APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1997, p. 1715–1720 Vol. 63, No. 5
0099-2240/97/$04.00+0

Purification and Properties of Methyl Formate Synthase, a Mitochondrial Alcohol Dehydrogenase, Participating in Formaldehyde Oxidation in Methylotrophic Yeasts

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Received 4 November 1996/Accepted 18 February 1997

Methyl formate synthase, which catalyzes methyl formate formation during the growth of methylotrophic yeasts, was purified to homogeneity from methanol-grown *Candida boidinii* and *Pichia methanolica* cells. Both purified enzymes were tetrameric, with identical subunits with molecular masses of 42 to 45 kDa, containing two atoms of zinc per subunit. The enzymes catalyze NAD^+-linked dehydrogenation of the hydroxyl group of the hemiacetal adduct [$CH_2(OH)OCH_3$] of methanol and formaldehyde, leading to the formation of a stoichiometric amount of methyl formate. Although neither methanol nor formaldehyde alone acted as a substrate for the enzymes, they showed simple NAD^+-linked alcohol dehydrogenase activity toward aliphatic long-chain alcohols such as octanol, showing that they belong to the class III alcohol dehydrogenase family. The methyl formate synthase activity of *C. boidinii* was found in the mitochondrial fraction in subcellular fractionation experiments, suggesting that methyl formate synthase is a homolog of *Saccharomyces cerevisiae* Adh3p. These results indicate that formaldehyde could be oxidized in a glutathione-independent manner by methyl formate synthase in methylotrophic yeasts. The significance of methyl formate synthase in both formaldehyde resistance and energy metabolism is also discussed.

In methylotrophic yeasts, methanol is oxidized to formaldehyde by alcohol oxidase (AOD; EC 1.1.3.13) (18), and then the assimilation pathway for C_1 compounds is initiated by formaldehyde fixation with xylulose 5-phosphate by dihydroxyacetone synthase (DHAS; EC 2.2.1.3) (16), leading to the formation of dihydroxyacetone and glyceraldehyde 3-phosphate. On the other hand, formaldehyde undergoes complete oxidation to CO_2, starting with oxidation by glutathione-dependent formaldehyde dehydrogenase (GSH-FaDH; EC 1.2.1.1) in the cytosol (28). These sequential reactions are considered to participate in the energy metabolism of methylotrophic yeasts (20). Thus, formaldehyde is the central intermediate of methylotrophic metabolism, being situated at the branching point of the assimilation and dissimilation pathways (20). However, since formaldehyde can be very toxic to cells by forming adducts with proteins and DNA (3), the level of formaldehyde should be kept under strict control. For example, the sudden addition of a large amount of methanol to a methanol-fed batch culture causes formaldehyde accumulation and in some cases leads to the cessation of cell growth. This could be a problem when using the methylotrophic yeast gene expression system for the industrial production of proteins for which high-cell-density cultivation (ca. 100 g of dry cell weight per liter) is necessary (4, 8, 24).

Methylotrophic yeasts are relatively more resistant to formaldehyde than are conventional yeast strains, and they are able to grow on media containing 16 mM formaldehyde or more (17). We have been studying formaldehyde metabolism in methylotrophic yeasts in relation to their formaldehyde resistance. So far, the formaldehyde resistance was mainly explained by (i) detoxification through formaldehyde-consuming reactions catalyzed by GSH-FaDH and DHAS and (ii) compartmentalization of the two enzymes responsible for the formaldehyde formation (AOD) and formaldehyde fixation (DHAS) reactions in a membrane-surrounded organelle, the peroxisome (5, 10, 11). Recently, we found a novel enzymatic activity responsible for formaldehyde consumption in diverse methylotrophic yeast strains and suggested that formaldehyde could be oxidized in a glutathione-independent manner concomitant with methyl formate synthesis (25). A significant amount of methyl formate accumulates during the growth of several methylotrophic yeast strains under normal physiological conditions, and the accumulation is stimulated by the exogenous addition of formaldehyde to the culture medium (25). In this study, we purified and characterized the enzyme responsible for this methyl formate accumulation, methyl formate synthase (MFS), from two species of methylotrophic yeasts, *Candida boidinii* and *Pichia methanolica*. MFS was found to be an NAD^+-linked alcohol dehydrogenase localized in mitochondria.

