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Pyridine Nucleotide-Linked Oxidation of Methanol in Methanol-Assimilating Yeasts

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An alcohol dehydrogenase linked to nicotinamide adenine dinucleotide and requiring glutathione has been isolated and partially purified from two methanol-assimilating yeasts. It differs from previously described methanol-oxidizing enzymes in pH optima, electron acceptor specificity, substrate specificity, inhibition pattern, and stability.

Several workers have investigated methanol oxidation in bacteria and none has reported evidence of a pyridine nucleotide-linked methanol-oxidizing enzyme (1-3). Tani et al. (6) described methanol oxidation in yeast that occurs via an oxidase. The data suggest that a flavine nucleotide was involved.

This paper describes the nicotinamide adenine dinucleotide (NAD)-linked methanol-oxidizing enzyme in methanol-assimilating yeasts, *Pichia pinus* and *Candida boidianii*.

A previous study showed that this enzyme is present in *P. pinus* and *Kloeckera* sp. 2201 (4).

MATERIALS AND METHODS

Organisms. *P. pinus* NRRL YB-4025 and *C. boidianii* NRRL Y-2332 were obtained from the Northern Regional Laboratory, Peoria, Ill. Cultures were maintained as described previously (4).

Growth conditions. Both cultures were grown as described previously (4) in a synthetic medium consisting of: NH_4NO_3, 0.5%; K_2HPO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.05%; yeast extracts, 0.2%; and methanol, 2%.

Chemicals: Methanol was purchased from Fisher Scientific Co., Fairlane, N.J. NAD and its phosphate form (NADP), dichlorophenol indophenol, and glutathione (GSH) were from Sigma Chemical Co., St. Louis, Mo.

Preparation of cell extracts. The cultures were centrifuged at 27,000 × *g* at 4 C and washed twice with deionized water. The washed cells (5 g, wet weight) were suspended in 30 ml of 0.001 M potassium phosphate buffer (pH 7.0) with 5 g of washed, superfine glass beads (Superbrite type 110, 3M Company, St. Paul, Minn.). The suspension, contained in a 100-ml beaker placed in dry ice, was treated in an ultrasonic disintegrator (Branson Sonifier, Stamford, Conn.) fitted with a 2.5-cm probe for 5 min at 1 C and then centrifuged at 27,000 × *g* for 30 min at 4 C. The clear supernatant was then assayed for the enzyme.

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Methanol dehydrogenase was assayed, as described previously (4), in a 1-ml system containing: potassium phosphate buffer, 100 μmol; NAD, 1 μmol; GSH, 1 μmol; and enzyme protein, 10 to 20 μg. To start the reaction, 20 μmol of methanol was added. Protein was measured by the method of Warburg and Christian (7).

RESULTS

Table 1 shows amount of methanol dehydrogenase activity in both yeasts at different pH values; pH 7.5 is optimum for both.

Cofactors required to oxidize methanol in a cell-free system. The results in Table 2 indicate that different cofactors affect methanol oxidation in a cell-free system. GSH stimulated the activity; this oxidation requires NAD (NADP cannot replace it).

Replacement of GSH by other thiol compounds. Several thiol compounds were tested for their ability to replace GSH. It was found that methanol oxidation in both yeasts depends strongly on GSH; dithiothreitol, cysteine, and sodium dithionite cannot replace it.

Partial purification of enzyme from P. pinus. A crude extract (40 ml) was applied to a column (95 by 2.5 cm) of diethylaminoethyl-cellulose that had been equilibrated with 0.01 M phosphate buffer. The charged column was treated with 300 ml of 0.01 M buffer, 200 ml of 0.1 M buffer, and 500 ml of 0.3 M buffer, each containing 0.001 M GSH. The enzyme was eluted when the column was washed with 0.3 M phosphate buffer. By this method, the enzyme was purified fourfold (Table 3). When purified further by ammonium sulfate fractionation or Sephadex G-200 column chromatography, it became inactive. By an alternate method (Table 3, no. 2) using ammonium sulfate fractionation, about fourfold purification was obtained.

TABLE 1. *Specific activities of methanol dehydrogenase in methanol-assimilating yeasts*

System (pH)	Activity[a]	
	P. pinus	*C. boidianii*
5.5	10.8	0
6.0	42.9	2.0
6.5	48.2	4.0
7.0	53.6	118.0
7.5	90.8	279.0
8.0	64.3	268.0
8.5	21.4	247.0
9.0	27.0	107.0

[a] Nanomoles of NADH formed per minute per milligram of protein.

