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MITOCHONDRIAL ACETALDEHYDE DEHYDROGENASE FROM *SACCHAROMYCES CEREVISIAE*

MYRON K. JACOBSON* and CARL BERNOFSKY**

Laboratory of Molecular Biology, Mayo Foundation and Mayo Graduate School of Medicine, Rochester, Minn. 55901 (U.S.A.)

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SUMMARY

Aerobic yeast mitochondria contain a unique isozyme of acetaldehyde dehydrogenase that accounts for the ability of yeast mitochondria to respire with acetaldehyde as a substrate. Mitochondrial acetaldehyde dehydrogenase has a high affinity for acetaldehyde, is active with NAD^+ and $NADP^+$, is stimulated by K^+, and appears to be the same acetaldehyde dehydrogenase previously isolated from yeast autolysates by Black ((1951) Arch. Biochem. Biophys. 34, 86–97) and Jakoby and coworkers (Steinman, C. R. and Jakoby, W. B. (1967) J. Biol. Chem. 242, 5019–5023; Steinman, C. R. and Jakoby, W. B. (1968) J. Biol. Chem. 243, 730–734; Clark, J. F. and Jakoby, W. B. (1970) J. Biol. Chem. 245, 6065–6071; Clark, J. F. and Jakoby, W. B. (1970) J. Biol. Chem. 245, 6072–6077; Bradbury, S. L. and Jakoby, W. B. (1971) J. Biol. Chem. 246, 1834–1840; Bradbury, S. L. and Jakoby, W. B. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2373–2376). The enzyme is inhibited by Li^+, Mg^{2+}, and Disulfiram. Mitochondrial acetaldehyde dehydrogenase appears to play a role in the oxidative metabolism of ethanol, especially when yeast cells are utilizing ethanol as a source of carbon and energy for growth. The enzyme is more than 99% repressed during the growth of yeast on glucose.

Yeast cells also contain a cytosol isozyme of acetaldehyde dehydrogenase that is active with $NADP^+$, is stimulated by Mg^{2+}, and is only partly repressed during growth on glucose. The cytosol isozyme resembles the acetaldehyde dehydrogenase previously prepared from yeast autolysates by Seegmiller ((1953) J. Biol. Chem. 201, 629–637).

The following enzyme terminology is used: alcohol dehydrogenase, (alcohol: NAD oxidoreductase; EC 1.1.1.1); acetaldehyde dehydrogenase, "Seegmiller enzyme", (aldehyde:NADP oxidoreductase; EC 1.2.1.4); acetaldehyde dehydrogenase, "Black–Jakoby enzyme", (aldehyde: NAD(P) oxidoreductase; EC 1.2.1.5); acetyl-CoA synthetase, (acetate:CoA ligase (AMP); EC 6.2.1.1); and pyruvate decarboxylase, (2-oxoacid carboxy-lyase; EC 4.1.1.1).

* Present address: Department of Chemistry, North Texas State University, Denton, Texas 76203, U.S.A.

** To whom requests for reprints should be directed.

INTRODUCTION

Growth of yeast on glucose results in the accumulation of ethanol, which arises as a consequence of glycolysis and the action of pyruvate decarboxylase and alcohol dehydrogenase (see refs 1 and 2 for reviews). When glucose or other fermentable substrate is absent from the culture medium, yeast can utilize ethanol as a source of energy and carbon [3, 4]. Thus, when yeast cells are cultured with glucose under aerobic conditions, two distinct stages of growth may be observed [4, 5].

The initial steps in the utilization of ethanol for growth involve the sequential transformation of ethanol to acetaldehyde, acetate, and finally acetyl-CoA. Energy may be derived through the mitochondrial oxidation of acetyl-CoA and of reduced pyridine nucleotides generated in the first two steps of this sequence. Carbon compounds with chain lengths greater than two carbon atoms may be derived from the metabolism of acetyl-CoA via the glyoxylate cycle [6, 7].

The involvement of mitochondria in the oxidative metabolism of ethanol is indicated by the correspondence of the second stage of growth on glucose (the ethanol phase) with the presence of functional mitochondria (see ref. 8 for review), the existence of a mitochondrial isozyme of alcohol dehydrogenase [9], and the ability of yeast mitochondria to respire on ethanol with the concomitant production of ATP [10]. As shown in the present communication, yeast mitochondria also possess an acetaldehyde dehydrogenase that enables them to oxidize acetaldehyde to acetate. Because yeast mitochondria contain acetyl-CoA synthetase [11, 12], which converts acetate to acetyl-CoA, and an active Krebs cycle, which further oxidizes acetyl-CoA, the mitochondria appear to contain all of the enzymes necessary for the complete oxidation of ethanol to CO_2 and H_2O.

Two isozymes of acetaldehyde dehydrogenase have previously been prepared from yeast. However, their intracellular location and metabolic significance have not been determined. The first isozyme was isolated by Black [13] and extensively characterized by Jakoby and coworkers [14–19]. The second isozyme was described by Seegmiller [20], but was not studied in detail because of instability [21]. The Black–Jakoby enzyme catalyzes the oxidation of acetaldehyde in the presence of either NAD^+ or $NADP^+$ and is activated by K^+ [13–19]. The Seegmiller enzyme is active only with $NADP^+$ and is stimulated by divalent cations, but not by K^+ [20]. Both enzymes have a high affinity for acetaldehyde.

