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Substrate specificity and stereoselectivity of fatty alcohol oxidase from the yeast *Candida maltosa*

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Summary. In *Candida maltosa* and other alkane-utilizing yeasts a membrane-bound fatty alcohol oxidase (FAOD) is induced by growth on *n*-alkanes. The oxidation of 1-alkanols to the corresponding aldehydes is accompanied by the stoichiometric consumption of 1 mol O_2 and formation of 1 mol hydrogen peroxide (H_2O_2). The FAOD of *C. maltosa* shows a broad substrate specificity. It catalyses the oxidation of 1-alkanols (C_4 to C_{22}), with a maximal activity of 1.85 μmol H_2O_2/min × mg protein for 1-octanol, as well as the transformation of 2-alkanols (C_8 to C_{16}) to ketones. Other compounds as α,ω-alkanediols, ω-hydroxypalmitic acid, phenylalkanols and terpene alcohols are substrates for the enzyme, although mostly with decreased activities. The oxidation of the racemic 2-alkanols by the FAOD proceeds with very high stereoselectivity for the *R*(−)-enantiomers only, leaving the *S*(+)-2-alkanol untouched.

Introduction

For *Candida* and other alkane-utilizing yeasts it has been shown that the initial step in the oxidative degradation of *n*-alkanes (Fig. 1) is carried out by a cytochrome P-450 monooxygenase system (AMOS). It catalyses the terminal hydroxylation of the *n*-alkane substrates to the corresponding fatty alcohols (for reviews see Käppeli 1986; Müller et al. 1991b).

It is well established now that the further oxidation of the 1-alkanol formed to the fatty aldehyde is mainly catalysed by a hydrogen-peroxide (H_2O_2)-producing fatty alcohol oxidase (FAOD), shown firstly by Ilchenko (1984) and Krauzova et al. (1984, 1985, 1986), and not by NAD(P)-requiring fatty alcohol dehydrogenases (FADH), as was previously assumed (Lebeault et al. 1970a, b; Sapozhnikova and Krauzova 1979; Yamada et al. 1980; Gmünder et al. 1981).

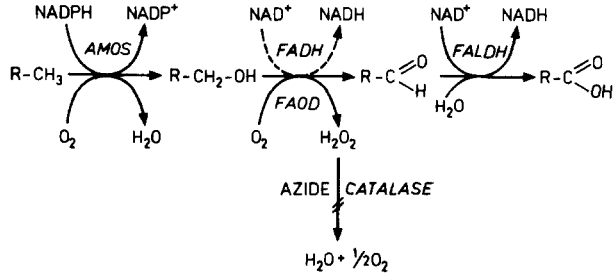

Fig. 1. First steps of alkane metabolism in yeast: *R*, alkyl chain, *AMOS*, alkane monooxygenase system; *FADH*, fatty alcohol dehydrogenase; *FAOD*, fatty alcohol oxidase; *FALDH*, fatty aldehyde dehydrogenase

On the other hand, the aldehyde formed is really oxidized by an NAD-dependent fatty aldehyde dehydrogenase (FALDH) to the fatty acid (Krauzova et al. 1985, 1986; Ilchenko and Tsfasman 1987), which, after activation to acyl-CoA esters undergoes β-oxidation or incorporation into lipids (Tanaka et al. 1988).

The aim of the present study was to investigate the substrate specificity and the stereoselectivity in 2-alkanol oxidation of the alkane-induced FAOD of *C. maltosa*.

Materials and methods

Yeast strains and cultivation conditions. They yeast *C. maltosa* EH15 (from the Institute of Biotechnology, Leipzig, FRG) was cultivated in a fermentor or in shake flasks in a mineral salt medium with *n*-alkanes (C_{11}–C_{19} mixture named Parex), glycerol or glucose as the only carbon sources, as described previously (Mauersberger et al. 1981, 1984). In comparative studies the following yeast strains were included: *C. maltosa* VSB 779 (Research Institute of Protein Biosynthesis, Moscow, USSR), *Arxula adeninivorans* CBS 8244, *Pichia guilliermondii* SBUG 50 (former H17), *Candida* sp. SBUG 440 (Section of Biology, University Greifswald, FRG), *Debaryomyces formicarius* VKM Y1555 (Institute of Biochemistry and Physiology of Microorganisms, Pushchino,

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USSR) and *Yarrowia lipolytica* H222 (Central Institute of Microbiology and Experimental Therapy, Jena, FRG).

Preparation of the crude membrane fraction. Cells of the late (linear) growth phase were harvested by centrifugation and preparation of the crude membrane fraction after mechanical cell disintegration with glass beads in a Dyno mill was performed according to earlier publications using the method of calcium-precipitation of membranes at 6000 *g* or high speed centrifugation (100000 *g*, 1 h) from the 6000 *g* supernatant (Riege et al. 1981; Mauersberger et al. 1987).

Enzyme purification. Highly purified FAOD preparations obtained by two different procedures from decane-grown *C. maltosa* VSB 779 were kindly provided by Drs. S. M. Avetisova and Y. I. Sokolov from the Research Institute of Protein Biosynthesis, Moscow, (details will be published elsewhere).

