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SUBSTRATE SPECIFICITY OF 17β-HYDROXYSTEROID DEHYDROGENASE FROM *PLEUROTUS OSTREATUS*

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ABSTRACT

We present evidence which suggests that *Pleurotus ostreatus* 17β-HSD is a pluripotent enzyme which can oxidize 17β-hydroxysteroids and even more so hydroquinone in the presence of NAD^+. The study of the reverse reaction indicates that the carbonyl reductase activity prevails over the hydroxysteroid dehydrogenase activity. Kinetic studies also reveal the presence of a separate acetoacetyl CoA reductase / β-hydroxybutyryl CoA dehydrogenase activity in *Pleurotus ostreatus*.

INTRODUCTION

The metabolism of xenobiotics and steroids has been intensively studied from the point of view of their oxidation by different cytochromes P-450. Recently, the reduction of these compounds by carbonyl reducing enzymes has attracted more attention. A group of enzymes, classified either as hydroxysteroid dehydrogenases (HSDs) or carbonyl reductases, was found to exhibit specificity towards both groups of compounds (1). 17β-hydroxysteroid dehydrogenases (17β-HSDs) can be taken as representatives of this group of enzymes since several 17β-HSDs from mammalian peripheral as well as steroidogenic tissues have been found to posses carbonyl reductase activity in addition to

hydroxysteroid dehydrogenase activity (2-7). In fungi the pluripotency has been established only for 17β-HSD from the filamentous *Cochliobolus lunatus* (8). The white-rot fungus *Pleurotus ostreatus* has so far been found to metabolize polycyclic aromatic hydrocarbons (9) as well as to oxidize androgens and estrogens (10). Since recently a preliminary study suggested several differences in the characteristics between 17β-HSDs from both fungi, we studied *Pleurotus ostreatus* 17β-HSD in greater detail.

EXPERIMENTAL

1) Fungal species

Pleurotus ostreatus G7 was obtained from the Microbial Culture Collection of the Chemical Institute , MZKIBK Ljubljana.

2) Growth conditions

Ten-day-old cultures on agar slants (2% agar in 6^0 Blg malt extract) were used to inoculate 100 ml of liquid media composed of 0.5% cornstep liquor, 1% oatmeal, 1% glucose, 4% tomato paste, 10ml/l mineral solution, pH 7 (mineral solution: 1g/l $FeSO_4$ x $7H_2O$, 1g/l $MnSO_4$ x0 H_2O, 0.2 g/l $ZnSO_4$ x $7H_2O$, 0.1 g/l $CaCl_2$ x $2H_2O$, 0.056 g/l H_3BO_3, 0.0025 g/l $CuC0_3$, 0.019 g/l $(NH_4)_6Mo_7O_{24}$ x $4H_2O$). Cultivation was performed in 500 ml Erlenmeyer flasks for six days at 25^0 C on a rotary shaker at 110 rpm.

3) Enzyme preparation

After six days of growth the mycelium of *Pleurotus ostreatus* was filtered and frozen by liquid nitrogen. The enzyme preparation used for kinetic measurements was prepared in 50 mM Tris/HCl, pH 9, 20% glycerol, as described previously (11).

4) Enzyme assays

a) Chromatographic method

During the partial purification procedure the enzyme activity was tested as described (11).

b) Spectrophotometric method

For the kinetic measurements a similar procedure was used as already described (8).

Measurements were performed against blanks without the substrate in the reaction mixture, or without the enzyme in the case of quinone. Reaction rates were expressed as relative enzyme activities (%) with different substrates oxidized or reduced by 100µl of the enzyme preparation. The enzyme activities with 100 µM testosterone and 100 µM NAD^+ in 50 mM Tris/HCl, pH 8.5 with 20 % glycerol for the oxidation or 100 µM androstenedione and 100 µM NADH in 50 mM Tris/HCl, pH 7.0 with 20 % glycerol for the reduction reaction were taken as 100 %.

