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Study of an Alcohol Dehydrogenase from *Rhizopus arrhizus* Fisher

JEAN FAVERO,* JEAN-CLAUDE MANI,* AND FRANÇOIS WINTERNITZ†

*Laboratoire de Biochimie des Membranes, ER CNRS n° 228, and †Laboratoire de Produits Naturels et de Biochimie Organique de Synthèse, ER CNRS n° 195, Ecole Nationale Supérieure de Chimie, 8 rue de l'Ecole Normale, F-34075 Montpellier Cedex, France

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The *in vivo* reduction of ketone I (1,2-3*H*-dihydro(3,2,1-*kl*)pyridophenothiazine-3-one) by *Rhizopus arrhizus* Fisher is due to a NADPH-dependent alcohol dehydrogenase. This cytosolic enzyme displays a narrow specificity for ketone I, its pH optimum being pH 8. Partially purified alcohol dehydrogenase has a good affinity for ketone I (K_m = 68 μM).

1. INTRODUCTION

Fungal transformations of xenobiotics have led to spectacular results, mainly in the steroid field. Many other compounds have been assayed as substrates, but their transformations were essentially performed *in vivo*, and little is known about the enzymatic systems involved in such processes. The main reasons for this lack of data arise from experimental problems (difficulties in obtaining efficient grinding of fungi, lability of isolated enzymes, etc.).

The study of steroid hydroxylases features one of the few examples of determination of fungal transformation mechanism (*1–7*). Several bacterial hydroxysteroid dehydrogenases were also reported, mainly by Talalay (*8–10*) and Ringold (*11, 12*). The alcohol–ketone interconversion of nonsteroidal xenobiotics was studied with variously purified hydroxysteroid dehydrogenase preparations, such as the reduction of adamantanone by 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (*12*) or by crystalline cortisone reductase from *Streptomyces hydrogenans* (*13*). Jakoby and Fredericks (*14*) demonstrated the oxidation of secondary alcohols by a *Pseudomonas* dehydrogenase. The first experiments with soluble alcohol dehydrogenase preparations from yeasts were run in 1933 (*15*), and the crystallization of this enzyme from brewer's yeast (*16–18*) was the first successful crystallization of a pyridine nucleotide-dependent dehydrogenase. Prelog studied the reduction of xenobiotics by numerous microorganisms (*19*) and by partially purified dehydrogenases; he showed in particular the specific reduction of stereoisomeric decalones by *Rhizopus nigricans* (*19*) and *Curvularia falcata* (*20*).

We reported earlier the *in vivo* transformation of various steroid (*21, 22*) and nonsteroid (*23*) compounds by some microorganisms. We describe in this paper the reduction of a neuroleptic ketone, 1,2-3*H*-dihydro(3,2,1-*kl*)pyrido-

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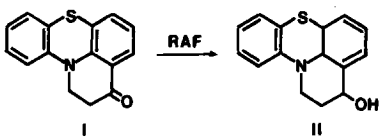
phenothiazine-3-one (I), to the corresponding 3-OH derivative (II) by *Rhizopus arrhizus* Fisher (RAF), and the partial purification and *in vitro* properties of an alcohol dehydrogenase from RAF which is involved in the reduction of ketone I. Optimal conditions for activity determination are described and kinetic constants evaluated; various analogs of ketone I are also assayed as substrates for this dehydrogenase.



I II

MATERIALS AND METHODS

Preparation of an active cell-free system. The *R. arrhizus* Fisher strain (ref CBS 127.08) was obtained from the Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands). The strain was maintained on malt extract agar at 4°C and transferred monthly to a fresh medium. The cultures used for transformations were conducted in a rich liquid medium prepared according to Kieslich (*24*): 3% glucose, 1% corn steep, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4$, 0.2% $NaNO_3$, 0.002% $FeSO_4$ in distilled water (pH 4.9). After 24 hr an inoculum from this primary culture was grown in the same medium. After 20 hr the mycelium from this secondary culture was collected on cheesecloth and thoroughly washed with 0.5% NaCl, filtered on a Büchner funnel, dried between filter paper sheets, then suspended (0.8 g/ml) in buffer A (10 m*M* EDTA, 50 m*M* Na_2HPO_4, 1 m*M* dithiothreitol, 3 mg/ml Tween 20, pH 7.1) according to Zuidweg (*1*), and ground with a Potter Elvehjem motor-driven homogenizer at 4°C. The crude homogenate was centrifuged at 900*g* (30 min), 14,000*g* (30 min), and 105,00*g* (60 min). Protein concentrations were determined by the Lowry method (*25*) using bovine serum albumin as standard.

