Isolation, Characterization, and Disruption of the Yeast Gene Encoding Cytosolic NADP-Specific Isocitrate Dehydrogenase[†,‡]

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ABSTRACT: The cytosolic isozyme of NADP-specific isocitrate dehydrogenase (IDP2) was purified from a *Saccharomyces cerevisiae* mutant containing a chromosomal disruption in the gene encoding the mitochondrial isozyme (IDP1). IDP2 was shown to be a homodimer with a subunit molecular weight of approximately 45 000 and an isoelectric point of 5.5. Amino acid sequences were obtained for tryptic peptides of IDP2 and used to plan polymerase chain reactions. A resulting 400 bp DNA fragment was used as a hybridization probe to isolate the *IDP2* gene from a yeast genomic DNA library. The complete nucleotide sequence of the *IDP2* coding region was determined and translated into a 412-residue amino acid sequence. IDP2 and IDP1 were found to be identical in 71% of the aligned residue positions. The identity of the *IDP2* gene was confirmed by genomic replacement with a disrupted *IDP2* coding region. Haploid yeast strains lacking either or both IDP2 and IDP1 were constructed by genetic crosses of mutant strains containing disruptions in chromosomal *IDP2* and *IDP1* loci. No dramatic differences in growth rates with common carbon sources could be attributed to these disruptions.

Three distinct isozymes of isocitrate dehydrogenase have been described for eucaryotic cells. All three catalyze the oxidative decarboxylation of isocitrate to form α-ketoglutarate. However, based on cofactor specificity and cellular localization, the three isozymes may have very different metabolic roles. The mitochondrial NAD-specific isozyme is a complex allosteric enzyme believed to catalyze a key regulatory step in the tricarboxylic acid cycle (Hathaway & Atkinson, 1963). Less clear are functions of the two NADP-specific isozymes located in mitochondria and in the cytosol. Their relative contribution to compartmental pools of NADPH and α-ketoglutarate is unknown. Also, since isocitrate and α-ketoglutarate can traverse the mitochondrial membrane via specific carriers (LaNoue & Schoolwerth, 1979), the isozymes may participate in an intercompartmental exchange of reducing equivalents, although there is no experimental evidence to support this hypothesis.

NADP-specific isocitrate dehydrogenases have been purified from several sources. The sizes of mammalian mitochondrial and cytosolic isozymes are similar and both are homodimers (Plaut & Gabriel, 1983), but the enzymes differ in several physical properties. For example, isoelectric points of 4.8 and 5.7 have been reported for the cytosolic isozymes from porcine corpus luteum and rat liver, respectively (Jennings et al., 1990; Fatania et al., 1993), while more basic values are characteristic of the mitochondrial isozymes (Haselbeck et al., 1992). The isozymes also differ in expression levels, with levels of the cytosolic enzyme being highly variable in mammalian tissues. Levels of the cytosolic isozyme are reported to be low in heart and kidney tissues relative to liver (Plaut & Gabriel, 1983), and dramatically elevated in lactating bovine mammary gland (Farrell et al., 1987) and in rat ovary during gonadotropin-induced development (Jennings & Stevenson, 1991). Because of these properties of expression, metabolic roles for cytosolic NADP-specific isocitrate dehydrogenase in NADPH production for fatty acid and sterol synthesis have been proposed.

Despite different properties and expression patterns, there has been no classical genetic evidence that the cytosolic and mitochondrial isozymes of NADP-specific isocitrate dehydrogenase are encoded by different genes. While the genes for the mitochondrial isozyme from yeast (Haselbeck & McAlister-Henn, 1992), porcine heart (Haselbeck et al., 1992), and bovine kidney (Huh et al., 1993) have been cloned and sequenced, there have been no reports of a cloned gene for a cytosolic isozyme. In this report, we describe cloning and sequence analysis of the yeast gene encoding the cytosolic isozyme, provide genetic proof for two distinct loci for the compartmentalized isozymes, and investigate the functions of the NADP-specific isocitrate dehydrogenases by gene disruption.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions. The parental yeast strains were S173–6B (*MATa, leu2-3,112, his3-1, ura3-57, trp1-289*; Botstein et al., 1979) and an isogenic diploid strain. The *IDP1* disruption strain used for purification of IDP2 was previously constructed by transformation of S173–6B (Haselbeck & McAlister-Henn, 1992). Yeast cells were grown in liquid or on 2% agar plates containing YP medium (1% yeast extract, 2% Bactopeptone) or YNB medium (0.17% yeast nitrogen base; 0.5% ammonium sulfate, pH 6.0) with nutritional supplements of 20 μg/mL to support growth of auxotrophs. Carbon sources were added to 2%. Yeast strain mating, sporulation, and tetrad dissection were conducted according to standard techniques (Rose et al., 1990). Plasmid amplifications were performed using the bacterial strain

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DH5αF′ (Raleigh et al., 1988) in liquid or on 2% agar plates containing Luria Broth and 0.05 mg/mL ampicillin. Cell growth in cultures was monitored spectrophotometrically at 600 nm.

Purification and Sequencing of Cytosolic NADP-Specific Isocitrate Dehydrogenase. NADP-specific isocitrate dehydrogenase activity was monitored as NADPH production at 340 nm in 1 mL assays containing 50 mM KPO_4 (pH 7.5), 10 mM $MgCl_2$, 0.25 mM $NADP^+$, and 2.5 mM isocitrate. Units are expressed as μmoles of NADPH produced per minute. The Bradford method (Bradford, 1976) with bovine serum albumin as the standard was used to quantitate protein.

