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Subunit Structure, Expression, and Function of NAD(H)-Specific Isocitrate Dehydrogenase in *Saccharomyces cerevisiae*

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Mitochondrial NAD(H)-specific isocitrate dehydrogenase was purified from *Saccharomyces cerevisiae* for analyses of subunit structure and expression. Two subunits of the enzyme with different molecular weights (39,000 and 40,000) and slightly different isoelectric points were resolved by denaturing electrophoretic techniques. Sequence analysis of the purified subunits showed that the polypeptides have different amino termini. By using an antiserum to the native enzyme prepared in rabbits, subunit-specific immunoglobulin G fractions were obtained by affinity purification, indicating that the subunits are also immunochemically distinct. The levels of NAD(H)-specific isocitrate dehydrogenase activity and immunoreactivity were found to correlate closely with those of a second tricarboxylic acid cycle enzyme, malate dehydrogenase, in yeast cells grown under a variety of conditions. *S. cerevisiae* mutants with defects in NAD(H)-specific isocitrate dehydrogenase were identified by screening a collection of yeast mutants with acetate-negative growth phenotypes. Immunochemical assays were used to demonstrate that one mutant strain lacks the 40,000-molecular-weight subunit (IDH1) and that a second strain lacks the 39,000-molecular-weight subunit (IDH2). Mitochondria isolated from the *IDH1* and *IDH2* mutants exhibited a markedly reduced capacity for utilization of either isocitrate or citrate for respiratory O_2 consumption. This confirms an essential role for NAD(H)-specific isocitrate dehydrogenase in oxidative functions in the tricarboxylic acid cycle.

The oxidative decarboxylation of isocitrate is considered a committed step in the tricarboxylic acid cycle because this reaction is essentially irreversible under physiological conditions (2). NAD(H)-specific isocitrate dehydrogenase is presumed to be the enzyme that catalyzes this reaction, since this complex allosteric mitochondrial enzyme is subject to multiple regulatory controls, the activity being particularly responsive to the positive effector AMP in *Saccharomyces cerevisiae* (14) and ADP in other eucaryotes (31). This observation is the basis for speculation that the cellular adenylate energy charge exerts inverse regulatory effects on the rates of glycolysis (at the level of the reaction catalyzed by phosphofructokinase) and of respiration [NAD(H)-specific isocitrate dehydrogenase]. Eucaryotic cells also contain NADP(H)-specific isozymes of isocitrate dehydrogenase that are structurally and catalytically more analogous to the tricarboxylic acid cycle enzyme in bacterial cells (32, 36). The metabolic functions of the NADP(H)-specific isozymes are unclear, although potential roles in respiration (32) and in production of α-ketoglutarate in yeast cells under anaerobic conditions (24) have been proposed.

The catalytically active form of NAD(H)-specific isocitrate dehydrogenase isolated from various organisms is reported to be an octomer (1, 15, 35), but there is some discrepancy regarding the subunit composition of these enzymes. Barnes et al. (1) reported that the enzyme from *S. cerevisiae* is composed of apparently identical subunits with molecular weights of 39,000, whereas Illingworth (15) described the resolution of two nonidentical subunits with similar molecular weights (41,000) by electrophoresis in 8 M urea. Results of kinetic and ligand-binding studies with the yeast enzyme (1, 21) indicating four binding sites for isocitrate and two each for NAD^+, AMP, and Mg^{2+} or Mn^{2+} are more consistent with functionally distinct subunits. As an initial step toward defining the functions of individual subunits, we have isolated the NAD(H)-specific isocitrate dehydrogenase from yeast cells and report here conclusive evidence for two independent subunits.

In addition to allosteric regulation, several previous reports suggest that isocitrate dehydrogenase activity in yeast cells varies with growth conditions (24, 33). For example, activity is reduced in cells grown with glucose as a carbon source, suggesting that levels of the enzyme may be subject to catabolite repression, as are many respiratory functions in *S. cerevisiae* (30, 33). In the current studies, an antiserum specific for yeast NAD(H)-specific isocitrate dehydrogenase was used to examine levels of the enzyme in extracts from cells grown under various conditions. Concomitant analyses of levels of mitochondrial malate dehydrogenase were conducted to determine whether various growth conditions result in coordinate or noncoordinate expression of these tricarboxylic acid cycle enzymes.

In recent years, examination of the structure and function of enzymes in the tricarboxylic acid cycle has been facilitated by isolation of genes from various organisms that encode these enzymes. The *S. cerevisiae* genes encoding mitochondrial isozymes of citrate synthase (*CIT1*), isolated following enrichment for mRNAs in polysomes bound to mitochondria (41), and of malate dehydrogenase (*MDH1*), isolated by using immunoscreens of a λgt11 expression library (26), were used to construct yeast strains containing chromosomal disruptions in these structural genes. The only dramatic growth phenotype attributable to strains lacking these tricarboxylic acid cycle functions is an inability to grow with acetate as a carbon source (16, 26). Based on this observation, we used immunoscreens to identify yeast mutants with defects in NAD(H)-specific isocitrate dehydrogenase in a collection of acetate-negative strains.