Enzyme assays. The FAOD activity was assayed by measuring the substrate-dependent O_2 consumption or H_2O_2 formation in the presence of sodium azide to inhibit catalase activity as described in detail earlier (Mauersberger et al. 1987; Blasig et al. 1988). The enzyme activity was determined at 30° C under magnetic stirring in 2.5 or 3 ml of 100 mM TRIS-HCl buffer, pH 8.8, in an open chamber equipped with an H_2O_2 electrode or in a closed chamber using an Clark type O_2 electrode (Blasig et al. 1988). After addition of the FAOD-containing protein sample the reaction was started with substrates dissolved in up to 150 µl acetone. The amount of protein sample added was varied from 0.1 to 1 mg protein for determination of initial rates and up to 5 mg for determination of total amounts of the co-substrate O_2 used or H_2O_2 formed during the reaction per substrate concentration added (measurements of stoichiometry). Saturation of the buffer with air resulted in an O_2 concentration of 240 µM under the conditions used. For H_2O_2 measurement the electrode was calibrated with H_2O_2 solutions defined by spectral determination at 240 nm.

The cytochrome P-450 content and the NAD-dependent alcohol- and aldehyde dehydrogenase (ADH and FALDH) activities were determined as described previously (Mauersberger et al. 1984). Protein concentration was assayed using the method of Lowry et al. (1951).

Product identification. To identify the products of alkanol oxidation the volume of the buffered reaction mixture described above was increased up to 10 ml, containing 25–50 mg protein of the crude membrane fraction. The reaction was started by addition of about 10 µmol substrate (1-alkanols or 2-alkanols) dissolved in 250–500 µl acetone. The incubations were continued until no further O_2 consumption was observed in a parallel 3 ml analytical assay, and stopped with 5 ml of 8% H_2SO_4. Control assays were stopped before addition of substrates. Both the stopped reaction and control mixtures were extracted according to the procedure of Dole and Meinerts (1960). The heptane extracts were analysed by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) as described by Blasig et al. (1988).

Further analysis of enantiomeric 2-alkanols was carried out by GLC (5890 Hewlett Packard, Avondale, Pa., USA; isotherm at 125° C or 150° C) after derivatization with isopropylisocyanate on a 30 m fused silica capillary coated with an XE-60 bonded tertiary butylvalinamide phase (König 1987). Peaks of the racemic mixture could be resolved to about 80%. However, the reproducibility was measured better than ±2% and the accuracy was estimated better than ±3% in the range of small amounts of the *R*(−)-enantiomers.

Materials. Primary and secondary alcohols, diols, hydroxy fatty acids and the other substrates used for the enzyme assays were purchased from Fluka (Buchs, Switzerland), Ferak (Berlin, FRG), Merck-Schuchardt (Hohenbrunn, FRG), Ventron (Karlsruhe, FRG) and from the Chemistry Department of te Mining Academy Freiberg (FRG) at least as pure and mostly analytical grade quality. All other chemicals used were of analytical grade.

Results

Occurrence and function of the FAOD in yeasts

Initial results on some properties and subcellular distribution of the FAOD activity of *C. maltosa* have been reported in previous papers (Mauersberger et al. 1987; Blasig et al. 1988). The activity of the FAOD can be measured in cell-free extracts or in membrane fractions of alkane-grown *C. maltosa* by its substrate-dependent O_2 consumption or H_2O_2 formation, respectively (Figs. 1, 3). In 100 mM TRIS-HCl buffer the FAOD showed an activity maximum at pH 8.7–9.0 with 1-decanol and 1-hexadecanol as substrates. The enzyme activity was not inhibited by 3 mM KCN, and the O_2 consumption rate was stimulated twice by addition of sodium azide, due to the inhibition of catalase present in the protein samples (Fig. 1).

The determination of real kinetic constants for the FAOD is complicated by the poor solubility (less than 10^{-5} M) of the most fatty alkanols in aqueous solutions. We obtained the best results with substrates dissolved in acetone. The initial alkanol oxidation rates determined were very dependent on the amount of acetone present in the assay. For middle-chain alkanols (C_8 to C_{12}) apparent Michaelis constant (K_m) values of 20–50 µM were determined with the membrane-bound FAOD by adding the substrates in 60–80 µl (2.0–2.7% in the assay) of acetone. For long-chain substrates such as 1-hexadecanol no clear saturation of the enzyme was achieved, even when the amount of acetone was increased up to 250 µl (8.3% in the assay). A higher acetone content in the assay decreased the enzyme activity considerably.

The FAOD and the FALDH activities of alkane-grown yeast cells are membrane-bound. In contrast to cytochrome P-450 AMOS localized in the endoplasmic reticulum (microsomes), these enzymes were found mainly in the peroxisomal fraction after cell fractionation of lysed spheroplasts, but not in mitochondria (Krauzova and Sharyshev 1987; Mauersberger et al. 1987). The partial localization of these activities in the microsomal fraction is very probably due to destruction of peroxisomes during the preparation procedure. The FAOD-containing membrane fraction after mechanical cell disruption used in this study contained besides microsomal constituents large amounts of mitochondrial and peroxisomal membrane fragments due to their disruption during cell disintegration and should be therefore regarded as a crude membrane fraction (Mauersberger et al. 1987).

Like the alkane-hydroxylating cytochrome P-450 system the two other enzymes, FAOD and FALDH, involved in the oxidation of the hydrocarbons to the corresponding fatty acids (Fig. 1) were induced by the growth of *C. maltosa* on *n*-alkanes in comparison with the substrate glycerol (Table 1). All these enzymes were found to be repressed significantly using glucose as growth substrate. A further increased FAOD activity was detected during O_2-limited growth of *C. maltosa* on alkanes (Table 1), although this O_2-mediated effect was