5) Competition between different substrates

Competition between the most effective substrates was studied for the oxidation reaction because of more comparable relative activities of selected substrates and because of higher initial velocities of testosterone oxidation in comparison to androstenedione reduction. It was tested by following the activity of 17β-HSD in the presence of each individual substrate and in the presence of two substrates simultaneously; the concentration of one substrate varied (testosterone from zero to 250 µM, β-hydroxybutyryl CoA from zero to 200µM, and hydroquinone from zero to 400 µM) while the concentration of the other was kept constant (testosterone 100µM, hydroquinone 150 µM and β-hydroxybutyryl CoA 75µM), and *vice versa*. The concentration of the enzyme was constant in all experiments. From the Michaelis-Menten curves fitted to the experimental results obtained in the presence of each individual substrate, we determined the apparent K_m and V_{max} for each individual substrate in the presence of 20 µM NAD^+. These parameters were then used for the calculation of the curves valid for the competition between two substrates for the same enzyme or/and for the parallel action of two different enzymes on two substrates (12).

RESULTS

A range of hydroxylated steroid and non-steroid compounds was used to investigate the substrate specificity of the 17β-HSD enzyme preparation. Table 1 shows that it has a broad specificity for hydroxylated substrates with NAD^+ required as a coenzyme.

Table 1. Relative oxidative activities of 17β-HSD enzyme preparation in the presence of different substrates and 100 μM coenzymes. The activities are normalized to the activity in the presence of 100 μM testosterone and 100 μM NAD^+, considered to be 100%.

SUBSTRATE	CONC.	COENZYME	RELATIVE ACTIVITIES (%)
testosterone	100μM	NAD^+	100
		$NADP^+$	0
estradiol	100μM	NAD^+	80
		$NADP^+$	0
hydroquinone	100μM	NAD^+	116
		$NADP^+$	0
DL-β-hydroxybutyryl CoA	100μM	NAD^+	506
		$NADP^+$	0
L-β-hydroxybutyrate	1mM	NAD^+	13
		$NADP^+$	0
p-nitrobenzyl alcohol	1mM	NAD^+	69
		$NADP^+$	0
L-malate	1mM	NAD^+	40
		$NADP^+$	0

0 = no detectable activity

β-Hydroxybutyryl CoA was found to be the best substrate while hydroquinone, testosterone, and estradiol were also readily oxidized. The relative activities for β-hydroxybutyrate, malate and p-nitrobenzyl alcohol were much smaller than for testosterone (100%). No enzyme activity could be detected in the presence of $NADP^+$.

In addition carbonyl compounds, including steroids, quinones, aromatic aldehyde and aliphatic ketones were tested as substrates for the reductase activity of 17β-HSD enzyme preparation (Table 2). Both nicotinamide nucleotides could serve as electron donors. Acetoacetyl CoA and benzoquinone were reduced at much higher relative activities than androstenedione in the presence of NADH (100%). No activity could be detected in the presence of the other tested compounds.

Table 2. Relative reductive activities of 17β-HSD enzyme preparation in the presence of different substrates and 100 μM coenzymes. The activities are normalized to the activity of 100 μM androstenedione and 100 μM NADH which was taken as 100%.

SUBSTRATE	CONC.	COENZYME	RELATIVE ACTIVITIES (%)
androstenedione	100μM	NADH NADPH	100 31.3
estrone	100μM	NADH NADPH	0 0
benzoquinone	100μM	NADH NADPH	3103 3737
acetoacetyl CoA	100μM	NADH NADPH	3108 728
acetoacetate	1mM	NADH NADPH	0 0
p-nitrobenzaldehyde	1mM	NADH NADPH	0 0

0 = no detectable activity

As the above mentioned results suggested that at least some of the reactions could be catalyzed by 17β-HSD, competition experiments between the most effective substrates testosterone, hydroquinone, and β-hydroxybutyryl CoA were performed. Testosterone was found to compete with hydroquinone for the active centre of 17β-HSD (Fig.1A). On the other hand, the results on Fig.1B suggest parallel oxidations of testosterone and β-hydroxybutyryl CoA catalyzed by two different enzymes. These results were confirmed by experimental data suggesting parallel oxidations also for hydroquinone and β-hydroxybutyryl CoA (data not shown).