MATERIALS AND METHODS

Strains and growth conditions. The haploid yeast strain Sg7 (*MATα leu2 his3-Δ1 trp1 gcr1-1* [26]) was used for isolation

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of NAD(H)-specific isocitrate dehydrogenase. Cell fractionation and expression studies were conducted with *S. cerevisiae* S173-6B (*MAT***a** *leu2-3 leu2-112 his3-Δ1 ura3-57 trp1-289* [3]). The parental *S. cerevisiae* strain MMY011 (*MATα ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-52*) and various acetate-negative mutant strains derived by UV mutagenesis of this strain were provided by M. T. McCammon. Yeast strains were cultivated on YP medium (1% yeast extract, 2% Bacto-peptone [Difco Laboratories]) containing as a carbon source 2% glucose, 2% glycerol plus 2% lactate, or 2% acetate. Cell growth was monitored spectrophotometrically, and cultures were routinely harvested in the log phase of growth at an A_{600} of 1.0 to 1.5.

Purification of NAD(H)-specific isocitrate dehydrogenase. Yeast strain Sg7 was cultivated at 30°C in YP medium containing 2% glycerol and 2% lactate. Cells were harvested by centrifugation at 3,000 × *g* and washed once with distilled water. The cell pellets were suspended (1 g of cells per 5 ml) in 0.25 M Tris hydrochloride (Tris-HCl, pH 8.3)–5 mM EDTA–5 mM β-mercaptoethanol–50% glycerol and stored at −70°C. For enzyme isolation, 100 g of frozen cells was thawed in 100 ml of buffer A (5 mM potassium phosphate [pH 7.6], 10 mM β-mercaptoethanol) containing 0.5 mM sodium citrate plus 2 mM phenylmethylsulfonyl fluoride and broken with glass beads in a Bead Beater (Biospec Products, Bartlesville, Okla.). The lysate was cleared by centrifugation at 10,000 × *g* for 10 min. A 2% solution of protamine sulfate was added to the cleared lysate to a final concentration of 0.25%, and the precipitate was removed by centrifugation. The supernatant was loaded directly onto a phosphocellulose column (2.5 by 40 cm) equilibrated with buffer A containing 0.5 mM citrate. The column was washed with 8 volumes of buffer A containing 10 mM citrate, and proteins were eluted with buffer A containing 50 mM citrate. Fractions containing NAD(H)-specific isocitrate dehydrogenase activity were pooled and dialyzed against buffer A containing 0.5 mM citrate. The dialysate was loaded onto a DEAE-cellulose column (1.5 by 20 cm) equilibrated in the same buffer. The column was washed with 8 volumes of buffer A containing 5 mM citrate, and proteins were eluted by increasing the citrate concentration to 10 mM. Pooled enzyme fractions were dialyzed against a buffer containing 0.1 M $NaHCO_3$, 10 mM β-mercaptoethanol, and 0.5 mM citrate and concentrated approximately fivefold by Amicon YM10 ultrafiltration. The concentrate was loaded onto a Bio-Gel A-1.5m gel filtration column (1.5 by 90 cm) equilibrated with the same buffer. Fractions containing NAD(H)-specific isocitrate dehydrogenase activity were pooled, an equal volume of 100% glycerol was added, and the purified enzyme was stored at −20°C.

NAD(H)-specific isocitrate dehydrogenase activity was measured as NADH production at A_{340} in 1-ml assay mixes containing 40 mM Tris-HCl (pH 7.6), 4 mM $MgCl_2$, 2.5 mM trisodium isocitrate, and 0.25 mM NAD^+. Malate dehydrogenase activity was measured as described previously (26), and NADP(H)-specific isocitrate dehydrogenase activity was measured in assay mixes containing 50 mM KPO_4 (pH 7.4), 10 mM $MgCl_2$, 2.5 mM trisodium isocitrate, and 0.25 mM $NADP^+$. Units are expressed as micromoles of NADH or of NADPH produced per minute. Assays for glucose-6-phosphate dehydrogenase (7) and for all other enzyme activities (37) were conducted as described before. Protein concentrations were determined by the method of Lowry et al. (21).

Amino acid sequence determinations. Samples (50 μg) of purified yeast isocitrate dehydrogenase were electrophoresed in each of four lanes on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon) with a graphite semidry electroblotter. The filter was stained for 5 min with Coomassie blue (0.1% in 50% methanol plus 10% acetic acid), destained for 5 min (50% methanol plus 10% acetic acid), rinsed in distilled water, and air dried. Portions of the filter containing Coomassie-stained bands corresponding to either the 40,000- or 39,000-molecular-weight subunit were used directly for amino-terminal sequence analysis with an Applied Biosystems 470-A gas-phase microprotein sequencer (Biotechnology Instrumentation Facility, University of California at Riverside).

Gel electrophoresis. Protein samples were electrophoresed on 10% SDS–polyacrylamide gels as described by Douglas et al. (9). Nondenaturing gels were 6 to 8% polyacrylamide gels with the same buffer systems but lacking SDS. Nondenaturing gels were stained for NAD(H)-specific isocitrate dehydrogenase by being submerged in a solution containing 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM isocitrate, 0.5 mM NAD^+, 0.4 mg of Nitro Blue Tetrazolium per ml, and 40 μg of phenazine methosulfate per ml for 20 to 30 min at room temperature. The staining reaction was terminated by transferring the gel to a solution of 7% acetic acid. Two-dimensional gel electrophoresis was conducted by the procedure of O'Farrell (27). Protein samples used in two-dimensional analyses were diluted 1:2 in a buffer containing 9.5 M urea, 2% Nonidet P-40, 2% pH 3 to 10 ampholytes, and 5% β-mercaptoethanol before being loaded onto isoelectric-focusing tube gels.