MATERIALS AND METHODS

Materials. An aqueous solution of formaldehyde was prepared by hydrolysis of paraformaldehyde and standardized by means of the reaction catalyzed by FaDH (Toyobo, Osaka, Japan) (15). All other reagents were commercially available and of analytical grade.

Yeast strains and cultivation. The methylotrophic yeasts *C. boidinii* S2 (AKU 4615) and *P. methanolica* (AKU 4262) were grown on methanol in MI medium as described previously (26). Formaldehyde at 2 mM was added after a 24-h cultivation.

Enzyme assays. MFS was assayed in a standard reaction mixture comprising 100 μmol of potassium phosphate buffer (pH 7.0), 50 μmol of formaldehyde, 500 μmol of methanol, 15 μmol of NAD^+, and an appropriate amount of enzyme, in a total volume of 1 ml. After incubation at 25°C for 10 min, an aliquot of the reaction mixture was directly subjected to gas chromatography. One unit of enzyme is defined as the amount of enzyme that catalyzes the production of 1 μmol of product in 1 min under the described conditions. The apparent K_m and V_{max} values of the enzymes were determined from Lineweaver-Burk plots. The

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following enzymes were assayed by previously described methods: alcohol dehydrogenase (EC 1.1.1.1) (7), AOD (EC 1.1.3.13) (30), GSH-FaDH (EC 1.2.1.1) (28), catalase (EC 1.11.1.6) (1), DHAS (EC 2.2.1.3) (16), and cytochrome *c* oxidase (EC 1.9.3.1) (31).

Analyses. The amount of protein was measured with a Bio-Rad protein assay kit (Japan Bio-Rad Laboratories, Tokyo, Japan) with bovine serum albumin as the standard (2). The specific absorbance at 280 nm of the enzyme at 1 mg/ml was determined by the method of van Iersel et al. (32). The specific absorbance of the purified fraction was calculated to be 0.88 for the *Candida* enzyme and 0.91 for the *Pichia* enzyme. The molecular mass of the native enzyme was determined by gel filtration with a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden) involving a Superdex 200 column equilibrated with 50 mM Tris-Cl buffer (pH 7.5) containing 0.1 M KCl. The standard protein markers were from Pierce (Rockford, Ill.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12.5% polyacrylamide gel by the method of Laemmli (22). The purified enzymes were partially digested with protease V8 (Sigma Chemical Co., St. Louis, Mo.), subjected to SDS-PAGE, and then blotted onto a polyvinylidene difluoride membrane. The amino acid sequence of the peptide in each band was determined by Edman's method with a gas-phase protein sequencer (Applied Biosystems model 761A). Methyl formate and methanol were determined by gas chromatography with a flame ionization detector under the following conditions: column, Porapak Q (3.2 mm by 2.1 m); carrier gas, N_2 (50 ml/min); column temperature, 170°C. Formaldehyde was measured by the method of Nash (23). The enzymatic determination of formate was performed principally by the method of Hopper and Knappe (12) with yeast formate dehydrogenase (Boehringer, Mannheim, Germany). The zinc content of the protein was determined with an atomic absorption/flame emission spectrophotometer (Shimadzu type AA-670), with the dialyzed enzyme solution being directly applied to the spectrophotometer. The dialysis buffer was previously treated with diphenylthiocarbazone to remove contaminating metal ions.

Enzyme inhibition. A mixture of the enzyme and a metal salt (or a reagent) in 50 mM potassium phosphate buffer (pH 7.0) was incubated at 25°C for 1 h, diluted 10-fold with the buffer, and then subjected to the assay under standard conditions.

Purification of MFS. The enzyme was purified from methanol-grown *C. boidinii* and *P. methanolica* cells. The procedures were performed at 0 to 4°C. All the buffers used for the purification contained 2 mM dithiothreitol.