For purification of the cytosolic isocitrate dehydrogenase (IDP2), a haploid yeast strain containing a disruption of the *IDP1* gene was grown to stationary phase in YP medium containing 2% glycerol and 0.5% glucose as carbon sources. The cells were harvested by centrifugation at 500 × g and stored at −70 °C until use. 200 g of cells were lysed by two rounds of agitation with 100 mL of buffer A (10 mM KPO_4 [pH 7.0], 5 mM $MgCl_2$, 2 mM potassium citrate, 2 mM β-mercaptoethanol, 5% glycerol, 2 mM phenylmethanesulfonyl fluoride, and 4 μg/mL aprotinin) in a Bead Beater apparatus (Biospec Products, Bartlesville, Oklahoma). Lysates were cleared by centrifugation at 27000 × g for 20 min, brought to 15% polyethylene glycol (molecular weight 6000) by addition of an equal volume of a 30% solution of polyethylene glycol in buffer A, and stirred on ice for 30 min The precipitate was collected by centrifugation for 20 min at 12000 × g and resuspended in 50 mL of buffer A. This was bound in batch to 60 mL of the dye-binding affinity medium, Matrix red A (Pharmacia) preequilibrated in buffer A. The suspension was poured into a 2.5 × 15 cm column and washed extensively with buffer A. Protein was eluted from the column with a linear 500 mL 0–1.0 M KCl gradient in buffer A. Fractions containing enzymatic activity were pooled and brought to 13% polyethylene glycol with a 26% solution in buffer A. The solution was stirred on ice for 30 min and cleared by centrifugation. The supernatant was combined with an equal volume of 100% ammonium sulfate. The upper aqueous phase was recovered and dialyzed against 4 L of buffer A containing 100 mM NaCl. The dialysate was brought to 20% polyethylene glycol using a 40% solution. The resulting precipitate was resuspended in 20 mL of buffer A, loaded onto a 1 × 10 cm DEAE cellulose column, and washed with buffer A. Protein was eluted with a linear 250 mL 0–1.0 M KCl gradient. Fractions containing isocitrate dehydrogenase activity were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Coomassie blue staining.

Gel filtration analysis of the purified enzyme was conducted using a 100 cm × 1 cm column of Sephacryl S-200 superfine (Pharmacia) with β-amylase (M_r = 200 000), bovine serum albumin (M_r = 66 000), and carbonic anhydrase (M_r = 29 000) as calibration standards. Isoelectric focusing was conducted with gels containing pH 3.0–10.0 or pH 6.0–9.0 ampholyte gradients using the Pharmacia Phast system.

To generate tryptic peptides for sequencing, a 200 μg aliquot of purified IDP2 was electrophoresed on a 10% polyacrylamide–sodium dodecyl sulfate gel and stained with Coomassie blue. The 45000 molecular weight polypeptide band was excised, crushed, and eluted for 16 h in 50 mM Tris (pH 8.0), 0.1% sodium dodecyl sulfate, 0.1 mM EDTA, and 200 mM NaCl. The eluate was cleared by centrifugation, concentrated to 250 μL with a Centricon 30 filter (Amicon), and precipitated with 4 volumes of cold acetone. The precipitate was washed with 80% acetone, allowed to dry, and resuspended for 16 h in 50 μL of 50 mM KPO_4 (pH 7.2) and 6 M urea. The urea was diluted to 1 M using 250 μL of 50 mM KPO_4, and trypsin was added at a 1:50 w/w ratio of trypsin:IDP2 for 2 h at 37 °C. A second aliquot of trypsin was added and incubation continued for 2 h. Peptides were resolved by reverse phase HPLC on a C_8 column (Rainin). Elution with a linear H_2O/acetonitrile gradient was monitored at 214 nm. Peptides were sequenced using an Applied Biosystems 475A protein sequencer (Biotechnology Instrumentation Facility, University of California, Riverside).

Recombinant DNA Methods. Plasmid DNA was prepared by the alkaline lysis method (Maniatis et al., 1982), and yeast genomic DNA was prepared as previously described (McAlister & Holland, 1982). DNA fragments were cloned into a Bluescribe minus plasmid (Stratagene) for nucleotide sequencing and into the shuttle vectors YEp351 (Hill et al., 1986) or pRS315 (Sikorski & Hieter, 1989) for transformation into yeast. Yeast transformations were performed by the method of Ito et al. (1983) and one-step gene replacements by the method of Rothstein (1983). Double stranded DNA sequence analysis was conducted using the dideoxynucleotide method (Wang, 1988) with a Sequenase kit (USB). Radiolabeled DNA probes for colony hybridization or for Southern blot analysis were prepared by the random primer method (Feinberg & Vogelstein, 1984).

Polymerase Chain Reaction. Polymerase chain reaction (PCR) was performed with yeast genomic DNA as a template and degenerate oligonucleotide primers (Operon, San Pablo, California) synthesized on the basis of peptide sequences. For the peptide sequence TDDMVA (*cf.* Results), the corresponding oligonucleotide was AC(A/C/G/T)GA(C/T)-GA(C/T)ATGGT(A/C/G/T)GC. The first residue in the peptide sequence was incorrectly called as threonine rather than isoleucine; however, this did not preclude successful use of the oligonucleotide. For the downstream sequence, EEFIDA, the corresponding anti-sense oligonucleotide, GC(A/G)TC(A/G/T)AT(A/G)AA(C/T)TC(C/T)TC, was used. Reactions were conducted using a DNA amplification kit (Perkin-Elmer Cetus) in an Ericomp thermal cycler. Genomic DNA (1 μg) was used for amplification with 0.4 nmol of each primer. An initial denaturing step of 5 min at 94 °C was followed by 30 cycles of 1 min at 94 °C, 30 s at 45 °C, and 1 min at 72 °C.

Immunoblotting. Protein samples were electrophoresed on polyacrylamide–sodium dodecyl sulfate gels and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) using a graphite semidry electroblotter. Membranes were blocked and washed as described by Burnette (1981) prior to incubation with a 1:500 dilution of rabbit anti-yeast IDP1 antiserum (Haselbeck & McAlister-Henn, 1983) followed by incubation with ^{125}I-labeled protein A.