Two isozymes of acetaldehyde dehydrogenase were also found in the present study. One isozyme is localized in mitochondria, is responsible for mitochondrial respiration with acetaldehyde, and appears to be the same enzyme described by Black [13] and Jakoby et al. [14–19]. The other isozyme is localized in the cytosol and resembles the enzyme described by Seegmiller [20]. A somewhat analogous distribution of acetaldehyde dehydrogenase isozymes seems to exist in rat liver [22, 23]. A preliminary account of the present work has been given [24].

MATERIALS AND METHODS

Growth of yeast cells

A strain of *Saccharomyces cerevisiae* isolated from commercial yeast (Red Star Yeast and Products Co., Milwaukee, Wisc.)was grown aerobically at 30 °C in a

Microferm Fermenter (New Brunswick Scientific Co., New Brunswick, N.J.), using the glucose-containing medium described by Duell et al. [25], supplemented with 30 ppm of silicone antifoam agent SAG-471. Unless otherwise specified, cultures were initiated with 0.01 vol. of a 24-h subculture grown in the same medium.

Cell density was measured as the apparent absorbance of the culture medium at 600 nm in a 1.00-cm light path cuvette with a Beckman DB spectrophotometer. Except where noted, the cells were cultured for 1–3 h after reaching the stationary phase. Cells were harvested by centrifugation in the cold at 1000 × *g* for 10 min, washed three times with water, and stored in the cold for not longer than 24 h.

Isolation of mitochondria; glass bead method

Packed cells (12.5 g, wet wt) were suspended in 30 ml of 100 mM Tris–H_2SO_4 (pH 8.6), 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM $MgCl_2$, centrifuged, and resuspended with 12 ml of the same buffer. All operations were conducted at 0–4 °C. The suspension was transferred to a homogenization flask containing 25 cm^3 of 0.5-mm diameter glass beads, and shaken at 4000 rev./min for 30 s in a Braun MSK cell homogenizer, cooled with liquid CO_2. The pH of the mixture after shaking was 7.8–8.0. The suspension was centrifuged at 1000 × *g* for 15 min, and the supernatant fraction was recentrifuged to obtain a cell-free extract. Mitochondria in the cell-free extract were sedimented at 12 000 × *g* for 15 min, resuspended in 10 ml of 50 mM Tris–H_2SO_4 (pH 8.0), 0.25 M sucrose, 1 mM EDTA, and 1 mM dithiothreitol, recentrifuged, and finally suspended in 2 ml of the same buffer. A Potter–Elvehjem homogenizer with a Teflon pestle rotating at 400 rev./min was used to suspend the mitochondria. The yield of mitochondrial protein was about 2.5 mg/g wet wt of cells.

Isolation of mitochondria; spheroplast method

A modification of the two-step procedure of Duell et al. [25] was used. Packed cells (100 g, wet wt) were suspended in 150 ml of 50 mM Tris–H_2SO_4 (pH 8.0), 90 mM β-mercaptoethylamine–HCl, and 25 mM EDTA, incubated with stirring at 32 °C for 45 min, and centrifuged at 1000 × *g* for 10 min at room temperature. The cells were resuspended with 80 ml of 50 mM Tris–maleic acid (pH 5.8), 1.2 M sorbitol, and 1 mM EDTA, centrifuged as above, and resuspended with the same buffer to 90 ml. Glusulase was added (usually 10 ml, Endo Laboratories, Inc., Garden City, N.Y.), and the incubation was continued until 90% of the cells were converted to spheroplasts as judged by phase contrast microscopy (40–50 min). All subsequent steps were conducted at 0–4 °C.

The spheroplasts were sedimented at 3300 × *g* for 15 min, resuspended with 200 ml of 50 mM Tris–H_2SO_4 (pH 8.0), 1.2 M sorbitol, and 1 mM EDTA, and sedimented again. The spheroplasts were washed again, suspended in 480 ml of 50 mM Tris–H_2SO_4 (pH 8.0), 0.25 M sucrose, and 1 mM EDTA, and lysed in a Waring Blendor, operated for 30 s and at 50 V by means of a variable transformer. The mitochondria were sedimented and washed as described above for the glass bead method, but without the dithiothreitol, quickly frozen in a mixture of solid CO_2 and Cellosolve, and stored at −30 °C until used. The yield of mitochondrial protein was about 3.6 mg/g wet wt of cells. Unless otherwise indicated, mitochondrial acetaldehyde dehydrogenase was obtained from mitochondria prepared by the spheroplast proce-

dure. Mitochondria from the glass bead procedure yielded the identical enzyme preparation.