Immunochemical methods. Antiserum against yeast NAD(H)-specific isocitrate dehydrogenase was obtained through a commercial source (Bethyl Laboratories, Montgomery, Tex.) by injecting rabbits with the purified native enzyme. An immunoglobulin G (IgG) fraction was prepared from the serum as described by Palacios et al. (29).

For immunoblot (Western) analyses, protein samples were electrophoresed on SDS-polyacrylamide gels and electrotransferred to nitrocellulose or PVDF filters. The filters were blocked and washed as described by Burnette (5) and incubated with anti-isocitrate dehydrogenase IgG diluted 1:100 in TBST (50 mM Tris-HCl [pH 8], 0.15 M NaCl, 0.1% Tween 20). Bound IgG was detected by autoradiography following a 1-h incubation of the washed filters with ^{125}I-labeled protein A.

To obtain subunit-specific IgG fractions, 80 μg of pure isocitrate dehydrogenase was electrophoresed (10 μg per lane) on a 10% SDS–polyacrylamide gel and electrotransferred to a nitrocellulose filter. The filter was stained with India ink (1 μl/ml [13]) in PBST (10 mM sodium phosphate [pH 7.0], 15 mM NaCl, 0.4% Tween-20) to visualize the resolved isocitrate dehydrogenase subunit bands. The filter was then blocked for 1 h with 5% bovine serum albumin in PBST and incubated overnight at 4°C with a 1:100 dilution of anti-isocitrate dehydrogenase IgG in PBST. After three 15-min washes in PBST, the individual subunit bands were excised and transferred to microfuge tubes. Subunit-specific IgG fractions were eluted with three 30-s washes in 500 μl of pH 2.3 buffer (42). Each 500 μl was immediately neutralized with 167 μl of 0.2 M dibasic sodium phosphate. The eluted IgG fractions were dialyzed against phosphate-buffered saline prior to use in immunoblot analyses.

Cell fractionation. Whole-cell protein extracts were prepared by glass bead lysis of cell pellets in a buffer containing 10 mM $NaPO_4$ (pH 7.4), 1 mM EDTA, 1 mM β-mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride. The

TABLE 1. Purification of yeast NAD(H)-specific isocitrate dehydrogenase

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	290	464	5,200	0.1	
Protamine sulfate	293	410	2,700	0.2	88
Phosphocellulose	45	232	82	2.8	50
DEAE-cellulose	1.3	104	8.5	12.0	22
Bio-Gel A-1.5m	11	98	4.9	20.0	21

lysates were cleared by microcentrifugation for 10 min, and the supernatants were used for enzyme and protein assays. Mitochondrial pellets and postmitochondrial supernatants were obtained by fractionation of harvested cells as described by Daum et al. (8), except that bovine serum albumin was eliminated in all buffers. Mitochondrial pellets were suspended either in a buffer containing 0.65 M mannitol, 10 mM KPO_4 (pH 6.5), 10 mM Tris-maleate (pH 6.5), 10 mM KCl, and 0.1 mM EDTA for O_2 consumption measurements or in the lysis buffer described above before glass bead lysis for enzyme and protein assays.

Rates of respiratory O_2 consumption for isolated mitochondria were measured with a Clarke-type polarographic O_2 electrode as described by Ohnishi et al. (28). All substrate and ADP solutions were adjusted to pH 6.5 prior to addition to mitochondrial suspensions. State III respiratory rates were measured in the presence of 167 μM ADP with 13.3 mM of one of the following substrates: α-ketoglutarate, isocitrate, citrate, or pyruvate (12.0 mM) plus malate (1.3 mM).

RESULTS

Isolation and characterization of *S. cerevisiae* NAD(H)-specific isocitrate dehydrogenase. The haploid yeast strain Sg7 was used for isolation of NAD(H)-specific isocitrate dehydrogenase. This strain contains a mutation (*gcr1*) that results in substantial reduction in the levels of otherwise very abundant glycolytic enzymes (7) that we have found to be major contaminants in isocitrate dehydrogenase preparations. Also, presumably due to a combination of low levels of glycolytic enzymes and to a dependence on oxidative metabolism, the specific activity of NAD(H)-specific isocitrate dehydrogenase is two- to threefold higher in extracts from Sg7 than in extracts from yeast strains lacking the *gcr1* mutation. A rapid and reproducible purification scheme was developed based on techniques described by Barnes et al. (1), including phosphocellulose and DEAE-cellulose chromatography followed by Bio-Gel A-1.5m gel filtration. These steps resulted in a 200-fold purification and yielded approximately 5 mg of the enzyme per 100 g of yeast cells (Table 1).

The profiles of proteins following each step in the purification procedure are shown in Fig. 1. The near homogeneity of the purified native enzyme was illustrated by the presence of a single major Coomassie blue-stained band following nondenaturing polyacrylamide gel electrophoresis (Fig. 1B, lane 2) that comigrated with a band detected by enzyme activity stain for NAD(H)-specific isocitrate dehydrogenase (Fig. 1B, lane 1). Following SDS-polyacrylamide gel electrophoresis, the purified enzyme was resolved into two Coomassie blue-stained bands representing apparently equivalent amounts of polypeptides with approximate molecular weights of 40,000 and 39,000 (Fig. 1A, lane 4).