RESULTS

Purification and Characterization of Yeast Cytosolic NADP-Specific Isocitrate Dehydrogenase. During previous purification of NADP-specific isocitrate dehydrogenase from yeast, it was noted that activity in cytosolic cellular fractions was extremely labile whereas the mitochondrial matrix enzyme (IDP1) proved to be stable and easily purified (Haselbeck & McAlister-Henn, 1992). It was empirically determined that the cytosolic activity could be stabilized by addition of 1–5 mM citrate or isocitrate to purification buffers. Previous work also established that an antiserum raised against the mitochondrial isozyme was cross reactive in immunoblots with a slightly smaller polypeptide which was highly enriched in

Table 1: Purification of Yeast Cytosolic NADP(H)-Specific Isocitrate Dehydrogenase

purification step	total activity (units)	total protein (mg)	specific activity (units/mg)	yield (%)
crude extract	720	5850	0.1	100
polyethylene glycol	200	950	0.2	28
matrix red A	257[a]	113	2.3	36[a]
polyethylene glycol	200	60	3.3	28
ammonium sulfate	80	20	4.0	11
DEAE-cellulose	34	1	28.3	5

[a] The apparent gain in activity and yield in this step may be due to partial refolding of the enzyme following a precipitation step.

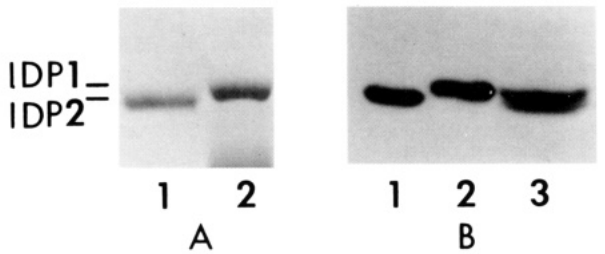

FIGURE 1: Electrophoretic and immunoblot analysis of yeast IDP2 and IDP1. Purified IDP2 (0.5 μg, lane A1, and 0.12 μg, lane B1), purified IDP1 (1.0 μg, lane A2, and 0.25 g, lane B2), and cellular proteins (100 μg, lane B3) from a wild type strain grown with glycerol and lactate as carbon sources were electrophoresed on a 10% polyacrylamide/sodium dodecyl sulfate gel. The resolved proteins were stained with Coomassie blue (panel A) or transferred to a filter for immunoblot analysis (panel B) conducted as described under Experimental Procedures.

cytosolic cellular fractions (Haselbeck & McAlister-Henn, 1993). Levels of the latter polypeptide and of cytosolic NADP-specific isocitrate dehydrogenase activity were found to be elevated in yeast cells grown on nonfermentable carbon sources. Therefore, to ensure purification of the cytosolic enzyme, a yeast strain containing a disruption of the locus (*IDP1*) encoding the mitochondrial isozyme was used, the cells were grown with glycerol as the primary carbon source, and citrate was included in all buffers.

A protocol for purification of yeast cytosolic NADP-specific isocitrate dehydrogenase was empirically developed and implemented as described under Experimental Procedures. The protocol employed bulk fractionation with both polyethylene glycol and ammonium sulfate, and chromatographic fractionation using Matrix red A and DEAE cellulose. These procedures resulted in a 234-fold increase in specific activity and yielded approximately 1.2 mg of the cytosolic enzyme from a 200 g yeast cell pellet (Table I). With polyacrylamide–sodium dodecyl sulfate gel electrophoresis, the purified enzyme migrated as a single polypeptide with an apparent molecular weight of 45 000 (Figure 1A, lane 1). The polypeptide designated IDP2 migrates slightly ahead of purified IDP1 (apparent M_r = 46 000; Figure 1A, lane 2). Both comigrate with polypeptides detected with an antiserum against IDP1 in immunoblots of cellular extracts from a wild type yeast strain grown on a nonfermentable carbon source (Figure 1B, lane 3). Using this antiserum and purified IDP1 and IDP2 in immunoblots (Figure 1B, lanes 1 and 2), we estimated that the reactivity of the antiserum for IDP2 is about 2-fold greater than for IDP1.

Gel filtration chromatography, conducted as described under Experimental Procedures, gave an estimated molecular weight of 95000 for catalytically active IDP2. This suggests that the native enzyme is a homodimer as is the yeast mitochondrial isozyme (Haselbeck & McAlister-Henn, 1992) and as are NADP-specific isocitrate dehydrogenases from mammalian sources (Plaut & Gabriel, 1983; Fatania et al., 1993). The two purified yeast isozymes were compared using isoelectric focusing as described under Experimental Procedures. A pI of 5.5 was obtained for IDP2 whereas IDP1 had a pI of 8.5 as previously reported (Haselbeck & McAlister-Henn, 1992). This suggests that the isozymes should have different chromatographic properties on ion-exchange resins, and we have found that chromatography on DEAE-cellulose is an effective method for separation of the isozymes.

Isolation of the IDP2 Gene. Attempts to obtain amino terminal sequence data using purified IDP2 were unsuccessful, suggesting that the amino terminus may be blocked. To obtain internal peptide sequence information, the IDP2 polypeptide was resolved by polyacrylamide–sodium dodecyl sulfate gel electrophoresis, eluted, and digested with trypsin as described under Experimental Procedures. Tryptic peptides were resolved by HPLC and partial amino acid sequences obtained for three peptides: (1) DQTNDQVTVDSATATL; (2) LTDDMVA; (3) SAYVTTEEFIDAVESR.

The peptide sequences from IDP2 were compared with the amino acid sequence for IDP1 and all three found to align with highly homologous regions of IDP1 (*cf.* Figure 4). For polymerase chain reaction (PCR), degenerate oligonucleotides based on peptide sequences 2 and 3 were synthesized and used as primers with yeast genomic DNA as the template. Assuming that the *IDP2* and *IDP1* genes would be similar in size, a PCR product of approximately 400 bp was predicted, and a DNA fragment of this size was obtained using conditions described under Experimental Procedures.