Ammonium sulfate precipitation. The 105,000*g* supernatant was fractionated by ammonium sulfate precipitation. Dehydrogenase activity was found in the fraction precipitating between 40 and 60% of ammonium sulfate saturation.

In vivo reduction of ketones by R. arrhizus Fisher. After 12 hr of secondary culture, dimethylformamide solutions of various ketones (20 mg/ml) were added to the medium (1 ml/100 ml medium). After 72 hr of incubation the various compounds were extracted with benzene. The organic solution was washed with water, dried on sodium sulfate, evaporated, and the compounds analyzed by thin-layer chromatography.

Determination of dehydrogenase activity. The dehydrogenase activity of the various centrifugation fractions was assayed by determining the reduction of ketonic substrates, using NADPH as a coenzyme. In a typical experiment the reaction mixture contained 0.8 m*M* NADPH, 2.3 m*M* glucose 6-phosphate, 0.17 U/ml glucose-6-phosphate dehydrogenase, 0.35 m*M* substrate in 50 m*M* phos-

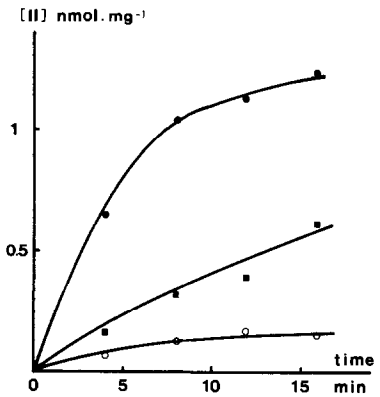


FIG. 1. NADPH specificity of soluble dehydrogenase activity from RAF. Dehydrogenase activity of a 105,000*g* supernatant was determined with either NADPH (●), NADH (■), or no added coenzyme (○); all other conditions were identical.

phate buffer (pH 7.1). The reaction started by addition of a cell-free fraction (about 1.8 mg protein/ml). After incubation at 25°C for various times, the reaction was stopped by heating 3 min at 100°C. After filtration through a Millipore filter, the clear solution was assayed for product formation. The relative amounts of ketonic substrate and alcoholic product were determined using high-pressure liquid chromatography (hplc), since spectroscopic assays of coenzyme or substrate were impossible to carry out (superposition of absorption spectra and interference of other NADPH-dependent enzymatic systems). High-pressure liquid chromatography was carried out on a Waters 6000A apparatus with a μ-Bondapack-packed analytical column (reverse phase), CH_3OH–H_2O (7 : 3) as a solvent (flow rate 2 ml/min), and uv detection. Chromatogram peak areas were standardized for substrate and product.

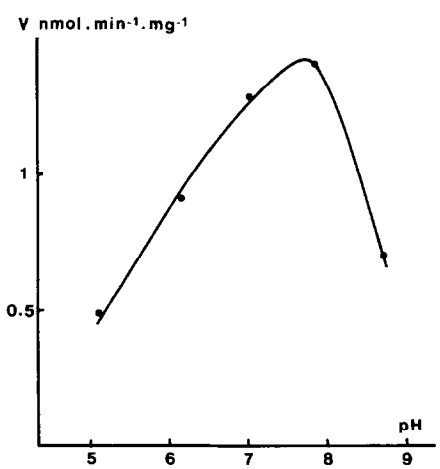


FIG. 2. pH effect on partially purified RAF dehydrogenase.

RESULTS

In vivo reduction of ketone I by R. arrhizus Fisher. Incubation of ketone I with a secondary culture of RAF led to alcohol II in high yield (95%). The benzene extract contained only products I and II, as evidenced by thin-layer chromatography. II was identified by its spectral and physical properties and by comparison with the alcohol resulting from borohydride reduction of ketone I. However, the bioconversion product II displayed optical activity ($[\alpha]_D^{20} = -32°$), while the alcohol resulting from chemical reduction was a racemate.

