained peroxygenase and CHol in following concentrations: 819 nM *Aae*UPO, 0.1–7.5 mM CHol; 41 nM *Mro*UPO, 0.1–5 mM CHol; 2 μM r*Cci*UPO, 0.1–7.5 mM CHol and 122 nM rNOVO, 0.1–5 mM CHol. The reactions were initiated with 0.5 mM H_2O_2 and stopped with 40 μL of 50 mM sodium azide solution after 10 s, at which time less than 5% of the substrate had been consumed. The resulting CHone was quantified by GC/MS as described above, and the apparent K_m value for CHol was obtained by nonlinear regression using the Michaelis–Menten model in the ANEMONA program [32].

2.5. Time series data

The experiments were performed in a total volume of 0.2 mL with CH (40 mM) and *Mro*UPO (2 U mL^{-1}, 2.62 μM) dissolved in a mixture of potassium phosphate buffer (10 mM, pH 7.0) and acetone (4%, v/v). The reaction was started with manually added H_2O_2 (4-times 5 μL of a 40 mM stock solution with a pipette, resulting in a final H_2O_2 concentration of 4 mM) and stopped at four time points (after 1, 2, 3, 4 min) by adding 40 μL sodium azide (50 mM). Unconsumed substrate and products were extracted with *n*-hexane (0.1 mL) by vigorous shaking prior to GC analysis. In a second experiment the total amount of H_2O_2 was doubled (8 mM) and 2 mM H_2O_2 were added at each time point instead of 1 mM.

2.6. Micro mixer setup

The micro-mixing device SIMM-V2 (Slit interdigital Micro Mixer Version 2; Institut für Mikrotechnik Mainz GmbH, Mainz, Germany) was fed with two inlets. A 2.2-m long PEEK tubing (internal diameter 0.16 mm; VWR, Darmstadt, Germany) was attached onto the outlet. The inlet flow rates were always held at a ratio of 1:3 (one part Inlet I: three parts Inlet II). To achieve variation of the outlet flow, the two inlet flows were changed in the same ratio. Changing the total flow rate at a constant outlet-capillary length gave a change in residence time. A solution of H_2O_2 was pumped into inlet I and a mixture of *Mro*UPO, cyclohexane, acetone and phosphate buffer pH 7 was pumped into inlet II. The outlet flow was directed into Eppendorf tubes filled with 2 μl catalase solution to stop the reaction by removing hydrogen peroxide. For analysis samples were treated in a similar way as described above: 200 μl samples were extracted with 100 μl cyclohexane and then analyzed with GC–MS.

3. Results and discussion

3.1. Conversion of CH with different peroxygenases

The conversion of CH with the four peroxygenases *Aae*UPO, *Mro*UPO, r*Cci*UPO and rNOVO gave an interesting picture. While *Aae*UPO and r*Cci*UPO produced mainly CHol and only traces of CHone (*Aae*UPO: 3.05 mM CHol, 0.05 mM CHone; r*Cci*UPO: 2.37 mM