Preparation of mitochondrial extracts

Mitochondria were disrupted in the cold by sonication for 40 s with a Heat Systems, Model J17-A ultrasonic generator (Heat Systems-Ultrasonics Inc., Plainview, N.Y.), and the disrupted mitochondria were centrifuged for 90 min at 150 000 × *g*. The supernatant fraction was mixed with an equal volume of glycerol and dialyzed at 0–4 °C for two 12-h periods, each against 100 vol. of 50% (v/v) glycerol, 25 mM Tris–H_2SO_4 (pH 8.0), 0.125 M sucrose, and 1 mM dithiothreitol. The preparation was stable for several months at −30 °C.

Spectrophotometric assay

Acetaldehyde dehydrogenase was assayed at 30 °C by following the reduction of $NADP^+$ or NAD^+ at 340 nm in 1.00-cm light path cuvettes using a Beckman DK-2 or Gilford Model 2000 spectrophotometer. Unless otherwise specified, the reaction mixture consisted of 50 mM Tris–H_2SO_4 (pH 8.0), 0.25 M sucrose, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 1 mM acetaldehyde, and 0.6 mM $NADP^+$ in a volume of 1.0 ml. Other additions are noted in the legends to Figs 3–6.

Acetaldehyde dehydrogenase activities in undialyzed extracts were corrected for low rates of pyridine nucleotide reduction observed in the absence of acetaldehyde. Such corrections were unnecessary with dialyzed extracts. Rates with mitochondrial extracts were linear for at least 10 min. However, cytosol preparations typically gave rapid initial rates lasting 2 min or less, followed by slower rates that were linear for about 5 min. The slower rates were used to determine acetaldehyde dehydrogenase activities.

Mitochondrial respiration

Respiration was determined polarographically at 30 °C with a Clark oxygen electrode as previously described [26]. The respiration buffer consisted of 1.5 ml 10 mM Tris–maleic acid (pH 6.8), 20 mM KH_2PO_4–K_2HPO_4 (pH 6.8), 0.6 M mannitol, 10 mM KCl, 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin, and substrate as listed in Table I. Calculations of oxygen uptake are based on a dissolved oxygen concentration of 0.196 mM [27].

Gel electrophoresis

Polyacrylamide gels were prepared as described by Dietz and Lubrano [28], except that 23.2 g of acrylamide and 0.8 g of *N,N'*-methylenebisacrylamide were used in the preparation of their solution C. Fresh ammonium persulfate was used for each set of gels. The undialyzed supernatant fraction from disrupted mitochondria was supplemented with bromphenol blue (10 μg/ml), and 20-μl aliquots (48 μg of protein) were layered directly on top of the gels. Electrophoresis was performed at 4 °C using an apparatus from Shandon Scientific Co. (Sewickley, Penn.). The buffer reservoirs contained 0.19 M glycine and 0.025 M Tris (final pH 8.3). The current was maintained at 2.5 mA per tube for 15 min, then increased to 5 mA per tube for an additional 30 min.

Gels were stained for enzymatic activity by incubation at 37 °C for 30 min in

the dark with 5 ml of 100 mM *N,N*-bis(2-hydroxyethyl)glycine-NaOH (pH 7.8), 1 mM EDTA, 0.05 mM 5-ethylphenazinium ethyl sulfate, and 0.4 mM nitroblue tetrazolium. Other additions (see Fig. 2) were 1 mM acetaldehyde, 1 mM NAD^+ or $NADP^+$, and 100 mM KCl. The gels were placed in 7.5% (v/v) acetic acid overnight, the acetic acid was changed, and the gels were stored at 0–4 °C. Protein was stained with Coomassie blue according to the procedure of Chrambach et al. [29].

Chemicals

Nucleotides, Tris, glycine, nitroblue tetrazolium, thiazolyl blue, malic acid, maleic acid, EDTA, β-mercaptoethylamine–HCl, sodium α-ketoglutarate, sodium pyruvate, and crystalline yeast alcohol dehydrogenase, Type A-7011, were obtained from Sigma Chemical Co. (St. Louis, Mo.). EDTA was used as the disodium salt. Coomassie brilliant blue R-250 was from Mann Research Labs. (New York, N.Y.), 5-ethylphenazinium ethyl sulfate was from Nutritional Biochemicals Corp. (Cleveland, Ohio), dithiothreitol, mannitol, sorbitol, and *N,N*-bis(2-hydroxyethyl)glycine were from Calbiochem (Los Angeles, Calif.), and crystalline bovine serum albumin (Pentex) was from Miles Laboratories, Inc. (Kankakee, Ill). SAG-471 and Disulfiram were generous gifts from Union Carbide Corp. (Minneapolis, Minn.) and Ayerst Laboratories, Inc. (New York, N.Y.), respectively. All other chemicals were reagent grade. Solutions were prepared with distilled water that was previously deionized. Before use, acetaldehyde was redistilled, diluted to 0.1 M, and stored in the dark at 0–4 °C. Nucleotides were standardized spectrophotometrically by established procedures [30].

Protein, glucose, ethanol

Protein was measured by the procedure of Lowry et al. [31], using bovine serum albumin as a standard. Glucose was determined with Glucostat according to instructions furnished by the supplier (Worthington Biochemical Corp. Freehold, N.J.), and ethanol was assayed by the method of Bonnischen [32].