Partial purification of R. arrhizus Fisher alcohol dehydrogenase. The dehydrogenase activity tested on ketone I was found in the 900*g*, 14,000*g*, and 105,000*g* supernatants; the specific activities of these fractions were 0.10, 0.15, and 0.20 n*M* · min^{-1} · mg^{-1}, respectively. The 105,000*g* supernatant activity was detected even in the absence of Tween 20 (Tween 20 was, however, kept in the extraction medium in order to maintain homogeneity during the enzymatic reaction, because of the low solubility of ketone I). Because we showed that mitochondria were not disrupted in the homogenization, these results suggest that RAF dehydrogenase is a soluble enzyme present in the cytosol.

The alcohol dehydrogenase activity precipitated from the 105,000*g* supernatant between 40 and 60% ammonium sulfate saturation (specific activity, 1.4 n*M* · min^{-1} · mg^{-1}; sevenfold purification). However, this enriched fraction was

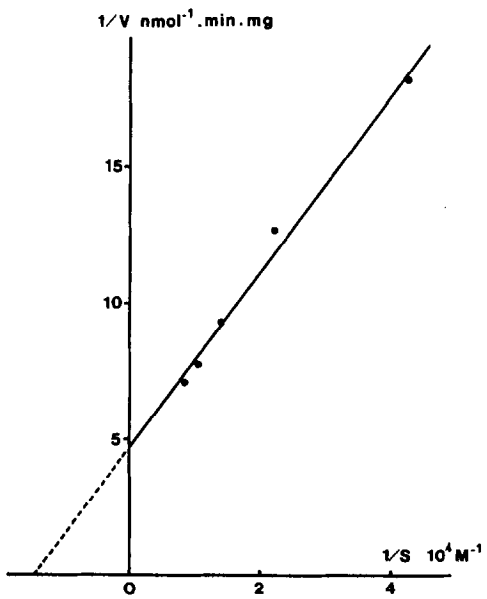


FIG. 3. Effect of substrate concentration on RAF dehydrogenase activity. Each assay (1 ml) contained 10.7 mg protein (105,000*g* supernatant), 28 μM NADPH, 2.3 m*M* glucose 6-phosphate, 0.14 U glucose-6-phosphate dehydrogenase, and various substrate (ketone I) concentrations, in phosphate buffer (pH 7.1). Lineweaver–Burk plots of the reduction rate are presented. Each point is the mean of five determinations. The standard deviation was about 10%.

very labile and lost 80% of its activity after 24 hr at 4°C or at liquid nitrogen temperature.

Temperature effects. Dehydrogenase activity was determined in parallel at 4°C and 25°C. The reaction rate was lower at 4°C and all subsequent experiments were carried out at 25°C.

Specificity for NADH. Activity curves obtained with the 105,000*g* supernatant under similar conditions, except for the nature of coenzyme (either NADPH, NADH, or no coenzyme), showed a much faster reaction with NADPH than with NADH (Fig. 1). The very low activity observed in the absence of coenzyme was probably due to the presence of small amounts of endogenous coenzyme in the supernatant, as this activity disappeared in the dialyzed supernatant. The dialyzed supernatant displayed the same specificity for NADPH.

pH effect. Figure 2 presents the variations of enzymatic activity as a function of pH between pH 4 and 9. RAF dehydrogenase displayed an optimum at pH 8.

Determination of the apparent kinetic constants. Figure 3 presents Lineweaver–Burk plots of dehydrogenase activity with various substrate (ketone I) concentrations. The apparent kinetic constants for ketone I reduction were determined from this curve: $K_m = 68\ \mu M$ and $V = 0.2$ nmol · min^{-1} · mg protein^{-1}.

Lineweaver–Burk plots of dehydrogenase activity for various NADPH concentrations are presented in Fig. 4. The curve is biphasic with a decrease in dehydrogenase activity at high coenzyme concentrations, which can be explained by an inhibition by NADPH excess. The apparent kinetic constants, obtained by extrapolation of the low-concentration part of this curve, were: $K_m = 4.3\ \mu M$ and $V = 0.11$ nmol · min^{-1} · mg protein^{-1}.