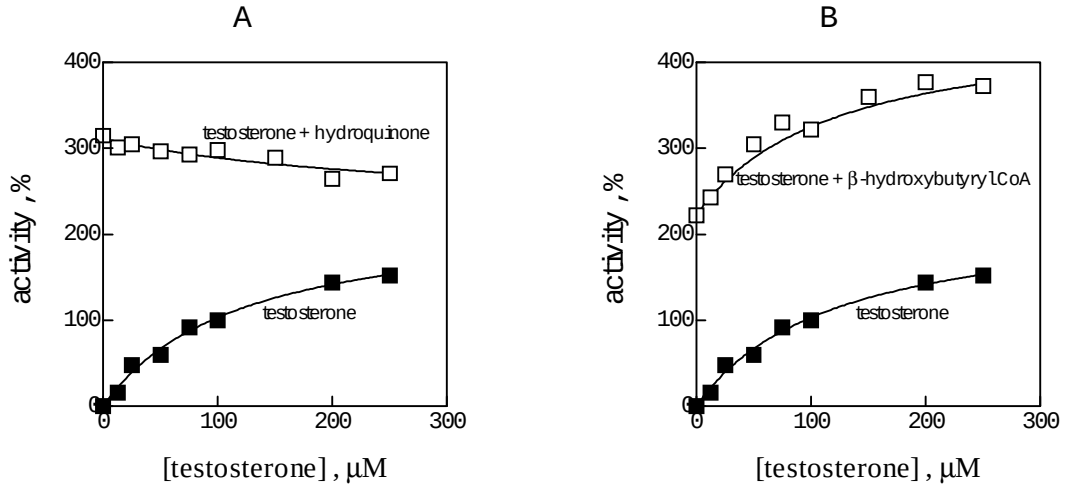

Fig.1.
A: Competition between substrates testosterone (variable concentration) and hydroquinone (150 μM) for the active centre of 17β-HSD.
B: Parallel oxidation of testosterone (variable concentration) and β-hydroxybutyrylCoA (75 μM) catalyzed by two different enzymes.
Points were obtained experimentally but the curves were calculated as described in Experimental, section 5.

CONCLUSIONS

While the important role of 17β-HSD in mammalian organisms is well established (13) the question about the role of these enzymes in primitive eukaryotes is not yet clear. It represents a challenge to those interested in fungal metabolism *per se* as well as the evolution of HSD and steroid hormone signalling system. In this sense, further characterization of fungal 17β-HSD seems desirable.

In the present study, the *Pleurotus osteratus* 17β-HSD enzyme preparation was found to have a broad substrate specificity catalyzing efficiently the oxidation of the steroid hormones testosterone and estradiol as well as the non-steroidal compounds hydroquinone and β-hydroxybutyryl CoA . NAD^+ is required as an electron donor. The results of the reverse reaction revealed the prevailing carbonyl reductase and acetoacetyl

reductase activity over 17β-HSD (reductase) activity. The competition experiments between the most effective substrates of 17β-HSD enzyme preparation suggested the presence of two separate enzymes, 17β-HSD and β-hydroxybutyryl CoA dehydrogenase / acetoacetyl CoA reductase. *Pleurotus osteratus* 17β-HSD was found to be pluripotent enzyme capable of testosterone and hydroquinone oxidation. It thus joins pluripotent HSDs whose role in detoxification of xenobiotic carbonyl compounds in addition to their role in the metabolism of endogenous steroids and quinones is only suspected (14).

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POVZETEK

Na osnovi kinetičnih študij, prikazanih v članku, lahko sklepamo, da je 17β-HSD iz glive *Pleurotus ostreatus* pluripotenten encim, ki lahko oksidira 17β-hidroksisteroide in hidrokinon v prisotnosti NAD^+. V smeri redukcije prevladuje karbonil-reduktazna aktivnost nad hidroksisteroidno reduktazno aktivnostjo. Rezultati kažejo, da je v glivi *Pleurotus ostreatus* prisotna tudi neodvisna acetoacetil CoA reduktaza / β-hidroksibutiril CoA dehidrogenaza.