The subunit composition of isolated NAD(H)-specific

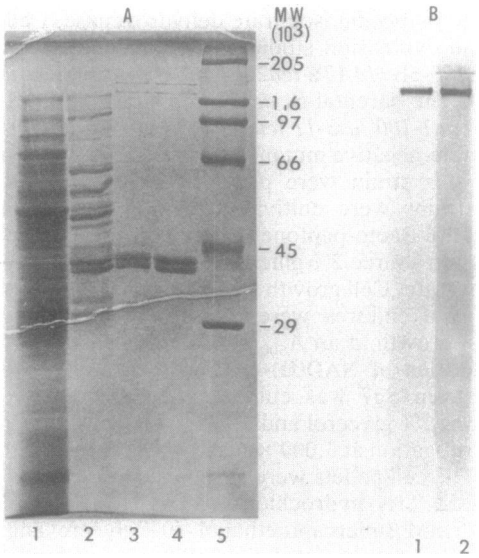

FIG. 1. Purification of yeast NAD(H)-specific isocitrate dehydrogenase. (A) Protein samples obtained during purification of NAD(H)-specific isocitrate dehydrogenase from yeast strain Sg7 were resolved on a 10% SDS–polyacrylamide gel and stained with Coomassie blue. Samples were taken following protamine sulfate precipitation (lane 1, 200 μg), phosphocellulose ion-exchange chromatography (lane 2, 50 μg), DEAE-cellulose chromatography (lane 3, 15 μg), and gel filtration (lane 4, 15 μg). In lane 5 are protein standards with molecular weights (MW) as shown. (B) Samples (10 μg) of the purified yeast isocitrate dehydrogenase were electrophoresed on a nondenaturing polyacrylamide gel and stained with an enzyme activity stain (lane 1) as described in Materials and Methods or with Coomassie blue (lane 2).

isocitrate dehydrogenase was also examined by the two-dimensional gel electrophoresis method of O'Farrell (27). Component polypeptides were resolved by electrophoresis in tube gels containing a pH 3 to 10 ampholyte gradient in 8 M urea followed by electrophoresis on a SDS-polyacrylamide slab gel. Two major equimolar polypeptide species were detected by Coomassie blue staining (Fig. 2). These had the predicted molecular weights of 39,000 and 40,000. The larger polypeptide had a slightly more basic isoelectric point. However, it was not possible from these data to assign

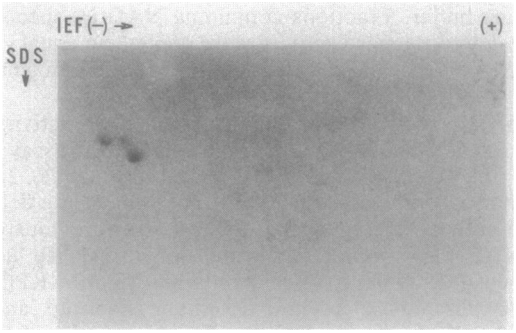

FIG. 2. Two-dimensional gel electrophoresis of purified yeast NAD(H)-specific isocitrate dehydrogenase. Purified yeast isocitrate dehydrogenase (15 μg) was subjected to isoelectric focusing (IEF) in a pH 3 to 10 ampholyte gradient in the first dimension and to electrophoresis on a 10% SDS–polyacrylamide slab gel in the second dimension. Only the portion of the gel (approximately one-fourth of the total area) containing the Coomassie-stained polypeptides is shown.

exact values, since the pH gradient near the basic end of the focusing gels was very shallow. The isoelectric points of both polypeptides fell within the range of pH 7 to 7.5 by this method.

The experimental data presented suggest that yeast NAD(H)-specific isocitrate dehydrogenase is composed of two distinct polypeptide subunits. In other experiments involving resolution on fast protein liquid chromatography columns and on other two-dimensional gel systems, we have observed multiple forms of both subunits, although never with different molecular weights (data not shown). These multiple forms were not obtained reproducibly and appeared to be more prevalent in aged enzyme preparations. To examine possible heterogeneity, the 39,000- and 40,000-molecular-weight subunits of the pure enzyme were resolved by SDS-polyacrylamide gel electrophoresis, transferred to PVDF filters, and independently subjected to amino-terminal amino acid sequence analysis as described in Materials and Methods. An 11-residue sequence of the 39,000-molecular-weight subunit and a 16-residue sequence of the 40,000-molecular-weight subunit were obtained: 39,000-molecular-weight subunit, Ala-Thr-Val-Lys-Gln-Pro-Ser-Ile-Gly-Gly-Tyr; 40,000-molecular-weight subunit, Ala-Thr-Ala-Ala-Gln-Ala-Glu-Gly-Thr-Leu-Pro-Lys-Lys-Tyr-Gly-Gly. These analyses provided no evidence for heterogeneity at the amino terminus of either subunit and clearly support the idea that the 39,000- and 40,000-molecular-weight subunits are distinct polypeptides.