(i) MFS from *C. boidinii*. The dialyzed cell extract of the yeast was applied to a DEAE-Toyopearl 650 M column (2.2 by 20 cm) equilibrated with 50 mM Tris-Cl buffer (pH 8.5). The column was washed with the equilibrating buffer, and then the enzyme was eluted with a linear gradient of increasing KCl concentrations (0 to 0.5 M). The active fractions collected were chromatographed on a butyl-Toyopearl 650 M column (2.2 by 20 cm), which was equilibrated with 50 mM Tris-Cl buffer (pH 8.5) containing 3 M NaCl, with a linear gradient of decreasing NaCl concentrations (3.0 to 0 M). The active eluate was concentrated by ultrafiltration (YM-30; Amicon, Beverly, Mass.) and then gel filtered through a HiLoad 16/60 Superdex 200 column (Pharmacia-Biotech) equilibrated with 50 mM Tris-Cl (pH 8.5) containing 0.5 M NaCl. Next, the NaCl concentration of the active fraction collected in the previous step was adjusted to 3 M, and the enzyme was chromatographed on a butyl-Sepharose 4FF column (1.2 by 9 cm) with a linear gradient of decreasing NaCl concentrations (3.0 to 0 M); the activity was found in the eluate containing 1.5 M NaCl. The purified enzyme was concentrated by ultrafiltration as described above, dialyzed against 50 mM Tris-Cl buffer (pH 8.5), and stored at 0°C.

(ii) MFS from *P. methanolica*. A cell extract was prepared in the same manner as for the *C. boidinii* enzyme. The enzyme was precipitated by the addition of ammonium sulfate to 40% saturation and then dialyzed against 50 mM Tris-Cl (pH 8.5). The enzyme purification was performed by sequential column chromatography on DEAE-Toyopearl 650 M, butyl-Toyopearl 650 M, and HiLoad 16/60 Superdex 200 under essentially the same conditions as described above. Finally, the purified enzyme was concentrated by ultrafiltration and stored at 0°C.

Subcellular fractionation. *C. boidinii* cells were grown on MI-YE medium as described previously, converted to spheroplasts, and then lysed by means of osmotic shock, and cell debris and nuclei were removed by centrifugation at $500 \times g$, as described previously (27). The resulting supernatant was subjected to centrifugation at $20,000 \times g$ for 20 min to obtain an organelle pellet containing mainly peroxisomes and mitochondria. The pellet was suspended in 1.0 M sorbitol–5 mM morpholineethanesulfonic acid (MES; pH 5.5) containing 1 mM phenylmethylsulfonyl fluoride. The obtained organelle suspension (3 ml) was loaded on 36.5-ml discontinuous sucrose gradients (6.5 ml of 30, 35, 40, 45, and 50% [wt/wt] sucrose and 4 ml of 60% [wt/wt] sucrose and then centrifuged for 5 h 30 min at 27,000 rpm ($100,000 \times g$) at 2°C in an SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The gradients were each fractionated into nine samples, as follows from top to bottom): no. 1, 2.0 ml; no. 2, 4.0 ml; no. 3 to 7; 6.0 ml; no. 8, 2.0 ml; no. 9, 1.5 ml. Catalase and cytochrome *c* oxidase were used as marker enzymes of peroxisomes and mitochondria, respectively.

RESULTS

Purification of MFS from *C. boidinii* and *P. methanolica*. The enzyme activity was measured by a direct assay of actual methyl formate formation. Since the enzyme activity increased on the addition of formaldehyde to the culture medium, the synthetic methanol medium for growing the cells contained 2 mM formaldehyde. As summarized in Tables 1 and 2, MFS was purified 94.6- and 57.8-fold from the cell extracts of *C. boidinii* and *P. methanolica*, respectively. Each purified enzyme gave a single band on SDS-PAGE, indicating apparent homogeneity of the protein. The specific activity was 209 U/mg of protein for the *Candida* enzyme and 62.6 U/mg of protein for the *Pichia* enzyme. The MFS activity was detected only when the purified MFS and all of the substrates, i.e., formaldehyde, methanol, and NAD^+, were present in the reaction mixture.