RESULTS

Growth of yeast

Fig. 1 shows the typical growth pattern. After an initial lag, the cells grow exponentially with a doubling time of 70–75 min. During the first exponential phase, glucose is removed from the medium and ethanol accumulates. When the glucose is depleted, growth ceases for approximately 1 h and then resumes with a doubling time of 160–170 min. During the second exponential phase, ethanol is consumed. When ethanol has been completely depleted, the cells enter the stationary phase.

Mitochondrial respiration with acetaldehyde

Table I shows respiratory rates of isolated, washed yeast mitochondria. The rates with ethanol and pyruvate plus malate are in the range reported by other workers [10, 25, 33]. However, the rate with α-ketoglutarate is lower than that reported by Ohnishi et al. [10]. The State 3 respiratory rate with 0.5 mM acetaldehyde is higher than that of the other substrates tested (Table I), indicating the presence of a very active acetaldehyde dehydrogenase. The dependence of oxidation on the respira-

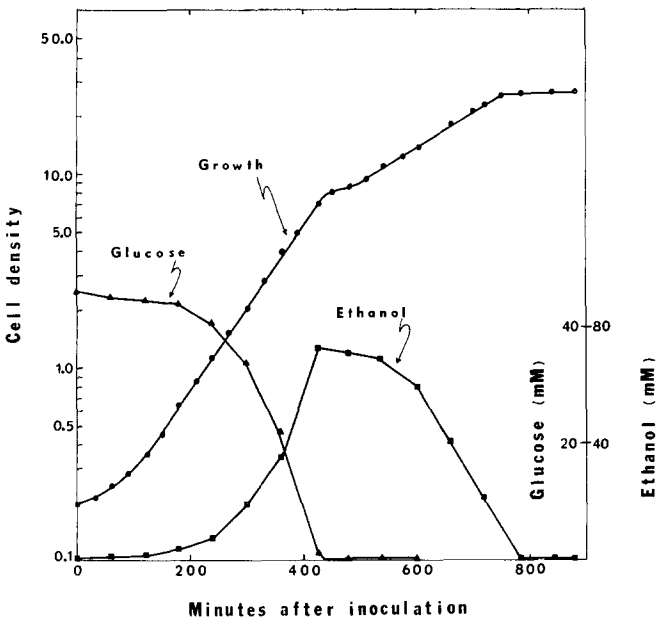

Fig. 1. Growth characteristics of yeast in a 12-l culture. Cell density, glucose, and ethanol were measured as described in Materials and Methods.

tory chain was demonstrated for each substrate by the complete inhibition of oxygen uptake by 2 mM NaCN (not shown).

Mitochondrial acetaldehyde dehydrogenase activity

Table II shows the acetaldehyde dehydrogenase activity of intact and disrupted yeast mitochondria. Cyanide was used to inhibit oxidation of reduced pyridine nucleotide via the respiratory chain. In all cases, activity is greatly stimulated by 100

TABLE I

OXIDATION OF SUBSTRATES BY ISOLATED YEAST MITOCHONDRIA

Mitochondria prepared from cells disrupted with glass beads were washed and assayed with an oxygen polarograph as described in Materials and Methods. Rates are corrected for an endogenous respiration of 15.6 natoms of oxygen per min per mg of mitochondrial protein. Assays were started by addition of 198 μg (10 μl) of mitochondrial protein, and State 3 respiration was initiated by addition of 10 μl of 30 mM ADP.

Substrate	Rate of oxygen consumption (natoms/min/mg protein)		Respiratory control ratio
	State 4 (−ADP)	State 3 (+0.2 mM ADP)	
Acetaldehyde (0.5 mM)	152	424	2.8
Pyruvate (10 mM) + malate (5 mM)	83	239	2.9
Ethanol (5 mM)	179	259	1.5
α-Ketoglutarate (10 mM)	9	106	12.0

TABLE II

ACETALDEHYDE DEHYDROGENASE ACTIVITY OF ISOLATED YEAST MITOCHONDRIA

Mitochondria prepared by the spheroplast procedure were disrupted and fractionated as described in Materials and Methods. The assay mixture contained, in a final volume of 3.0 ml, 100 mM Tris–H_2SO_4 (pH 8.0), 0.59 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 2 mM NaCN, 0.5 mM acetaldehyde, 0.5 mM $NADP^+$ or NAD^+, 100 mM KCl as indicated, and 23–94 μg of mitochondrial protein. Reactions were initiated with pyridine nucleotide and monitored at 340 nm with a DK-2 spectrophotometer. Rates shown are corrected for low rates of pyridine nucleotide reduction observed in the absence of acetaldehyde.

Preparation	Protein (mg/ml)	KCl (mM)	Acetaldehyde-dependent pyridine nucleotide reduction (nmoles/min/ml)	
			$NADP^+$	NAD^+
Intact	9.4	0	67	29
mitochondria		100	125	420
Sonically disrupted	9.4	0	53	96
mitochondria		100	1310	3620
Supernatant fraction from	2.3	0	116	236
disrupted mitochondria		100	1600	4640

mM KCl (Table II). Sonic disruption of mitochondria results in a 10-fold increase of K^+-stimulated activity, which is then recovered quantitatively in the supernatant fraction (Table II).