TABLE 2. *Cofactor requirement for oxidation of methanol by a cell-free system of methanol-assimilating yeasts*[a]

System	Activity[b]	
	P. pinus	*C. boidianii*
Complete	90.8	279.0
Minus GSH	32.0	30.0
Minus NAD	0	0
Minus methanol	10.0	15.0
Minus cell-free extract	0	0
Minus NAD plus NADP	0	0

[a] The complete system contained (in a total volume of 1 ml): potassium phosphate buffer (pH 7.0), 200 μmol; NAD, 1 μmol; GSH, 1 μmol; and enzyme protein, 10 to 20 μg. Reaction was initiated with 20 μmol of methanol.

[b] Nanomoles of NADH formed per minute per milligram of protein.

TABLE 3. *Partial purification of the methanol dehydrogenase of P. pinus*

Method	Step	Vol (ml)	Total protein (mg)	Total activity	Sp act[a]	Purification (fold)
1	Crude	40	320	32,000	100	1.0
	DEAE-cellulose (0.3 M PO_4 peak pooled)[b]	224	60	24,120	402	4.0
2	Crude	55	1,100	99,000	90	1.0
	40–80% (NH_4)-SO_4 fraction	7	290.5	98,000	337.3	3.7

[a] Nanomoles of NADH formed per minute per milligram of protein.

[b] DEAE, Diethylaminoethyl.

Stability. At 4 C the enzyme lost all ability to oxidize methanol within 24 h after fourfold purification by ammonium sulfate fractionation or diethylaminoethyl-cellulose chromatography; at 50 C it lost its activity within 5 min. Methanol-oxidizing activity did not stabilize when dithiothreitol, GSH, substrate, or a combination of GSH and substrate was added during purification, possibly because protecting proteins were removed and sulfhydryl groups oxidized rapidly at the active sites. An inhibition study supported this observation.

Inhibitor studies. *Para*-chloromercuric benzoate or iodoacetamide at a concentration of 10^{-6} M inhibited methanol-oxidizing activity from both yeasts after the enzyme was preincubated for 15 min.

Substrate specificity of a partially purified enzyme from P. pinus. The partially purified enzyme showed broader substrate specificity for various primary and secondary alcohols (Table 4).

Electron acceptor specificity. Methanol dehydrogenase activity from the partially purified enzyme is specific for NAD and GSH but not for NADP, potassium ferricyanide, dichlorophenol indophenol, and phenazine methosulfate.

Effect of metal ions. The effect of various metal ions on the oxidation of methanol by the enzyme is shown in Table 5. None of the metal ions tested stimulated the activity, and it was completely inhibited by Cu and Hg at a concentration of 10^{-4} M. Zn gave 50% inhibition at this concentration.

TABLE 4. *Oxidation of various alcohols by the partially purified enzyme of P. pinus*

Substrate	Relative rate (%)
Methanol	100
Ethanol	277
n-Propanol	144
n-Butanol	50
n-Pentanol	6
Isopropanol	633
Isobutanol	6

TABLE 5. *Effect of metal ions on enzyme activity of P. pinus*[a]

Metal ion	Relative activity (%)
None	100
$MgCl_2$	90
$ZnSO_4 \cdot 7H_2O$	50
$CuSO_4 \cdot 5H_2O$	0
$MnCl_2 \cdot 4H_2O$	102
$HgCl_2$	0
$CaCl_2 \cdot 2H_2O$	85

[a] The enzyme was preincubated at a concentration of 10^{-4} M for 1 h at room temperature and then assayed for activity.

DISCUSSION

The results of this study demonstrate that the methanol-assimilating yeasts *P. pinus* and *C. boidicnii* contain NAD-specific and GSH-requiring alcohol dehydrogenases that oxidize methanol.

In contrast, methanol dehydrogenases of *Pseudomonas* strains M27, AM1, and RJ1 were shown to be independent of pyridine nucleotides, and required phenazine methosulfate and dichlorophenol indophenol (1-3). An oxidase enzyme that utilizes molecular oxygen has been described for *Kloeckera* sp. 2201 and *C. boidianii* (5, 6). The pH optima are 8.5 to 9.0 for the methanol dehydrogenase enzyme in bacteria (2, 3), 7.5 to 9.5 for the methanol oxidase in yeasts, and 7.5 for the enzyme described here.

The methanol-oxidizing enzyme in bacteria (1, 2) and yeast (5, 6) is quite stable, but the partially purified *Pichia* methanol-oxidizing enzyme is very unstable.

Methanol dehydrogenases reported in the literature have broad specificity toward primary alcohols; the enzyme described here also exhibits similar broad specificity.

An unusual affinity was observed with the *Pichia* enzyme in that isopropanol was oxidized very rapidly. This reaction has not been observed with other alcohol dehydrogenases.

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