TABLE 1. Purification of MFS from *C. boidinii* S2

Procedure	Total activity (U)	Total amt of protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Cell extract	3,115	1,409	2.21	1	100
DEAE-Toyopearl 650M	1,652	492	3.36	1.52	53.6
Butyl-Toyopearl 650M	677	13.4	50.4	22.8	21.7
Superdex 200	199	2.04	97.5	44.1	6.39
Butyl-Sepharose 4FF	186	0.890	209	94.6	5.97

Molecular mass, subunit structure, and partial amino acid sequences. The apparent molecular mass of the *Candida* enzyme was estimated to be 45 kDa by SDS-PAGE or 174 kDa by gel filtration. Thus, the enzyme is composed of four subunits with identical molecular masses. The N terminus was resistant to Edman degradation, being blocked by a residue. Two partial amino acid sequences of internal peptides obtained on protease V8 digestion were determined. These sequences shared highly conserved amino acid stretches with several yeast alcohol dehydrogenases (Fig. 1).

The apparent molecular mass of the *Pichia* enzyme was 42 kDa by SDS-PAGE. Gel filtration of the purified enzyme gave two peaks corresponding to molecular masses of 169 and 42 kDa, with the specific activities of the two preparations being almost the same. When the higher-molecular-mass fraction was subjected to gel filtration, two peaks corresponding to 169 and 42 kDa were again observed. The estimated molecular mass of the latter peak corresponds to the molecular mass estimated by SDS-PAGE, and both peak fractions gave the same N-terminal amino acid sequence showing high similarity to those of several yeast alcohol dehydrogenases (Fig. 1). This suggests that the subunit structure of the native *Pichia* enzyme is a homotetramer, which is able to dissociate into the monomeric form.

TABLE 2. Purification of MFS from *P. methanolica*

Procedure	Total activity (U)	Total amt of protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Cell extract	9,910	9,000	1.1	1	100
Ammonium sulfate	7,130	3,130	1.7	1.6	71.9
DEAE-Toyopearl 650M	2,240	121	18.5	17.1	22.6
Butyl-Toyopearl 650M	1,190	34.5	34.5	31.8	12.0
Superdex 200	650	10.4	62.6	57.8	6.56