To obtain the full-length *IDP2* gene, the 400 bp PCR product was labeled by the random primer method (Feinberg & Vogelstein, 1984) and used as a probe to screen bacterial colonies transformed with a yeast genomic DNA library cloned into a multicopy 2 micron plasmid containing a selectable yeast *LEU2* gene (Naysmyth & Tatchell, 1980). Of approximately 5000 colonies, seven potential positives were identified. Plasmid DNA was prepared from the seven isolates and used to transform the yeast *IDP1* disruption strain. Individual Leu^+ transformants were isolated and cultivated in medium with glycerol and lactate as carbon sources. Cellular extracts prepared as described under Experimental Procedures were used to measure NADP-dependent isocitrate dehydrogenase activity. Extracts from one of the seven yeast transformants exhibited a 16.5-fold elevation in specific activity and immunoblot analysis confirmed a concomitant elevation in levels of the 45000 molecular weight IDP2 polypeptide (data not shown).

The 2 micron plasmid which produced elevated levels of IDP2 in yeast transformants was used for Southern blot analysis with the 400 bp PCR product as a probe. A 3.0 kb *Sal*I restriction fragment which hybridized with the probe was identified and subcloned into a Bluescribe vector for nucleotide sequence analysis. A partial restriction map of the subcloned fragment and the sequencing strategy are illustrated in Figure 2.

The nucleotide sequence of the *IDP2* gene and deduced amino acid sequence are shown in Figure 3. The gene contains an open reading frame encoding a polypeptide of 412 amino acid residues terminating with a TAA stop codon. It is not known if the initiating methionine is present on the mature polypeptide since no amino terminal sequence data could be obtained. The polypeptide has a predicted molecular weight of 46 535, a value similar to that obtained from electrophoresis.

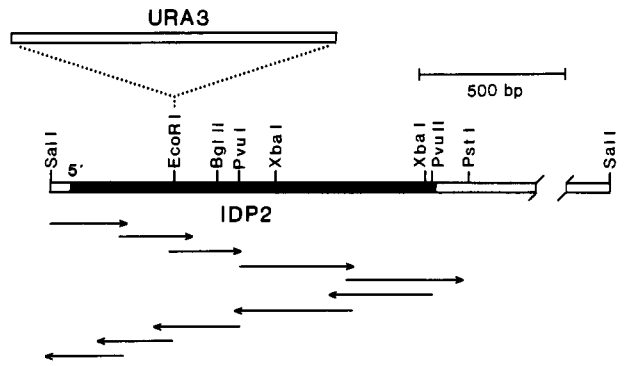

FIGURE 2: Strategies for nucleotide sequence analysis and disruption of the yeast *IDP2* gene. A partial restriction map for a 3.0 kb *Sal*I restriction fragment containing the *IDP2* gene is shown. The direction and extent of nucleotide sequences obtained from various subclones or with internal oligonucleotide primers are indicated by arrows. For gene disruption, the yeast *URA3* gene was inserted into the *Eco*RI site withing the *IDP2* coding region (solid line) as indicated.

The amino acid sequences of IDP1 and IDP2 are compared in Figure 4. The two polypeptides are identical in 71% of the residue positions. Despite this similarity and identity in total residue numbers, a two-residue gap in IDP2 (between positions 162 and 163) and two single residue gaps in IDP1 (positions 175 and at the carboxy terminus) must be introduced to maximize alignment. The amino terminus of IDP2 is very similar to the amino terminus of the mature mitochondrial form of IDP1, but it lacks the 16-residue presequence which is removed upon mitochondrial import of IDP1. This is consistent with cytosolic localization of IDP2. The overall amino acid compositions of IDP2 and IDP1 are quite similar. However, calculation of net charge (number of arginine plus lysine residues *vs* number of aspartate plus glutamate residues) gives a value of −8 for IDP2 and of +3 for IDP1. These values are generally consistent with the pI values measured for the proteins.

Chromosomal Location of the IDP2 Gene. During the course of these studies, it was communicated to us by Drs. Bobby Baum (Bethesda, MD) and John Carbon (Santa Barbara, CA) that a recently published nucleotide sequence for a yeast centromere binding protein (CBF5p; Jiang et al., 1993) contained a 5′ upstream open reading frame with extensive similarity to the published *IDP1* gene sequence. Comparison of restriction maps and nucleotide sequences confirmed that this upstream region represents the 3′ end of the *IDP2* coding region. The *IDP2* and *CBF5* genes are transcribed in the same direction and the last codon in *IDP2* is located 258 bp upstream from the initiator methionine of the *CBF5* coding region. The *CBF5* gene was localized to a position adjacent to and centromere proximal from *CDC42* on yeast chromosome XII (Jiang et al., 1993), thus establishing the chromosomal location of *IDP2*. The yeast *IDP1* gene was previously located to chromosome IV (Haselbeck & McAlister-Henn, 1993).

Disruption of the IDP2 Gene. To confirm the identity of the *IDP2* gene, the coding region within the 3.0 kb *Sal*I fragment was disrupted by insertion into the internal *Eco*RI site of a 1.1 kb DNA fragment containing the yeast *URA3* gene (Figure 2). The modified *Sal*I fragment was used for transformation and one-step gene replacement (Rothstein, 1983). Ura$^+$ transformants of a wild-type diploid yeast strain were obtained and sporulated to produce haploid tetrads. The Ura$^+$ phenotype was found to segregate in a 2:2 fashion with (a) replacement of a 1.8 kb *Pst*I/*Sal*I genomic *IDP2* DNA fragment by a 2.9 kb fragment containing the disrupted *IDP2*

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-68       -60         -50         -40         -30         -20
GTCGACTA  CGTCGGTGCT  CAGGCACGAG  AATAGGAGGT  AAGGTAACGT  ACGTATATAT