Gel electrophoresis

A single band of acetladehyde-dependent activity is observed which requires

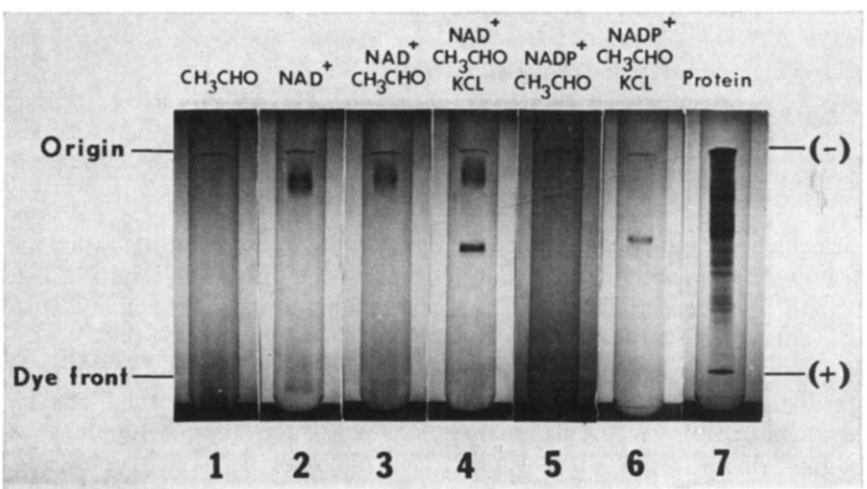

Fig. 2. Electrophoresis of mitochondrial acetaldehyde dehydrogenase on polyacrylamide gels. See Materials and Methods for explanation of conditions. Dye front indicates migration of bromphenol blue in sample.

the presence of K^+ and either NAD^+ or $NADP^+$ (Fig. 2, gels 4 and 6). A slower migrating band is also obtained when NAD^+ is present (gels 2, 3, and 4). This band is not observed with $NADP^+$ (gels 5 and 6), does not require acetaldehyde for staining (gel 2), and appears to represent an NAD-specific dehydrogenase capable of oxidizing some component of the buffer system [34]. Gel 7 demonstrates the proteins in the mitochondrial extract.

Dependence on acetaldehyde and K^+

The dialyzed supernatant fraction from disrupted mitochondria was used as the source of mitochondrial acetaldehyde dehydrogenase. Assays were conducted with $NADP^+$ to avoid interference by mitochondrial alcohol dehydrogenase.

Fig. 3 shows that mitochondrial acetaldehyde dehydrogenase has a high affinity for acetaldehyde, but is inhibited at relatively low levels of substrate. Maximal activity is observed at an acetaldehyde concentration of 0.35 mM. The results of Fig. 3 are similar to those reported by Black [13], who observed maximal activity at an acetaldehyde concentration of 0.17 mM.

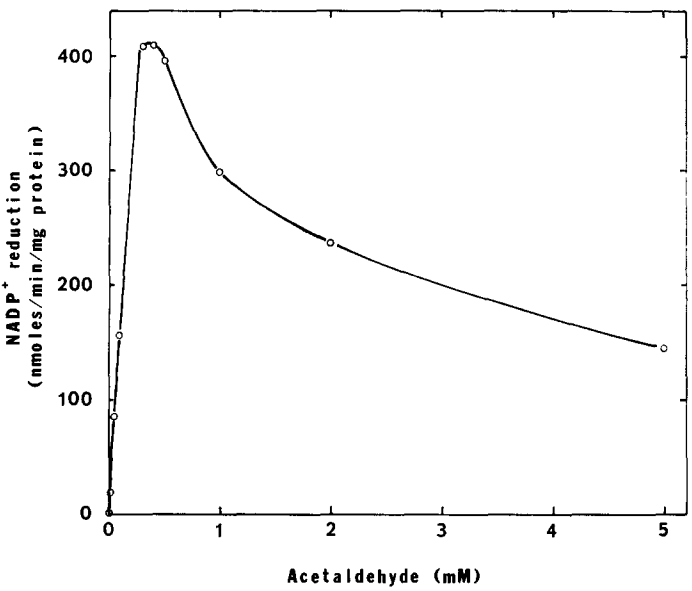

Fig. 3. Dependence of mitochondrial acetaldehyde dehydrogenase on acetaldehyde. Assays were conducted as described in Materials and Methods, except that they contained 100 mM KCl. The source of enzyme was the dialyzed supernatant fraction from disrupted mitochondria. Assays were initiated with 48 μg (50 μl) of protein.

Stimulation of mitochondrial acetaldehyde dehydrogenase by K^+ is shown in Fig. 4. Na^+ does not substitute for K^+, and Li^+ partially inhibits the small activity observed in the absence of K^+. The apparent K_m for K^+-activation is 1.18 mM, as determined from double-reciprocal plots (see Figs 5 and 6).