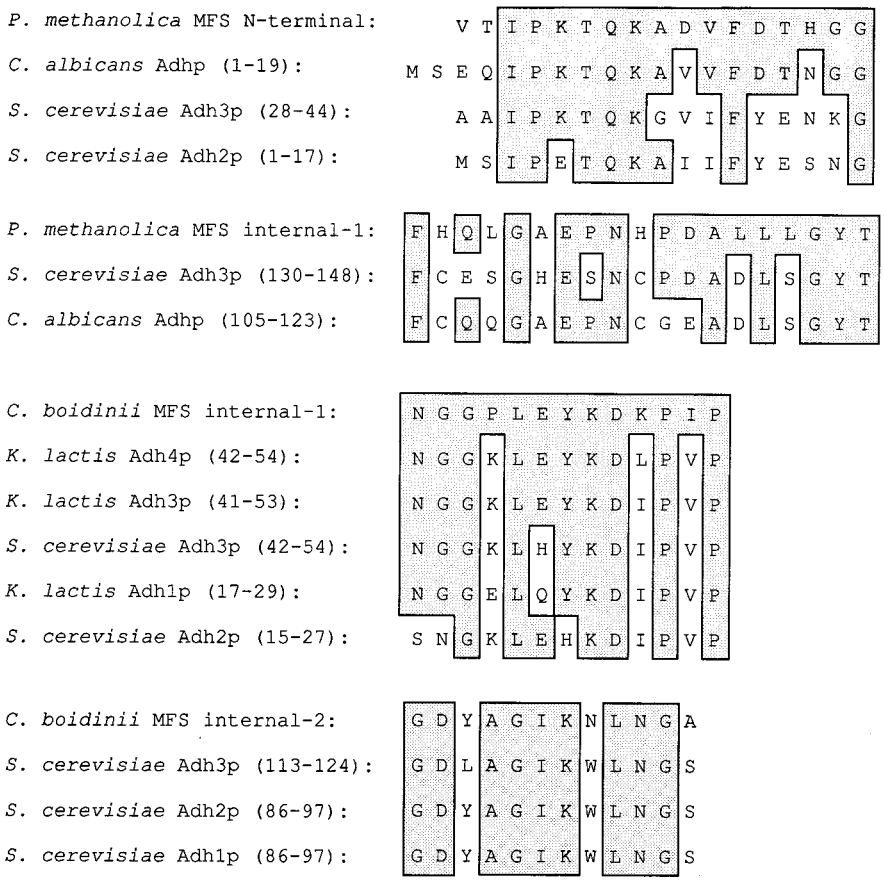

FIG. 1. Similarity of the partial amino acid sequences obtained for the purified MFS to those of other yeast zinc-containing alcohol dehydrogenases. Identical amino acid residues are shadowed. The amino acid positions in parentheses begin from the initiator methionine. The Swiss-Prot accession numbers are as follows: *S. cerevisiae* Adh1p (cytosolic), P00330; *S. cerevisiae* Adh2p (cytosolic), P00331; *S. cerevisiae* Adh3p (mitochondrial), P07246; *Kluyveromyces lactis* Adh1p (cytosolic), P20369; *K. lactis* Adh3p (mitochondrial), P49384; *Candida albicans* Adhp, P43067.

The enzyme preparations from both yeasts exhibited an absorption maximum at 280 nm but none in the visible region.

Mechanism of the enzyme reaction. Enzymatic synthesis of methyl formate occurred only in the complete reaction mixture containing formaldehyde, methanol, and NAD^+. The stoichiometry of the enzyme reaction was established with the *Candida* enzyme. Under standard conditions, the amounts of formaldehyde consumed (397 nmol), NADH formed (405 nmol), and methyl formate formed (402 nmol) were closely equivalent. The amount of methanol consumed was not measured because it was present in excess in the reaction mixture. NAD^+-dependent dehydrogenation of formaldehyde or methanol was not found with the purified enzyme from either yeast, even when reduced GSH (2 mM) was added. The apparent K_ms of the *Candida* enzyme for methanol and formaldehyde were 36 and 48 mM, respectively. Relatively high concentrations of formaldehyde and methanol were needed for methyl formate synthesis. The affinities of the substrate to the *Pichia* enzyme were almost the same as above. Since the hemiacetal adduct [$CH_2(OH)OCH_3$] is present in equilibrium with methanol and formaldehyde in an aqueous solution, the enzyme catalyzes the dehydrogenation of the hydroxyl group of the hemiacetal adduct as follows:

$$CH_2(OH)OCH_3 + NAD^+ \rightarrow CH(O)OCH_3 + NADH + H^+$$

The reverse reaction, i.e., NADH-dependent formation of methanol and formaldehyde, did not occur. Both enzymes catalyzed the formation of methyl acetate from 50 mM acetaldehyde and 500 mM methanol and the formation of ethyl formate from 50 mM formaldehyde and 500 mM ethanol, with the activities being 38 and 45%, respectively, under the standard conditions for methanol and formaldehyde. No ester was formed from the combinations of methanol and propionaldehyde, formaldehyde and 1-propanol, or acetaldehyde and ethanol. On the other hand, the enzyme exhibited NAD^+-dependent dehydrogenase activity toward several primary alcohols but not methanol (Table 3). The kinetic data showed that primary aliphatic alcohols with longer carbon chains were preferred as substrates by both enzymes.

Effects of temperature and pH on the enzyme activity. The enzymes from *C. boidinii* and *P. methanolica* exhibited maximum activity at 25°C, 40% of maximum activity at 40°C, and no activity at 55°C. The enzymes were most active at pH 7.0 (50 mM potassium phosphate) and exhibited 55% of maximum activity at pH 6.0 (50 mM potassium phosphate) and 8.0 (50 mM glycine–NaOH).

Effects of metal salts and reagents. Both the methyl formate-synthesizing and alcohol (ethanol)-dehydrogenating activities of the *Candida* and *Pichia* enzymes were completely

TABLE 3. Apparent kinetic parameters for primary alcohols of the enzymes from *C. boidinii* S2 and *P. methanolica*

Alcohol	*Candida* enzyme			*Pichia* enzyme		
	K_m (mM)	V_{max} (U[a]/mg)	V_{max}/K_m	K_m (mM)	V_{max} (U[a]/mg)	V_{max}/K_m
Ethanol	469	22.1	0.047	478	21.8	0.046
1-Propanol	387	28.0	0.072	393	27.3	0.069
1-Butanol	107	24.8	0.23	93.8	23.5	0.25
1-Pentanol	34.8	18.7	0.54	36.1	21.8	0.60
1-Hexanol	8.19	50.2	6.1	7.41	43.7	5.9
1-Octanol	6.25	73.4	12	5.19	68.1	13

[a] The NAD^+-dependent alcohol dehydrogenase was assayed by the method of Fujii and Tonomura (7). 1 U = 1 μmol of NADH produced/min.