-10                    15                  30                  45
ATAAATCGTA  ATG ACA AAG ATT AAG GTA GCT AAC CCC ATT GTG GAA ATG GAC GGC
            Met Thr Lys Ile Lys Val Ala Asn Pro Ile Val Glu Met Asp Gly

                60                  75                  90
GAT GAG CAA ACA AGA ATA ATC TGG TCA TTT AAT CAG GAC AAG TTA GTC TTG CCC
Asp Glu Gln Thr Arg Ile Ile Trp Ser Phe Asn Gln Asp Lys Leu Val Leu Pro

    105                 120                 135                 150
TAT CTT GAC GTT GAT TTG AAG TAC TAC GAT CTT TCC GTG GAG TAT CGT GAC CAG
Tyr Leu Asp Val Asp Leu Lys Tyr Tyr Asp Leu Ser Val Glu Tyr Arg Asp Gln

            165                 180                 195
ACT AAT GAT CAA GTA ACT GTG GAT TCT GCC ACC GCG ACT TTA AAG TAT AGG GTA
Thr Asn Asp Gln Val Thr Val Asp Ser Ala Thr Ala Thr Leu Lys Tyr Arg Val

210                 225                 240                 255
GCT GTC AAA TGC GCG ACT ATT ACA CCC GAT GAG GCA AGG GTC GAG GAA TTT CAT
Ala Val Lys Cys Ala Thr Ile Thr Pro Asp Glu Ala Arg Val Glu Glu Phe His

        270                 285                 300                 315
TTG AAA AAG ATG TGG AAA TCT CCA AAT GGT ACT ATT AGA AAC ATT TTG GGT GGT
Leu Lys Lys Met Trp Lys Ser Pro Asn Gly Thr Ile Arg Asn Ile Leu Gly Gly

                330                 345                 360
ACA GTG TTC AGA GAA CGT ATT ATT ATC CCT AGA ATT CCA GGG CTA GTT CCT CAA
Thr Val Phe Arg Glu Pro Ile Ile Ile Pro Arg Ile Pro Arg Leu Val Pro Gln

    375                 390                 405                 420
TGG GAG AAG CCC ATC ATC ATT GGG AGA CAC GCA TTC GGC GAT CAG TAC AAA GCT
Trp Glu Lys Pro Ile Ile Ile Gly Arg His Ala Phe Gly Asp Gln Tyr Lys Ala

            435                 450                 465
ACC GAT GTA ATA GTC CCT GAA GAA GGC GAG TTG AGG CTT GTT TAT AAA TCC AAG
Thr Asp Val Ile Val Pro Glu Glu Gly Glu Leu Arg Leu Val Tyr Lys Ser Lys

480                 495                 510                 525
AGC GGA ACT CAT GAT GTA GAT CTG AAG GTA TTT GAC TAC CCA GAA CAT GGT GGG
Ser Gly Thr His Asp Val Asp Leu Lys Val Phe Asp Tyr Pro Glu His Gly Gly

        540                 555                 570                 585
GTT GCC ATG ATG ATG TAC AAC AAT ACA GAT TCG ATC GAA GGG TTT GCG AAG GCC
Val Ala Met Met Met Tyr Asn Asn Thr Asp Ser Ile Glu Gly Phe Ala Lys Ala

                600                 615                 630
TCC TTT GAA TTG GCC ATT GAA AGG AAG TTA CCA TTA TAT TCC ACT ACT AAG AAT
Ser Phe Glu Leu Ala Ile Glu Arg Lys Leu Pro Leu Tyr Ser Thr Thr Lys Asn

    645                 660                 675                 690
ACT ATT TTG AAG AAG TAT GAT GGT AAA TTC AAA GAT GTT TTC GAA GCC ATG TAT
Thr Ile Leu Lys Lys Tyr Asp Gly Lys Phe Lys Asp Val Phe Glu Ala Met Tyr

            705                 720                 735
CTA GAA GTT ATA AAG AGA AGT TTG GAA TCC CTT GGC ATC TGG TAC GAG CAC CGT
Leu Glu Val Ile Lys Arg Ser Leu Glu Ser Leu Gly Ile Trp Tyr Glu His Arg

750                 765                 780                 795
TTA ATT GAT GAT ATG GTG GCC CAA ATG TTG AAA TCT AAA GGT GGA TAC ATA ATG
Leu Ile Asp Asp Met Val Ala Gln Met Leu Lys Ser Lys Gly Gly Tyr Ile Met

        810                 825                 840                 855
GCC ATG AAA AAT TAC GAC GGT GAC GTA GAA TCA GAT ATT GTT GCA CAA GGA TTT
Ala Met Lys Asn Tyr Asp Gly Asp Val Glu Ser Asp Ile Val Ala Gln Gly Phe

                870                 885                 900
GGC TCC TTG GGG TTA ATG ACA TCT GTG TTG ATA ACG CCG GAC GGT AAA ACC TTT
Gly Ser Leu Gly Leu Met Thr Ser Val Leu Ile Thr Pro Asp Gly Lys Thr Phe

    915                 930                 945                 960
GAA AGC GAC CGT GCC CAC GGT ACA GTA ACA AGA CAT TTG ACA GAC TAC GAC AAG
Glu Ser Asp Arg Ala His Gly Thr Val Thr Arg His Leu Thr Asp Tyr Asp Lys

            975                 990                1005
GGA AGG GAG ACG TCA ACA AAT TCC ATT GCA TCA ATT TTC GCG TGG ACT AGA GGT
Gly Arg Glu Thr Ser Thr Asn Ser Ile Ala Ser Ile Phe Ala Trp Thr Arg Gly

1020                1035                1050                1065
ATT ATT CAA AGG GGT AAA CTT GAT AAT ACA CCA GAT GTA GTT AAG TTC GGC CAA
Ile Ile Gln Arg Gly Lys Leu Asp Asn Thr Pro Asp Val Val Lys Phe Gly Gln