Expression of NAD(H)-specific isocitrate dehydrogenase in *S. cerevisiae*. Antiserum against isocitrate dehydrogenase was prepared by injection of rabbits with the purified native yeast enzyme. The IgG fraction from the antiserum prepared as described in Materials and Methods quantitatively precipitated NAD(H)-specific isocitrate dehydrogenase activity from yeast cellular protein extracts and reacted well in immunoblot analyses of the purified enzyme resolved by SDS-polyacrylamide gel electrophoresis (Fig. 3A, lane 1). In immunoblot analyses of yeast cellular extracts (Fig. 3A, lane 2), the IgG reacted strongly with isocitrate dehydrogenase polypeptides of 39,000 and 40,000 molecular weight and also with two smaller polypeptides, one with an apparent molecular weight of 35,000. In two-dimensional gel analyses, the latter polypeptide comigrated with glyceraldehyde-3-phosphate dehydrogenase (25) (data not presented). Since the level of this enzyme was significantly reduced in the yeast strain used to purify isocitrate dehydrogenase and was negligible in the pure enzyme preparation (Fig. 1A, lane 4), this immunoreactivity may reflect structural similarities between the enzymes. It should be stated, however, that this cross-reactivity was limited, since the levels of glyceraldehyde-3-phosphate dehydrogenase polypeptides in wild-type cell extracts, as estimated by Coomassie blue staining of two-dimensional gels, exceeded those of isocitrate dehydrogenase polypeptides by more than 10-fold (data not shown).

The anti-isocitrate dehydrogenase IgG prepared against the native enzyme appeared to be equally immunoreactive with both subunits of the enzyme in purified or whole-cell protein samples (Fig. 3A, lanes 1 and 2, respectively). To determine whether this reflected cross-reactivity, subunit-specific IgG fractions were isolated as detailed in Materials and Methods by elution of IgG adsorbed to each polypeptide. The eluted IgGs were then used for immunoblot analyses of yeast cellular protein extracts. Each subunit-selected IgG was found to interact specifically with only the cognate 40,000- or 39,000-molecular-weight subunit (Fig. 3B, lanes 1 and 2, respectively). Thus, the isocitrate dehydrogenase subunits appear to be immunochemically distinct.

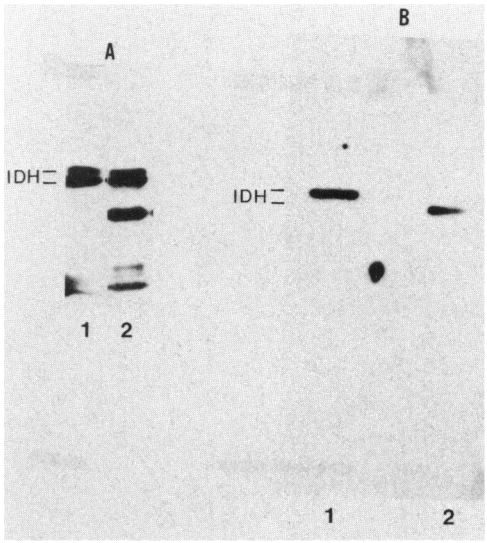

FIG. 3. Immunoreactivity of anti-isocitrate dehydrogenase IgG. A 1:100 dilution of anti-isocitrate dehydrogenase (IDH) IgG followed by ^{125}I-labeled protein A was used for immunoblot analysis of 500 ng of the purified enzyme (panel A, lane 1) and for 50 μg of an extract from yeast cells grown on 2% glycerol plus 2% lactate (panel A, lane 2). Subunit-specific fractions of the IgG obtained by preadsorption to the 40,000-molecular-weight subunit (panel B, lane 1) or to the 39,000-molecular-weight subunit (panel B, lane 2) as described in Materials and Methods were also used in immunoblot analyses of glycerol-lactate-grown whole-cell extracts.

To examine expression of NAD(H)-specific isocitrate dehydrogenase in diverse metabolic states, yeast strain S173-6B was grown in rich YP medium with one of the following carbon sources: 2% glucose, 2% glycerol plus 2% lactate, or 2% acetate. The cells were harvested, and mitochondrial fractions were isolated as described in Materials and Methods. The specific activities of isocitrate dehydrogenase and of malate dehydrogenase were determined for each cellular fraction and are compared in Table 2. Under these growth

TABLE 2. Activities of isocitrate and malate dehydrogenases in yeast cells grown on various carbon sources[a]

Cell fraction	Carbon source	Sp act (U/mg)	
		NAD(H)-specific isocitrate dehydrogenase	Malate dehydrogenase
Total cell extract	Glucose	0.03	0.08
	Glycerol-lactate	0.06	0.58
	Acetate	0.08	1.34
Mitochondria	Glucose	0.19	0.53
	Glycerol-lactate	0.29	1.28
	Acetate	0.38	1.43
Postmitochondrial supernatant	Glucose	0	0.02
	Glycerol-lactate	0	0.18
	Acetate	0	1.02

[a] Isocitrate and malate dehydrogenase activities were determined as described in Materials and Methods. The values shown represent an average of three independent determinations. Cell fractionations were conducted as described in Materials and Methods with freshly harvested cells from logarithmically growing cultures. Yeast cells were cultivated on YP medium containing the indicated carbon source(s) added to 2%.