TABLE 4. Effect of pH on the release of enzymes from pellets containing peroxisomes and mitochondria of methanol-grown *C. boidinii* S2

Enzyme	% Enzyme activity recovered in pellet after treatment at[a]:	
	pH 5.5	pH 8.0
Methyl formate synthase	95	97
Cytochrome *c* oxidase	>99	>99
Alcohol oxidase	91	35
Catalase	74	18
Dihydroxyacetone synthase	85	22

[a] An organellar pellet containing mainly peroxisomes and mitochondria was incubated in 5 mM MES buffer (pH 5.5) or 30 mM Tris-Cl buffer (pH 8.0) on ice overnight and then centrifuged at 20,000 × *g* (27). The total enzyme activities at pH 5.5 and pH 8.0 were 3.13 and 2.89 for MFS, 1.56 and 1.42 for cytochrome *c* oxidase, 47.8 and 10.5 for alcohol oxidase, 15.2 and 14.8 for catalase, and 0.871 and 0.748 for DHAS, respectively.

inhibited by the metal salts $HgCl_2$, $CuCl_2$, AgCl, $NiCl_2$, and $FeCl_3$, all at 1 mM, and a sulfhydryl reagent, *p*-chloromercuribenzoate, also at 1 mM. This suggests that a sulfhydryl group of the enzyme is related to both activities. The enzymes were susceptible to some metal-chelating agents: 1 mM *o*-phenanthroline (100% inhibition), 1 mM potassium cyanide (61 to 48%), 1 mM α,α′-dipyridyl (51 to 39%), and 5 mM EDTA (85%). On analysis of the enzymes by atomic absorption spectroscopy, only zinc was detected in a significant amount. The amounts of zinc in the *Candida* and *Pichia* enzymes were 8.32 and 8.28 atoms of Zn/mol of enzyme protein, respectively, so that each enzyme subunit was calculated to contain 2 mol of zinc. When the *Candida* and *Pichia* enzymes were incubated with 1 mM *o*-phenanthroline as above, the Zn contents decreased to 3.21 and 3.10 mol/mol of enzyme, respectively, with the initial activity being completely lost. When each *o*-phenanthroline-treated preparation was incubated with 10 mM $ZnCl_2$, about 70% activity was restored, with the Zn content being about 67% of the initial value. Mn^{2+}, Mg^{2+}, Ca^{2+}, or Co^{2+} could not restore the activity which was lost on *o*-phenanthroline treatment. These observations suggest that zinc is essential for the enzyme activity. It is not clear whether both zinc atoms per subunit participate directly in the enzyme activity. Pyrasol, a competitive inhibitor of alcohol, did not inhibit the activity.

MFS activity comigrates with a mitochondrial marker enzyme, cytochrome *c* oxidase, on sucrose gradient ultracentrifugation. Characterization of the purified MFS provided evidence that MFS indeed has alcohol dehydrogenase activity. In *Saccharomyces cerevisiae*, alcohol dehydrogenases are found in both the cytosol and mitochondria. In a previous study involving *P. methanolica*, only one peak of alcohol dehydrogenase separated on a Blue-Sepharose CL6B column exhibited MFS activity, whereas the other two peaks did not (25). Since *C. boidinii* has been used as a model organism for peroxisome biogenesis in our and other laboratories (10, 11, 27, 35), the subcellular fractionation method was well established with this organism. To determine the localization of MFS in *C. boidinii*, methanol-grown cells were spheroplasted and cell debris and nuclei were removed by low-speed centrifugation (500 × *g*) twice. The resulting supernatant was centrifuged at 20,000 × *g* at pH 5.5 to obtain a pellet of an organelle fraction containing mainly peroxisomes and mitochondria. Enzyme assays (cytochrome *c* oxidase as a mitochondrial marker and AOD, catalase, and DHAS as peroxisomal markers) were performed on the pellet and the supernatant fraction. As shown in Table 4, more than 95% of the MFS activity was recovered in the pellet fraction, as was 99% of the cytochrome *c* oxidase, 91% of the AOD, 74% of the catalase, and 85% of the DHAS activities. Since peroxisomes are known to be extremely unstable at pH 8.0 and the mitochondrial integrity is not affected under the same conditions (11), the same experiment was performed after the 500 × *g* supernatant suspension had been exposed to 30 mM Tris-Cl (pH 8.0). Although the recoveries of the three peroxisomal enzymes were greatly reduced, the recoveries of both cytochrome *c* oxidase and MFS were not affected. To confirm that MFS is a mitochondrial enzyme, the 20,000 × *g* pellet obtained at pH 6.0 was further fractionated by discontinuous sucrose gradient ultracentrifugation (Fig. 2). The ac-