        1080                1095                1110                1125
ATA TTG GAA AGC GCT ACG GTA AAT ACG GTG CAA GAA GAT GGA ATC ATG ACT AAA
Ile Leu Glu Ser Ala Thr Val Asn Thr Val Gln Glu Asp Gly Ile Met Thr Lys

            1140                1155                1170
GAT TTG GCG CTC ATT CTC GGT AAG TCT GAA AGA TCC GCT TAT GTC ACT ACC GAG
Asp Leu Ala Leu Ile Leu Gly Lys Ser Glu Arg Ser Ala Tyr Val Thr Thr Glu

    1185                1200                1215                1230
GAG TTC ATT GAC GCG GTG GAA TCT AGA TTG AAA AAA GAG TTC GAG GCA GCT GCA
Glu Phe Ile Asp Ala Val Glu Ser Arg Leu Lys Lys Glu Phe Glu Ala Ala Ala

     1,240       1,250       1,260       1,270       1,280       1,290
TTG TAAA  CTTACTCAAT  GACAGATTGA  TTTATTTATA  TTATATATCC  TTTTTATAGG
Leu

     1,300       1,310
AAATTTAGTA  TTTCTACTCG
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FIGURE 3: Nucleotide sequence and deduced amino acid sequence of the yeast *IDP2* gene.

gene as determined by Southern blot analysis (Figure 5) and with (b) loss of the IDP2 polypeptide in whole cell extracts as determined by immunoblot analysis (data not shown). In extracts from haploid cells containing the *IDP2* disruption, NADP-specific isocitrate dehydrogenase activity was decreased 50–60%, suggesting that the two NADP-specific isocitrate dehydrogenase isozymes contribute approximately equally to total cellular activity in yeast cells grown on

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IDP2                                                  M T K I K V A N P I V E M D G D E Q T R I I W S F N Q D
IDP1 (M S M L S R R L F S T S R L A A) F S * * * * K Q * V * * L * * * * M * * * * * D K I K K

K L V L P Y L D V D L K Y Y D L S V E Y R D Q T N D Q V T V D S A T A T L K Y R V A V K C
* * I * * * * * * * * * * * * * * * * S * * A * S * K I * Q * A * E * I K * * G * G I * *

A T I T P D E A R V E E F H L K K M W K S P N G T I R N I L G G T V F R E P I I I P R I P
* * * * * * * * * * K * * N * H * * * * * * * * * * * * * * * * * * * * * * V * * * * *

R L V P Q W E K P I I I G R H A F G D Q Y K A T D V I V P E E G E L R L V Y K S K S G T -
* * * * R * * * * * * * * * * * H * * * * * * * * T L I * G P * S * E * * * * P S D P * T

- H D V D L K V F D Y P E H G G V A M M M Y N N T D S I E G F A K A S F E L A I E R K L P
A Q P Q T * * * Y * * K G - S * * * * A * * * T D E * * * * * * H S * * K * * * D K * * N

L Y S T T K N T I L K K Y D G K F K D V F E A M Y L E V I K R S L E S L G I W Y E H R L I
* F L S * * * * * * * * * * * * R * * * I * Q E V * E A Q Y * S K F * Q * * * H * * * * * *

D D M V A Q M L K S K G G Y I M A M K N Y D G D V E S D I V A Q G F G S L G L M T S V L I
* * * * * * * I * * * * * F * * * L * * * * * * * Q * * * * * * * * * * * * * * * * I * V

T P D G K T F E S D R A H G T V T R H L T D Y D K G R E T S T N S I A S I F A W T R G I I
* * * * * * * * * E A * * * * * * * * * Y R K * Q * * E * * * * * * * * * * * * * * S * * L L

Q R G K L D N T P D V V K F G Q I L E S A T V N T V Q E D G I M T K D L A L I L G K S E R
K * * E * * * * * * A L C * * A N * * * * * * L * * * * Q * * * * * * * * * * * A C * N N * *