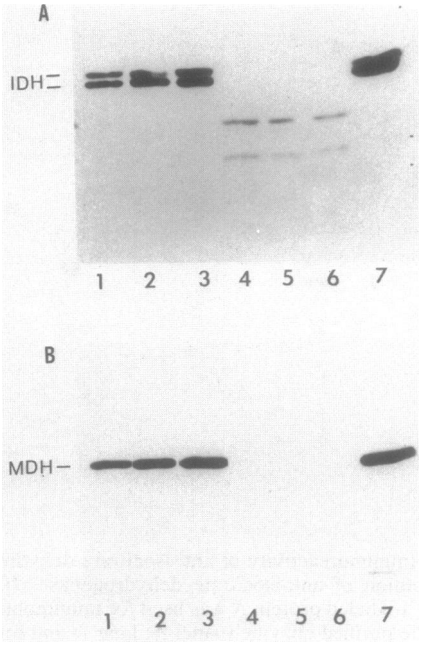

FIG. 4. Mitochondrial levels of isocitrate and malate dehydrogenases in yeast cells grown on different carbon sources. Mitochondrial (lanes 1 to 3) and postmitochondrial supernatant fractions (lanes 4 to 6) from yeast cells grown on glucose (lanes 1 and 4), on glycerol plus lactate (lanes 2 and 5), or on acetate (lanes 3 and 6) were electrophoresed on two 10% SDS–polyacrylamide gels and electrotransferred to PVDF filters. The filters were incubated with anti-isocitrate dehydrogenase (IDH) IgG (A) or anti-malate dehydrogenase (MDH) IgG (B). Bound IgG was detected by autoradiography following incubation with ^{125}I-labeled protein A. Samples (50 μg) of each cellular fraction and 1-μg samples of the purified enzymes (lanes 7) were loaded on the gels.

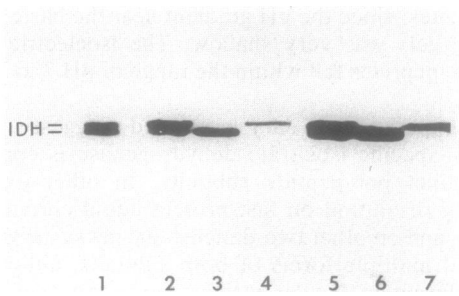

FIG. 5. Immunoblot analysis of protein extracts from yeast isocitrate dehydrogenase (IDH) mutants. Yeast strains MMY011 (lanes 2 and 5) and acetate-negative derivative strains designated A10-4, from complementation group A (lanes 3 and 6), and A14-3, from complementation group L (lanes 4 and 7), were grown on YP medium containing 2% glycerol, and cells were harvested during logarithmic growth (A_{600} of 1.0). Samples (100 μg) of total cellular protein extracts (lanes 2 to 4) and 50-μg samples of mitochondrial protein extracts (lanes 5 to 7) were prepared as described in Materials and Methods. The samples were electrophoresed on 10% polyacrylamide–SDS gels and transferred to PVDF membranes for Western blot analysis with anti-isocitrate dehydrogenase IgG and ^{125}I-labeled protein A. Lane 1 contained 1 μg of purified yeast NAD(H)-specific isocitrate dehydrogenase.

conditions, levels of both mitochondrial enzyme activities were repressed two- to threefold by growth on 2% glucose relative to levels with growth on nonfermentable carbon sources. In addition, both were repressed an additional two- to threefold by growth on 5% glucose compared with 2% glucose as the carbon source (data not shown). The 7- and 17-fold higher levels in total cellular malate dehydrogenase activity in cells grown on glycerol-lactate or acetate, respectively, relative to glucose levels are largely due to increased specific activity of the cytoplasmic malate dehydrogenase enzyme (postmitochondrial supernatant values [26]). The relative specific activities of the two mitochondrial tricarboxylic acid cycle enzymes correlated closely with levels of the proteins detected by immunoblot analyses (Fig. 4). Both enzymes were localized exclusively in mitochondria, as shown by the comparison of immunochemical levels in mitochondrial (lanes 1 to 3) versus postmitochondrial supernatant fractions (lanes 4 to 6). Thus, under these conditions, the tricarboxylic acid cycle enzymes exhibit closely coordinate expression.

Identification of NAD(H)-specific isocitrate dehydrogenase mutants. An inability to grow in medium with acetate as a carbon source is a phenotype observed for yeast mutants with defects in some tricarboxylic acid cycle enzymes, including citrate synthase (16) and malate dehydrogenase (26), and for some mutants with defects in glyoxylate or peroxisomal pathways (M. T. McCammon, personal communication). Representative strains from a collection of acetate-negative yeast mutants in 13 complementation groups isolated by M. T. McCammon and J. M. Goodman (University of Texas Southwestern Medical Center, Dallas, Tex.) were examined for mutants with potential defects in NAD(H)-specific isocitrate dehydrogenase. Enzyme assays with whole-cell lysates revealed two mutants from independent complementation groups (designated A and L) with no detectable NAD(H)-specific isocitrate dehydrogenase compared with levels ranging from 0.02 to 0.03 U/mg in extracts from the parental wild-type strain or from mutants representing other complementation groups in the collection.

Protein extracts from whole-cell samples and from mitochondria isolated following cellular fractionation (8) of the two apparent isocitrate dehydrogenase mutants and of the parental strain were examined by Western immunoblot analysis with the IgG specific for yeast NAD(H)-specific isocitrate dehydrogenase. As shown in Fig. 5, cellular and mitochondrial extracts from the parental strain (lanes 2 and 5, respectively) contained both the 40,000- and 39,000-molecular-weight subunits found in the purified yeast enzyme (lane 1). Cellular and mitochondrial extracts from the mutant strain representing complementation group A (lanes 3 and 6) lacked the 40,000-molecular-weight subunit, and similar extracts from the mutant strain representing complementation group L (lanes 4 and 7) lacked the 39,000-molecular-weight subunit of NAD(H)-specific isocitrate dehydrogenase. The mutant alleles were thus respectively designated *idh1-1* and *idh2-1*. The fact that the only immunoreactive polypeptide retained in the *IDH1* and *IDH2* mutants was the other NAD(H)-specific isocitrate dehydrogenase subunit substantiates the biochemical analyses of subunit structure given above.