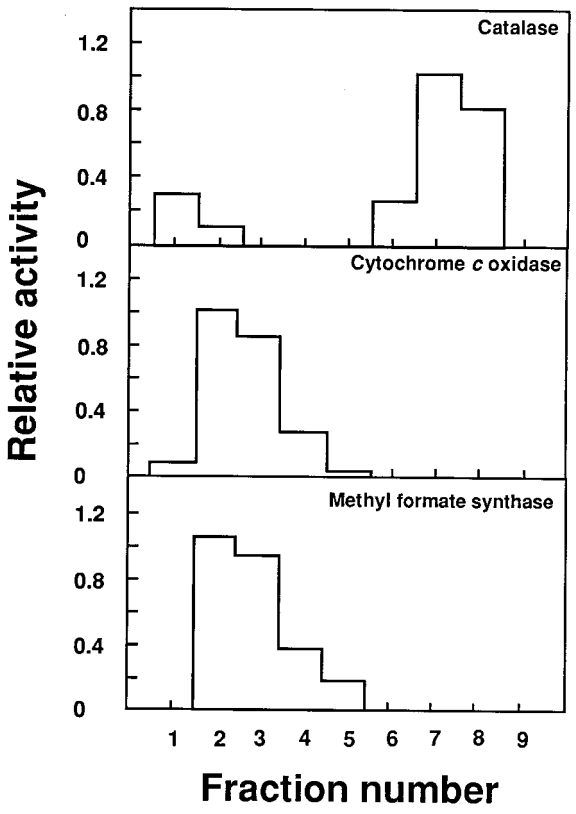

FIG. 2. Discontinuous sucrose gradient fractionation of organelle pellets derived from methanol-grown *C. boidinii* S2. The organelle pellets were prepared and fractionated on a sucrose gradient as described in Materials and Methods. A relative activity of 1.0 corresponds to 60.74 U/ml for catalase, 3.12 U/ml for cytochrome *c* oxidase, and 11.9 U/ml for MFS.

tivities of MFS and cytochrome *c* oxidase were detected primarily in fractions 1 to 3, and that of catalase was found in fractions 6 to 8. These results show that MFS is compartmentalized in mitochondria.

DISCUSSION

In this study, we purified and characterized MFS in an attempt to understand the GSH-independent oxidation of formaldehyde in methylotrophic yeasts. Analysis of the purified MFSs of *C. boidinii* and *P. methanolica* showed the close resemblance of the properties of the two enzymes. The enzymatic synthesis of methyl formate occurred in the reaction mixture containing methanol, formaldehyde, and NAD^+ as substrates. The amount of methyl formate formed was stoichiometrically equivalent to the amount of formaldehyde consumed and the amount of NAD^+ reduced. The enzyme was assumed to be a class III alcohol dehydrogenase (21) from the following results: (i) it catalyzed the dehydrogenation of a variety of primary aliphatic alcohols, having low affinity for ethanol and high affinity for long-chain alcohols such as octanol; (ii) it was composed of tetrameric subunits, which each had two atoms of zinc, at least one of which was related to the catalytic activity; (iii) it was insensitive to pyrasol; and (iv) the amino acid sequences obtained for the purified enzymes showed significant similarity to those of class III alcohol dehydrogenases from several other organisms. Methanol-grown *P. methanolica* cells contained at least three alcohol dehydrogenases, which were fractionated on Blue Sepharose CL-6B column chromatography, but only one fraction showed methyl formate-synthesizing activity (25). This means that MFS activity is not common to all NAD^+-dependent alcohol dehydrogenases but that it is a distinct catalytic feature of this enzyme. GSH-FaDH and class III alcohol dehydrogenase in rat liver were reported to be identical (21). In contrast MFS did not exhibit GSH-FaDH activity, and the two enzymes were completely separated from each other on column chromatography (25). MFS is also distinct from *Pseudomonas putida* formaldehyde dehydrogenase (EC 1.2.1.46), which does not require methanol or a cofactor such as GSH for formaldehyde oxidation (13).