Inhibition by Li^+ and Mg^{2+}

As shown in Fig. 5, Li^+ is a competitive inhibitor of the K^+-stimulated

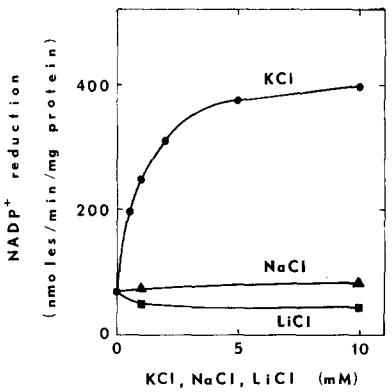

Fig. 4. Effect of monovalent cations on the activity of mitochondrial acetaldehyde dehydrogenase. Assays were conducted as described in Materials and Methods, except that they contained KCl, NaCl, or LiCl as noted in the figure. Rates (V) are given as $\Delta A_{340\ nm}$ per min. The source of enzyme was the dialyzed supernatant fraction from disrupted mitochondria. Assays were initiated with 65 μg (50 μl) of protein.

activity, with a K_i of 18.7 mM. Black [13] also reported an inhibition by Li^+ of K^+-stimulated acetaldehyde dehydrogenase.

Fig. 6 shows the inhibitory effect of Mg^{2+} on the K^+-stimulated activity of mitochondrial acetaldehyde dehydrogenase. The affinity of the enzyme for K^+ does not appear to be strongly dependent on the concentration of Mg^{2+}. Thus, the apparent K_m of K^+-stimulation in the absence of Mg^{2+} is 1.18 mM, while in the presence of 5–50 mM Mg^{2+}, the apparent K_m values of K^+-stimulation range from 1.28 to 1.57

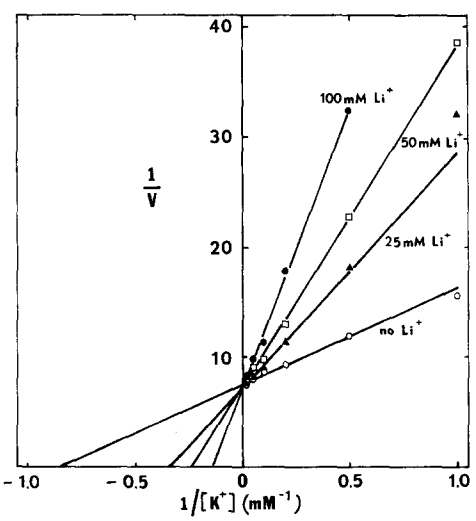

Fig. 5. Effect of Li^+ on the K^+-stimulation of mitochondrial acetaldehyde dehydrogenase. Assays were conducted as described in Materials and Methods, except that they contained KCl and LiCl as noted in the figure. Rates (V) are given as $\Delta A_{340\ nm}$ per min and are corrected for the rates observed in the absence of added K^+. The source of enzyme was the dialyzed supernatant fraction from disrupted mitochondria. Assays were initiated with 55 μg (50 μl) of protein.

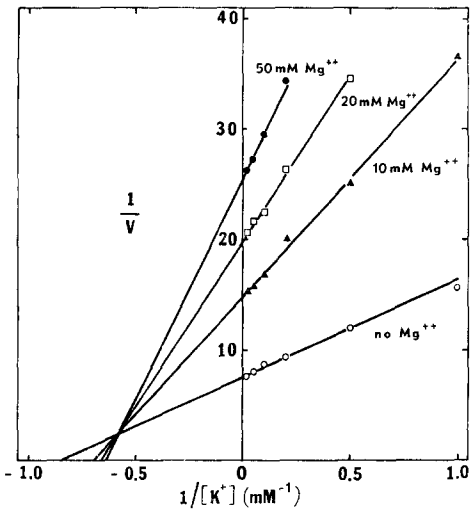

Fig. 6. Effect of Mg^{2+} on the K^+-stimulation of mitochondrial acetaldehyde dehydrogenase. Assays were conducted as described in Materials and Methods, except that they contained KCl and $MgCl_2$ as noted in the figure. Rates (V) are given as $\Delta A_{340\ nm}$ per min and are corrected for the rates observed in the absence of added K^+. The source of enzyme was the dialyzed supernatant fraction from disrupted mitochondria. Assays were initiated with 55 μg (50 μl) of protein.

mM. A plot (not shown) of the reciprocal acetaldehyde dehydrogenase activity versus the concentration of Mg^{2+}, according to the method of Dixon [35], indicates that Mg^{2+} inhibits the enzyme in a non-competitive manner with a K_i of 16 mM.