Metabolic effects in isocitrate dehydrogenase mutants. Since the levels of other mitochondrial enzymes have been reported to be dramatically altered by the absence of a single tricarboxylic acid cycle enzyme in *S. cerevisiae* (18), we assayed a number of enzymes in mitochondrial extracts and in postmitochondrial supernatants obtained from the *IDH1* and *IDH2* mutants. As shown in Table 3, no NAD(H)-specific isocitrate dehydrogenase activity was measurable in

TABLE 3. Activities of enzymes in yeast isocitrate dehydrogenase mutants[a]

Preparation	Strain	Sp act[b] (mU/mg)							
		NAD-IDH	G6PDH	NADP-IDH	MDH	CS	ACON	FUM	αKGDC
Mitochondrial extracts	Wild type	27		40	1,129	169	132	121	16
	IDH1 mutant	0		24	1,212	391	140	106	16
	IDH2 mutant	0		36	1,138	407	164	123	14
Postmitochondrial supernatants	Wild type		54	2	24	1			
	IDH1 mutant		63	4	26	1			
	IDH2 mutant		58	4	32	1			

[a] Yeast strains were grown on rich YP medium with 2% glycerol, 2% lactate, and 0.5% glucose as carbon sources. Cell fractionation was performed essentially as described by Daum et al. (8).

[b] The enzymes, assayed as described in Materials and Methods, were NAD(H)-specific isocitrate dehydrogenase (NAD-IDH), NADP(H)-specific isocitrate dehydrogenase (NADP-IDH), malate dehydrogenase (MDH), citrate synthase (CS), aconitase (ACON), fumarase (FUM), α-ketoglutarate dehydrogenase (αKGDC), and glucose-6-phosphate dehydrogenase (G6PDH). Values are averages of results for assays performed on two independently isolated cell fractions.

mitochondrial extracts from either mutant strain. These data, along with similar results from assays of whole-cell extracts, suggest that both subunits are required for activity to be measurable in soluble extracts. Levels of other enzymes measured in these assays, including most of the tricarboxylic acid cycle enzymes, appeared to be largely unaffected in extracts from the mutants compared with levels in extracts from the parental strain. The only reproducible differences obtained in both *IDH1* and *IDH2* mutants were twofold increases in the specific activities obtained for citrate synthase in mitochondrial extracts and for NADP(H)-specific isocitrate dehydrogenase in postmitochondrial supernatants.

To determine the contribution NAD(H)-specific isocitrate dehydrogenase function to mitochondrial respiratory capacity, O_2 consumption measurements were conducted with mitochondria isolated from the *IDH1* and *IDH2* mutants and from the parental strain. Pyruvate plus catalytic amounts of malate, an oxidative substrate indicative of malate dehydrogenase function (40), was chosen as the reference substrate because mitochondrial levels of malate dehydrogenase are approximately equivalent in these strains (Table 3). The rates of utilization of this substrate and the relative rates of utilization of α-ketoglutarate as an oxidative substrate were essentially equivalent for mitochondria from the mutant and wild-type strains (Table 4). In contrast, relative utilization of isocitrate was dramatically impaired (a 16-fold decrease) in mitochondria from the *IDH1* strain and reduced by approximately 3-fold in mitochondria from the *IDH2* strain compared with rates measured for mitochondria from the wild-type strain. The difference between rates measured for the mutant strains suggests that the 40,000-molecular-weight subunit of NAD(H)-specific isocitrate dehydrogenase may have some residual function in the *IDH2* strain. There are precedents for this result in that certain mutant forms of yeast mitochondrial malate dehydrogenase and citrate synthase that have no measurable activities in soluble assays have been found to retain significant function in respiration in intact mitochondria (17, 41). Importantly, mitochondria from both the *IDH1* and *IDH2* mutants had no capacity for utilization of citrate as an oxidative substrate (Table 4). This result implies that the normal route for utilization of citrate for respiration requires delivery of reducing equivalents by NAD(H)-specific isocitrate dehydrogenase to the electron transport chain. NAD(H)-specific isocitrate dehydrogenase is therefore critical for this tricarboxylic acid cycle function, and its role is not compensated for by the activity of mitochondrial NADP(H)-specific isocitrate dehydrogenase. The activities measured for the latter enzyme in mitochondrial extracts from the wild-type, *IDH1*, and *IDH2* strains were comparable (Table 3).

TABLE 4. O_2 consumption of mitochondria isolated from yeast isocitrate dehydrogenase mutants

Strain	Relative rate of mitochondrial O_2 consumption[a]		
	α-Ketoglutarate	Isocitrate	Citrate
Wild type	0.91	0.49	1.53
IDH1 mutant	1.03	0.03	0.01
IDH2 mutant	0.79	0.17	0.02

[a] Mitochondria were isolated from yeast strains grown on YP medium with 2% glycerol, 2% lactate, and 0.5% glucose as carbon sources. Measurements of O_2 consumption in the presence of various oxidative substrates were conducted as described in Materials and Methods with a Clarke-type polarographic O_2 electrode. Values represent the rate of utilization of the indicated substrate divided by the rate for the control substrate (pyruvate plus malate).