S A Y V T T E E F I D A V E S R L K K E F E A A A L
* * * * * * * * * L * * * * K * * Q * * I K S I E -
```

FIGURE 4: Amino acid sequence comparison of yeast IDP2 and IDP1. The amino acid sequence of IDP2 is compared with that previously reported for IDP1 (Haselbeck & McAlister-Henn, 1992). Sequences determined for tryptic peptides of IDP2 are underlined. The mitochondrial targeting presequence of IDP1 is shown in parentheses. Numbers refer to residue positions in IDP2. Identities are indicated by asterisks and gaps introduced to optimize alignment are indicated by dashes.

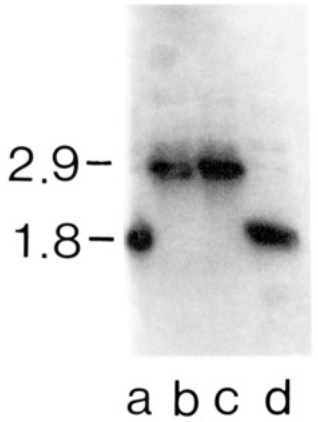

FIGURE 5: Southern blot analysis of the *IDP2* genomic disruption. Disruption of the *IDP2* coding region was conducted by transformation of a diploid yeast strain with the *IDP2*::*URA3* disruption construct described in the text. The diploid was sporulated and genomic DNA was isolated from two Ura$^+$ haploid segregants (lanes b and c) and two Ura$^-$ haploid segregants (lanes a and d) from a typical tetrad. Southern blot analysis was performed with 10 μg aliquots of the genomic DNAs digested with *Pst*I and *Sal*I using as a ^{32}P-labeled probe the 1.8 kb *Pst*I/*Sal*I fragment from *IDP2*.

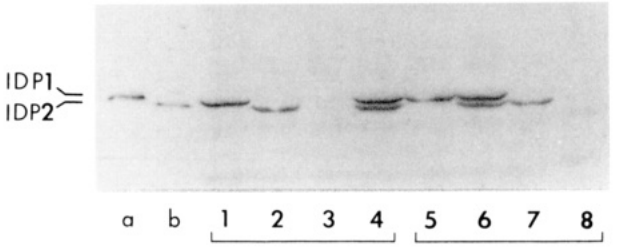

FIGURE 6: Immunoblot analysis of *IDP2* and *IDP1* disruption mutants. Haploid yeast strains containing each *IDP* gene disruption were mated and sporulated. Protein extracts (100 μg) from haploid segregants of two representative tetrads (lanes 1–4 and lanes 5–7) were electrophoresed for immunoblot analysis using anti-yeast IDP1 antiserum (Haselbeck & McAlister-Henn, 1993). Each tetrad contained an *IDP2* disruption mutant (lanes 1 and 5), an *IDP1* disruption mutant (lanes 2 and 7), a double disruption mutant (lanes 3 and 8), and a wild type strain (lanes 4 and 6). Lanes a and b contained 0.25 μg of purified IDP1 and IDP2, respectively.

nonfermentable carbon sources. Representative tetrads were plated on YP agar plates containing a variety of fermentable and nonfermentable carbon sources. In these initial tests, no discernible growth phenotype could be attributed to the *IDP2* disruption.

To obtain a haploid mutant with disruptions in both *IDP1* and *IDP2* genes, haploid strains containing each chromosomal disruption were mated and resulting diploids sporulated for tetrad dissection. Segregation of the disrupted genes was monitored by Southern blot analysis (data not shown) and by immunoblot analysis (Figure 6). Two tetrads (represented by lanes 1–4 and 5–8 in Figure 6) containing haploid segregants of the wild type, the two single mutants, and the double mutant were chosen for further analysis. Enzyme assays confirmed the absence of NADP-specific isocitrate dehydrogenase activity in mutants containing both gene disruptions. Similar assays of extracts from single mutant and wild type strains produced average values of approximately 30 and 70% of total cellular activity for IDP2 and IDP1, respectively. These values correlate well with relative levels of immunoreactivity in Western blot analysis (Figure 6). However, these relative activities differ somewhat from previous measurements for segregants from the single *IDP2* disruption cross and may be due to slight differences in cultivation procedures. Disruption of either *IDP* gene had no effect on expression of the other isozyme, indicating independent expression of the *IDP1* and *IDP2* genes. The strains from the *IDP1*/*IDP2* disruption cross

were tested for growth on YP agar plates containing various carbon sources (glucose, glycerol, acetate, or ethanol) and on minimal YNB plates in the presence or absence of glutamate supplements. Under these conditions, no dramatic differences in viability or growth were detected for mutant strains lacking either or both IDP isozymes.

DISCUSSION

In this report, we describe cloning and analysis of the yeast gene (*IDP2*) encoding the cytosolic isozyme of NADP-specific isocitrate dehydrogenase. These results, in combination with previous isolation of the yeast gene (*IDP1*) encoding the corresponding mitochondrial isozyme (Haselbeck & McAlister-Henn, 1992), conclusively demonstrate the independent genetic origin of these compartmentalized isozymes in eucaryotic cells. The yeast genes are located on different chromosomes and, despite extensive similarity between derived primary sequences of the proteins, do not cross-hybridize in Southern blot analyses under stringent conditions. The purified yeast isozymes also exhibit different physical properties: the cytosolic isozyme has a more acidic isoelectric point and is much more labile than the mitochondrial isozyme under most buffer conditions. Despite different physical properties, preliminary kinetic analyses demonstrate no dramatic differences between the isozymes.

In addition to genes encoding the NADP-specific isocitrate dehydrogenases, the two yeast genes (*IDH1* and *IDH2*) encoding subunits of mitochondrial NAD-specific isocitrate dehydrogenase have been cloned (Cupp & McAlister-Henn, 1991; Cupp & McAlister-Henn, 1992). The primary sequences of the subunits of the NAD-specific enzyme are 42% identical to each other but both are essentially unrelated (<20% identity) to those of the NADP-specific enzymes. Thus, it might be assumed that this lack of relatedness reflects differences in cofactor specificity and in quaternary structure (the NAD enzyme is an octamer and the NADP enzymes are dimers). However, this assumption is questionable when the primary sequence for isocitrate dehydrogenase from *Escherichia coli* is also considered. The bacterial enzyme is NADP-specific and a homodimer (Reeves et al., 1972; Burke et al., 1974), but its primary sequence is much more similar to those of the subunits of the yeast NAD-specific enzyme (32% identity, Cupp & McAlister-Henn, 1991) than to those of the NADP-specific isozymes. This suggests possible evolutionary conservation of structure for function in the tricarboxylic acid cycle.