Reduction of ketone I analogs. Various tetrahydroquinolinones which are structurally related to the phenothiazinic ketone I were synthesized according to

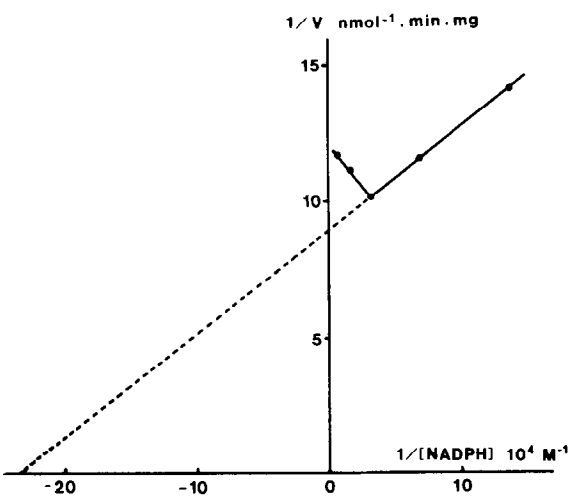


FIG. 4. Effect of NADPH concentration on RAF dehydrogenase activity. Assay conditions were similar to those of Fig. 3, except for ketone I concentration (256 μM) and the use of various coenzyme concentrations. Results are presented as Lineweaver–Burk plots. Each point is the mean of five determinations. The standard deviation was about 10%.

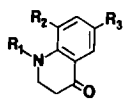
TABLE 1

REDUCTION OF KETONE I ANALOGS BY PARTIALLY PURIFIED RAF ALCOHOL DEHYDROGENASE AND BY WHOLE RAF

	Substrate[a]			Transformation extent by RAF after 72 hr (%)	Transformation by purified dehydrogenase
	R_1	R_2	R_3		
III	Phenyl	H	H	60	+
IV	H	OCH_3	H	60	–
V	H	H	OCH_3	0	–
VI	H	CH_3	H	10	–
VII	H	H	CH_3	10	–

[a] Substrate concentrations ranged between 50 and 300 μM.

Note. These analogs are various substituted 4-oxo-tetrahydroquinolines and possess the general formula:



Merchant (*26*) and Cookson's (*27*) methods. These compounds were incubated either with RAF or with the 105,000*g* supernatant, or with ammonium sulfate-purified dehydrogenase. The results are summarized in Table 1.

1-Phenyl-4-oxo-1,2,3,4-tetrahydroquinoline (III), which has the best structural analogy with ketone I, was the only compound reduced both *in vivo* by whole RAF and *in vitro* by the ammonium sulfate-purified dehydrogenase. This compound partially inhibited the reduction of ketone I. *o*-Methoxytetrahydroquinolinone (IV), which was readily reduced *in vivo* by RAF, was not affected by the purified dehydrogenase and did not inhibit ketone I reduction. These results suggest that two different dehydrogenases are involved in the reduction of ketones I and III on the one hand, and ketone IV on the other. A narrow structural specificity appears between these two enzymatic systems. Other well-known dehydrogenases (alcohol dehydrogenase from baker's yeast or horse liver, 3-OH-steroid dehydrogenase) did not reduce ketone I.

DISCUSSION

R. arrhizus Fisher is a microorganism largely used as a steroid hydroxylating agent (*28*), although a few papers reported the reduction of ketones to alcohols by this fungus (*29*). However, in spite of its extensive use in xenobiotic transformations, *in vitro* transformations using acellular extract or purified proteinic fractions from RAF have never been reported.

The excellent yield (95%) of the *in vivo* transformation of ketone I (1,2-3*H*-dihydro(3,2,1-*kl*)pyridophenothiazine-3-one) by RAF prompted us to study the alcohol dehydrogenase involved in this transformation. The experimental difficulties encountered in this study were: (1) finding grinding conditions leading

to noticeable dehydrogenase activity in the acellular supernatant; (2) measuring this enzymatic activity (hplc was the only technique which gave reliable results).

We showed that the RAF acellular extract contains an NADPH-dependent alcohol dehydrogenase which reduces ketone I to the corresponding 3-OH derivative. This cytosolic enzyme displays a narrow specificity for ketone I as evidenced by the results in Table 1. Its pH optimum was found around pH 8. Partial purification of this dehydrogenase was obtained by ammonium sulfate precipitation of a 105,000*g* supernatant; a sevenfold enhanced activity was found in the fraction precipitating between 40 and 60% ammonium sulfate saturation. This partially purified enzyme has a good affinity for ketone I (K_m = 68 μM). However, the high lability of this fraction prevents further purification of this dehydrogenase.

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