DISCUSSION

The presumed role of NAD(H)-specific isocitrate dehydrogenase in controlling cellular respiratory rates is the basis for interest in the structure and regulation of this enzyme. A particular focus is determination of the structure and function of individual subunits of this complex allosteric enzyme. In addition to functions in catalysis and allosteric regulation by adenylate nucleotides, specific interactions with other mitochondrial proteins, including α-ketoglutarate dehydrogenase, have been described (34) and implicated in the control of tricarboxylic acid and isocitrate–α-ketoglutarate shuttle cycle function. Such analyses would be particularly instructive in *S. cerevisiae* not only because many of the definitive kinetic analyses by Atkinson and his colleagues were conducted with the yeast enzyme (1, 2), but also because this eucaryote is metabolically flexible and amenable to extensive molecular genetic analyses.

Our initial biochemical characterization of the purified yeast NAD(H)-specific isocitrate dehydrogenase suggests that the native enzyme has a quaternary structure of $\alpha_4\beta_4$ (where α is the 39,000-molecular-weight subunit and β is the 40,000-molecular-weight subunit). The purified yeast enzyme retains the property of positive allosteric regulation of AMP in the presence of subsaturating concentrations of isocitrate as originally reported (1) (data not shown). The molecular weight differences for the subunits were not observed in previous studies of the yeast enzyme (1, 15), although similar differences in isoelectric points for the subunits were reported by Illingworth (15). We observed the same molecular weights and isoelectric characteristics for the subunits on immunoblots of either total cellular or

mitochondrial extracts; therefore, these physical differences are not artifacts of the purification protocol. Our data further suggest that the two subunits have unique primary sequences and immunochemical properties. The identification of *IDH1* and *IDH2* mutants also supports the conclusion that yeast NAD(H)-specific isocitrate dehydrogenase is composed of two subunits. This is an important point, since Ramachandran and Colman (35) have reported a quaternary structure for the enzyme from pig heart of $\alpha_4\beta_2\gamma_2$ (with α as a 39,000- and β and γ as 41,000-molecular-weight subunits).

Under all metabolic conditions which require cyclic function of the tricarboxylic acid cycle in *S. cerevisiae* the mitochondrial tricarboxylic acid cycle enzymes isocitrate dehydrogenase and malate dehydrogenase are maintained at constant relative levels. Both enzymes are subject to catabolite repression to a similar extent as yeast mitochondrial citrate synthase (16) and numerous other mitochondrial functions in yeast cells cultivated on glucose as a carbon source. Under all conditions examined, levels of the 40,000- and 39,000-molecular-weight subunits are also coordinately maintained, although repeated use of portions of the anti-isocitrate dehydrogenase IgG can differentially deplete the IgG of immunoreactive components for one or the other subunit band. One question of interest for future studies is the basis for coordinate expression and localization of the isocitrate dehydrogenase subunits in mitochondria.

Control of tricarboxylic acid cycle function during growth of yeast cells on acetate as a carbon source is clearly, and not unexpectedly, different from control at the level of isocitrate dehydrogenase as described for *Escherichia coli*. Activity of the bacterial enzyme, an NADP(H)-specific dimeric enzyme (4, 36), is decreased 80% by phosphorylation during acetate growth, allowing effective competition for isocitrate by isocitrate lyase, an enzyme with a substantially higher K_m for that substrate (20). Not only is the yeast tricarboxylic acid cycle enzyme apparently not subject to any significant modification by phosphorylation (unpublished observation), the specific activity of NAD(H)-specific isocitrate dehydrogenase increases substantially in acetate-grown cells. It seems more likely that any competition to direct isocitrate to the cytosolic glyoxylate pathway or to the tricarboxylic acid cycle would be controlled in *S. cerevisiae* by allosteric mechanisms and/or by compartmentation of the substrate and pathways.

Two lines of evidence presented in this study support the conclusion that the mitochondrial NAD(H)-specific isozyme of isocitrate dehydrogenase is essential for tricarboxylic acid cycle function in eucaryotic cells. First, yeast mutants lacking either the 40,000- or 39,000-molecular-weight subunit of that enzyme have the same acetate-negative growth phenotype observed for mutants lacking mitochondrial malate dehydrogenase (26) or mitochondrial citrate synthase (16). Second, mitochondria isolated from *IDH1* or *IDH2* mutant strains exhibit severe impairment in the capacity for utilization of either isocitrate or citrate to support respiratory functions. The inability of mitochondrial NADP(H)-specific isocitrate dehydrogenase to compensate for these functions may be due to the absence of transhydrogenase activity in yeast mitochondria (39).

Both subunits of yeast NAD(H)-specific isocitrate dehydrogenase appear to be critical for catalytic activity in soluble assays. The *IDH2* mutant may retain some marginal enzymatic function in vivo, however, since mitochondrial respiration with isocitrate as an oxidative substrate is apparently reduced to a lesser extent than in the *IDH1* mutant. These mutants provide an opportunity for isolation of the corresponding genes through complementation and also for the independent purification of each subunit to examine the functional and regulatory properties contributed by each subunit to the holoenzyme.

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