The only three-dimensional structure currently available for an isocitrate dehydrogenase was obtained for the *E. coli* enzyme (Hurley et al., 1989, 1990). Despite their overall dissimilarity, many of the residues implicated in metal–isocitrate binding (Hurley et al., 1991) in the bacterial enzyme are conserved at similar relative positions in the yeast NADP-specific isozymes. These include *E. coli* residues Arg 119, Arg 153, Tyr 160, Lys 230, Asp 307, Asp 311, and Glu 336 which correspond to IDP2 residues Arg 109, Arg 132, Tyr 139, Lys 212, Asp 274, Asp 278, and Asp 306. Much less conserved are residues in the bacterial enzyme which have been implicated in NADP binding (Hurley et al., 1991).

Because of the cross-reactivity of IDP2 with antiserum raised against IDP1, we were able in previous studies (Haselbeck & McAlister-Henn, 1993) to simultaneously examine expression of both isozymes in whole cell extracts. IDP1 was found to be constitutively expressed with various carbon sources. In contrast, IDP2 levels were elevated in cells grown on nonfermentable carbon sources and undetectable in cells grown on glucose. This suggests that expression of IDP2 is subject to catabolite repression as is expression of yeast NAD-specific isocitrate dehydrogenase (Keys & McAlister-Henn, 1990).

To assess cellular functions of the eucaryotic NADP-specific isocitrate dehydrogenases, we have examined growth phenotypes of mutant yeast strains containing chromosomal disruptions of either or both *IDP1* and *IDP2*. In tests of growth with various carbon sources and for glutamate auxotrophy, we have observed no dramatic growth deficiencies relative to a wild type strain. This suggests that the remaining mitochondrial NAD-specificic isocitrate dehydrogenase is sufficient for α-ketoglutarate production. However, as previously reported (Haselbeck & McAlister-Henn, 1993), disruption of both *IDP1* and *IDH2* produced a unique growth phenotype, glutamate auxotrophy on glucose. The ability of this double disruption mutant to grow on nonfermentable carbon sources was attributed to the presence of residual cytosolic IDP2 under these cultivation conditions. Thus, it will be necessary to similarly utilize all possible combinations of isocitrate dehydrogenase gene disruptions to conclusively define functions of the individual isozymes. It will also be necessary to test additional growth conditions. For example, Machado et al. (1975) speculated that NADP-specific isocitrate dehydrogenase is essential for anaerobic growth of yeast cells, but the role of each compartmentalized isozyme was not established. Finally, it will be instructive to examine the relative contributions of the NADP-specific isozymes in production of NADPH relative to other cellular enzymatic sources of these reducing equivalents.

REFERENCES

Botstein, D., Falco, S. C., Stewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K., & Davis, R. W. (1979) *Gene (Amsterdam) 9,* 12–24.

Bradford, M. M. (1976) *Anal. Biochem. 72,* 248–254.

Burke, W. F., Johanson, R. A., & Reeves, H. C. (1974) *Biochim. Biophys. Acta 351,* 333–340.

Burnette, W. N. (1981) *Anal. Biochem. 112,* 195–203.

Cupp, J. R., & McAlister-Henn, L. (1991) *J. Biol. Chem. 266,* 22199–22205.

Cupp, J. R., & McAlister-Henn, L. (1992) *J. Biol. Chem. 267,* 16417–16423.

Farrell, H. M., Jr., Deeney, J. T., Tubbs, K. A., & Walsh, R. A. (1987) *J. Dairy Sci. 70,* 781–788.

Fatania, H., Al-Nassar, K. E., & Sidhan, V. (1993) *FEBS Lett. 320,* 57–60.

Feinberg, A. P., & Vogelstein, B. (1984) *Anal. Biochem. 137,* 266–267.

Haselbeck, R. J., Colman, R. F., & McAlister-Henn, L. (1992) *Biochemistry 31,* 6219–6223.

Haselbeck, R. J., & McAlister-Henn, L. (1992) *J. Biol. Chem. 266,* 2339–2345.

Haselbeck, R. J., & McAlister-Henn, L. (1993) *J. Biol. Chem. 268,* 12116–12122.

Hathaway, J. A., & Atkinson, D. E. (1963) *J. Biol. Chem. 238,* 2875–2881.

Hill, J. E., Myers, A. M., Koerner, T. J., & Tzagoloff, A. (1986) *Yeast 2,* 163–167.

Huh, T-L., Ryu, J-H., Huh, J-W., Sung, H-C., Oh, I-U., Song, B. J., & Veech, R. L. (1993) *Biochem. J. 292,* 705–710.

Hurley, J. H., Dean, A. M., Sohl, J. L., Koshland, D. E., Jr., & Stroud, R. M. (1990) *Science 249,* 1012–1016.

Hurley, J. H., Dean, A. M., Koshland, D. E., Jr., & Stroud, R. M. (1991) *Biochemistry 30,* 8671–8678.

Hurley, J. H., Thorsness, P. E., Ramalingam, V, Helmers, N. H., Koshland, D. E., Jr., & Stroud, R. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 8635–8639.

Ito, H., Fukuda, Y., Murata, K., & Kimura, A. (1983) *J. Bacteriol. 153,* 163–168.

Jennings, G. T., Sadleir, J. W., & Stevenson, P. M. (1990) *Biochim. Biophys. Acta 1034,* 219–227.

Jennings, G. T., & Stevenson, P. M. (1991) *Eur. J. Biochem. 198,* 621–625.

Jiang, W., Middleton, K., Yoon, H.-J., Fouquet, C., & Carbon, J. (1993) *Mol. Cell. Biol. 13,* 4884–4893.

Keys, D. A., & McAlister-Henn, L. (1990) *J. Bacteriol. 172,* 4280–4287.

LaNoue, K. F., & Schoolwerth, A. C. (1979) *Annu. Rev. Biochem. 48,* 871–922.

Machado, A., de Castro, N., & Mayor, F. (1975) *Mol. Cell. Biochem. 6,* 93–100.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McAlister, L., & Holland, M. J. (1982) *J. Biol. Chem.* 257, 7181–7188.

Naysmyth, K. A., & Tatchell, K. (1980) *Cell 19,* 753–764.

Plaut, G. W. E., & Gabriel, J. L. (1983) in *Biochemistry of Metabolic Processes* (Lennon, D. L. F., Stratman, F. W., & Zahlten, R. N., Eds.) pp 285–301, Elsevier Science Publishing Co., Inc., New York.

Raleigh, E. A., Murray, N. E., Revel, H., Blumenthal, R. M., Westaway, D., Reith, A. D., Rigby, P. W. J., Elhai, J., & Hanahan, D. (1988) *Nucleic Acids Res. 16,* 1563–1575.

Reeves, H. C., Daumy, G. O., Lin, C. C., & Houston, M. (1972) *Biochim. Biophys. Acta 238,* 27–39.

Rose, M. D., Winston, F., & Hieter, P. (1990) *Methods in Yeast Genetics. A Laboratory Course Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Rothstein, R. J. (1983) *Methods Enzymol. 101,* 202–211.

Sikorski, R. S., & Hieter, P. (1989) *Genetics 122,* 19–27.

Wang, Y. (1988) *Biotechniques 6,* 843–845.