Interestingly, MFS activity was found in an organelle pellet and comigrated with mitochondrial cytochrome *c* oxidase on sucrose gradient ultracentrifugation. The following observations are also consistent with the fact that MFS is a counterpart of *S. cerevisiae* Adh3p in methylotrophic yeasts. (i) The *ADH3* expression is repressed by growth on glucose (14). Similarly, MFS activity is not found in methylotrophic yeasts grown on glucose as the sole source of carbon but is induced in the cells by methanol or formaldehyde (25). (ii) Amino acid sequences obtained for proteolytic digests of MFS show significant similarity to those of yeast mitochondrial alcohol dehydrogenases (Fig. 1).

So far, the physiological function of mitochondrial alcohol dehydrogenase (*S. cerevisiae ADH3*) is unclear. However, our previous studies and the present one show that MFS (mitochondrial alcohol dehydrogenase) seems to be related to the methylotrophic metabolism in two respects: energy metabolism and formaldehyde resistance. Sibirny et al. (29) reported that a mutant of *Hansenula polymorpha* deficient in both GSH-FaDH and formate dehydrogenase activities still retained the ability to grow on methanol, and they proposed that the energy for methylotrophic growth of the mutant is supplied via the tricarboxylic cycle. From our results, MFS should be able to function as an energy producer through the oxidation of formaldehyde, providing NADH in mitochondria, where the reducing equivalence can be directly introduced to oxidative phosphorylation. GSH-FaDH in *Hansenula polymorpha* was reported to participate in the detoxification of formaldehyde under physiological conditions (29). By analogy, the resistance of *S. cerevisiae* (not a methylotroph) to formaldehyde was shown to be enhanced on the overproduction of an enzyme such as GSH-FaDH (9). However, judging from the kinetic characteristics of GSH-FaDH from *C. boidinii* (19), the enzyme does not seem to be suitable for instantaneous scavenging of a large amount of formaldehyde produced through unbalanced methanol oxidation, because the activity is strictly controlled by a feedback mechanism involving one of the products, NADH (19). From these kinetic data and the observation that the accumulation of methyl formate responded quickly to the addition of formaldehyde (25), MFS can be considered to contribute significantly to the detoxification of formaldehyde.

Factor-dependent formaldehyde dehydrogenase has been found in *Rhodococcus erythropolis* (6) and *Amycolatopsis methanolica* (34). The factor of the bacterial enzyme can be replaced by a high concentration of methanol, although no evidence of methyl formate formation under physiological conditions has been reported. By analogy with this, a cofactor which can be replaced with methanol was searched for in methylotrophic yeasts, but no positive results have been obtained so far. The heat-treated cell extract of methanol-grown *A. methanolica* was also inactive with our enzyme (27a). Although it cannot be excluded that our enzyme exhibits such factor-dependent activity, the genuine substrate of the enzyme is assumed to be the hemiacetal adduct of methanol and formaldehyde under physiological conditions. With respect to the carbon flow in methylotrophic yeasts, methyl formate is assumed to be hydrolyzed by an esterase and then the formate formed is subjected to oxidation to CO_2 by formate dehydrogenase or the ester may be oxidized by an ester dehydrogenase, as found in *A. methanolica* (33).

Based on the results of this work, an alternative GSH-independent pathway for formaldehyde oxidation involving MFS can be assumed to be of significant value in formaldehyde detoxification, with a large part of methyl formate leaking into the medium. It is a general metabolic event that carbon flow is switched over to an alternative pathway with a change in the physiological conditions. The relationship between the conventional pathway involving GSH-FaDH and an alternative pathway involving MFS is worth further metabolic studies involving cloning and disruption of the genes encoding the enzymes involved in formaldehyde metabolism.

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