Inhibition by Disulfiram

The drug Disulfiram (bis(diethylthiocarbamoyl)disulfide) is a well known in-

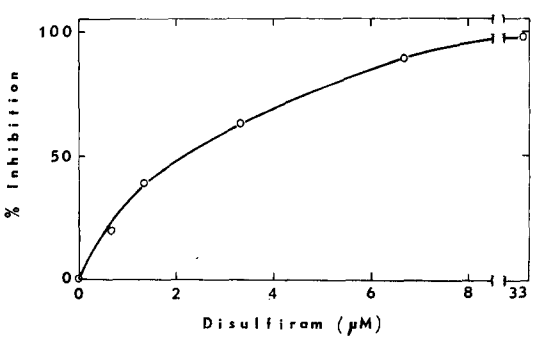

Fig. 7. Effect of Disulfiram on the activity of mitochondrial acetaldehyde dehydrogenase. The assay mixture (3.0 ml) contained 50 mM Tris–H_2SO_4 (pH 8.0), 0.25 M sucrose, 1 mM EDTA, 2 mM NaCN, 100 mM KCl, 50 μg/ml bovine serum albumin, 0.67 mM acetaldehyde, 0.5 mM $NADP^+$, and Disulfiram as indicated in the figure. Assays were initiated with 64 μg (50 μl) of protein. The source of enzyme was the dialyzed supernatant fraction from disrupted mitochondria as described in Materials and Methods, except that dithiothreitol was omitted from the dialysis buffer. Various concentrations of Disulfiram were prepared in dimethylsulfoxide, and 1-μl aliquots were added to the assay mixtures by means of a microliter syringe (Hamilton Co., Whittier, Calif.). Dimethylsulfoxide alone did not affect the rate. The uninhibited activity was 686 nmoles of $NADP^+$ reduced per min per mg of protein.

hibitor of mammalian acetaldehyde dehydrogenase [23, 36, 37]. As shown in Fig. 7, Disulfiram is also a potent inhibitor of yeast mitochondrial acetaldehyde dehydrogenase, 50% inhibition being observed at 2 μM. Dithiothreitol protects the enzyme from Disulfiram inhibition (Jacobson, M. K. and Bernofsky, C., unpublished).

Intracellular localization of acetaldehyde dehydrogenase activity

Packed spheroplasts (4 g wet wt) were lysed in the cold in a Potter–Elvehjem homogenizer with 50 ml of 50 mM Tris–H_2SO_4 (pH 8.0), 0.25 M sucrose, 1 mM EDTA, and 1 mM dithiothreitol. Five passes of the pestle were used. A cell free extract (140 mg of protein) was obtained by two successive centrifugations at 1000 $\times$ g for 15 min, and mitochondria were isolated by centrifuging the cell-free extract at 12 000 $\times$ g for 15 min. Before the mitochondria were assayed, they were washed twice with the above buffer and then disrupted. The supernatant fraction remaining after removal of the mitochondria is considered to be the cytosol fraction.

Table III shows that 92% of the K^+-stimulated acetaldehyde dehydrogenase

TABLE III

INTRACELLULAR DISTRIBUTION OF ACETALDEHYDE DEHYDROGENASE ACTIVITY

Mitochondrial and cytosol fractions were prepared from lysed spheroplasts as described in text. The assay mixture contained, in a final volume of 3.0 ml, 50 mM Tris–H_2SO_4 (pH 8.0), 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 0.67 mM acetaldehyde, 0.5 mM $NADP^+$, 100 mM KCl as indicated, and 15–384 μg of protein. Reactions were initiated with $NADP^+$ and monitored at 340 nm with a DK-2 spectrophotometer. Rates shown are corrected for low rates of $NADP^+$ reduction observed in the absence of acetaldehyde. Total activity in each fraction represents that activity derived from 100 mg of protein in the cell-free extract. K^+-stimulated activities are obtained by subtracting the rates in the absence of KCl from the rates in the presence of 100 mM KCl.

Fraction	Protein (mg/fraction)	KCl (mM)	Acetaldehyde-dependent $NADP^+$ reduction (nmoles/min/fraction)	
			Total	K^+-stimulated
Mitochondria	10	0	366	
	10	100	4540	4170
Cytosol	90	0	1080	
	90	100	1460	385
	Total K^+-stimulated activity: 4560			

activity is localized in the mitochondrial fraction, and that the specific activity of this activity is 100-fold greater in the mitochondrial fraction than in the cytosol fraction. These results indicate that the K^+-stimulated acetaldehyde dehydrogenase is essentially a mitochondrial enzyme.

Table III also shows that the cytosol fraction contains considerable acetaldehyde dehydrogenase activity that is not dependent upon K^+. In separate experiments (not shown), the K^+-independent activity was not sedimented at 150 000 $\times$ g for 90 min, nor was it affected by dialysis to remove endogenous K^+. However, the K^+-independent activity was found to be activated about 3-fold by 6 mM Mg^{2+}. The stim-

ulation by Mg^{2+} is a characteristic of the acetaldehyde dehydrogenase described by Seegmiller [20].

Glucose repression of mitochondrial acetaldehyde dehydrogenase

Glucose repressed cells were obtained by inoculating a 12-l culture with 8 ml of subculture and allowing growth to proceed for 8 h, at which time the cells were in the first exponential phase of growth ($A_{600\ nm} = 2.0$). The cells were harvested, washed three times with 0.8% (w/v) glucose, and once with 100 mM Tris–H_2SO_4 (pH 8.6), 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM $MgCl_2$. Stationary phase cells were prepared as described in Materials and Methods.

Both preparations of cells were homogenized with glass beads, and cell-free extracts were obtained by centrifuging the homogenates twice at $1000 \times g$ for 15 min. Mitochondrial and cytosol fractions were prepared as described under intracellular localization. The cell free extracts contained 87.8 and 842 mg of protein per l of culture medium from the glucose repressed and stationary phase cells, respectively.

Table IV shows that cells in the first exponential phase of growth contain

TABLE IV

ACETALDEHYDE DEHYDROGENASE ACTIVITY OF EXPONENTIAL AND STATIONARY PHASE CELLS

Mitochondrial and cytosol fractions were prepared from cells in the first exponential phase and stationary phase of growth as described in text. Acetaldehyde dehydrogenase was assayed as described in Table III, except that cytosol fractions were assayed in the presence of 6 mM $MgCl_2$. Activities are expressed as in Table III.

Growth stage	Fraction	Protein (mg/fraction)	KCl (mM)	Acetaldehyde-dependent NADP$^+$ reduction (nmoles/min/fraction)	
				Total	K^+-stimulated
First exponential phase	mitochondria	2	0	<0.01	
		2	100	20	20
	cytosol	98	0	4 900	
		98	100	4 900	<0.1
			Total K^+-stimulated activity:		20
Stationary phase	mitochondria	6	0	115	
		6	100	2 190	2080
	cytosol	94	0	9 600	
		94	100	10 700	1130
			Total K^+-stimulated activity:		3200

<1% of the K^+-stimulated acetaldehyde dehydrogenase activity present in stationary phase cells. However, cells in the first exponential phase contain nearly one-half of the K^+-independent, cytosol acetaldehyde dehydrogenase present in stationary phase cells (Table IV).

Cytosol acetaldehyde dehydrogenase activity in stationary cells appears to be substantially greater in Table IV than in Table III. The higher activity in Table IV

may be attributed to the presence of $MgCl_2$ in the assay mixture and homogenization medium. In addition to serving as an activator, $MgCl_2$ stabilizes the cytosol isozyme (Jacobson, M. K. and Bernofsky, C., unpublished).

The presence in the cytosol of considerable K^+-stimulated acetaldehyde dehydrogenase following disruption of stationary phase cells with glass beads (Table IV) is probably caused by damage of the mitochondria and a partial solubilization of the mitochondrial isozyme.

DISCUSSION

The results of the present study show that, during the ethanol phase of growth (see Fig. 1), yeast cells contain a mitochondrial isozyme of acetaldehyde dehydrogenase not present during the glucose phase of growth (Table IV). The presence of this enzyme would permit the mitochondria to utilize acetaldehyde for the production of energy (ATP) and the generation of reducing equivalents (NADPH) for intramitochondrial biosynthetic reactions. The following properties of the mitochondrial isozyme identify it with the acetaldehyde dehydrogenase described by Black [13] and Jakoby and coworkers [14–19]: (1) activation by K^+, (2) inhibition by Li^+, (3) ability to reduce either NAD^+ or $NADP^+$, the former at a more rapid rate, (4) high affinity for acetaldehyde, and (5) substrate inhibition by acetaldehyde.

The concentration of acetaldehyde in mitochondria is probably very low, even during the ethanol phase of growth, because the position of equilibrium in the alcohol dehydrogenase reaction greatly favors the formation of ethanol [38]. In order for ethanol to be an effective substrate for growth, acetaldehyde would have to be efficiently converted to a product with which it does not readily equilibrate. Two properties of acetaldehyde dehydrogenase may enable the enzyme to meet this condition. These are: the great affinity of the enzyme for acetaldehyde [13, 15], and the apparent inability of acetaldehyde dehydrogenase to catalyze the reduction of acetate [13, 18].

The exact physiological significance of the K^+ activation of mitochondiral acetaldehyde dehydrogenase is unclear. However, Maitra and Estabrook [39] have demonstrated that yeast cells specifically take up K^+ from the medium in exchange for H^+ during respiration on acetaldehyde. As shown by the equation: acetaldehyde + $NAD(P)^+ \rightarrow$ acetic acid + NAD(P)H + H^+, the oxidation of acetaldehyde results in the formation of acid. The requirement of mitochondrial acetaldehyde dehydrogenase for K^+ could represent, in part, a mechanism for ensuring the presence of a suitable counter ion to help balance excess acid production in mitochondria. It is known that acetic acid is produced when ethanol is oxidized by yeast cells [40, 41], and that during this process, K^+ is taken up by the cells in exchange for H^+ [42].

The presence of both alcohol dehydrogenase and acetaldehyde dehydrogenase in yeast mitochondria gives rise to an apparent NADP-specific alcohol dehydrogenase activity in crude mitochondrial preparations (Jacobson, M. K. and Bernofsky, C., unpublished). Thus, oxidation of ethanol by NAD^+ forms acetaldehyde and NADH, and subsequent oxidation of NADH [10] leads to the continued generation of acetaldehyde. When $NADP^+$ is present, NADPH is formed through the action of mitochondrial acetaldehyde dehydrogenase. NADPH would accumulate under these conditions because it is only poorly oxidized by mitochondrial preparations (Jacobson, M. K. and Bernofsky, C., unpublished). A similar artifactual NADP-specific alcohol

dehydrogenase activity in cell-free preparations of *Acetobacter peroxydans* has been described by Atkinson and Serat [43].

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