Expression and Gene Disruption Analysis of the Isocitrate Dehydrogenase Family in Yeast†

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ABSTRACT: Mammalian and yeast cells contain three isozymes of isocitrate dehydrogenase: mitochondrial NAD- and NADP-specific enzymes and a cytosolic NADP-specific enzyme. Independent metabolic functions of these enzymes in *Saccharomyces cerevisiae* were examined by analyses of expression and of phenotypes displayed by mutants containing all possible combinations of isozyme gene disruptions. All three isocitrate dehydrogenases are expressed at high levels with growth on nonfermentable carbon sources, whereas the mitochondrial NADP-specific enzyme constitutes the major cellular activity with growth on glucose. Distinct growth phenotypes are observed for mutants expressing a single isozyme, and expression of at least one isozyme is necessary for glutamate-independent growth. The NADP-specific tricarboxylic acid cycle isocitrate dehydrogenase from *Escherichia coli* was expressed in mitochondrial and cytosolic compartments of the yeast disruption mutants using plasmids carrying gene fusions of yeast promoters and a mitochondrial targeting presequence with the bacterial coding sequence. The bacterial enzyme is competent for restoration of NADP-specific functions in either compartment but does not compensate for function of the yeast NAD-specific tricarboxylic acid cycle enzyme.

The oxidative decarboxylation of isocitrate to α-ketoglutarate is catalyzed by three differentially compartmentalized isozymes in eucaryotic cells. Mitochondrial NAD-specific isocitrate dehydrogenase in yeast and mammalian cells is allosterically regulated by adenine nucleotides and NADH (Hathaway & Atkinson, 1963; Barnes et al., 1972), making this a sensitive point for control of flux through the tricarboxylic acid cycle. This isozyme is an octamer with an $\alpha_4\beta_4$ subunit composition in yeast (Keys & McAlister-Henn, 1990) and an $\alpha_4\beta_2\gamma_2$ subunit composition in mammals (Ramachandran & Colman, 1980). Genes encoding the two yeast subunits [IDH1 (M_r = 38 001), Cupp & McAlister-Henn, 1992; and IDH2 (M_r = 37 755), Cupp & McAlister-Henn, 1991] and cDNAs for the α and γ subunits from mammalian sources (Nichols et al., 1993; Kim et al., 1995) have been isolated. The yeast subunits share 42% primary sequence identity and the mammalian subunits 44% identity. The mammalian α subunit is 55% identical with yeast IDH2, which is believed to be the catalytic subunit (Cupp & McAlister-Henn, 1993).

The other two eucaryotic isocitrate dehydrogenases are mitochondrial and cytosolic NADP-specific enzymes. These enzymes are homodimers and, although not subject to allosteric regulation, exhibit patterns of highly regulated expression. For example, the cytosolic mammalian enzyme is expressed at high levels in rat ovary with the onset of ovulation (Jennings, & Stevenson, 1991) and in bovine mammary gland with lactation (Farrell et al., 1987). In yeast, levels of the cytosolic isozyme (IDP2, polypeptide M_r = 46 535) are elevated with nonfermentable growth conditions whereas levels of the mitochondrial isozyme (IDP1, polypeptide M_r = 46 400) are apparently constitutive (Haselbeck & McAlister-Henn, 1993). Sequences for cloned genes and cDNAs (Haselbeck & McAlister-Henn, 1991; Loftus et al., 1994; Haselbeck et al., 1992; Jennings et al., 1994) indicate a high degree of conservation between mitochondrial and cytosolic isozymes (approximately 70% sequence identity in yeast and in mammals) as well as between similarly compartmentalized isozymes from different species.

Beyond conservation of scattered residues with putative roles in catalysis, there is little primary sequence conservation between NAD- and NADP-specific polypeptides. The putative catalytic residues are assigned based on comparison with *Escherichia coli* isocitrate dehydrogenase, the only enzyme in this group with a defined X-ray crystal structure (Hurley et al., 1991).

Gene disruption studies in yeast have confirmed that the mitochondrial NAD-specific enzyme functions in the tricarboxylic acid cycle and that the mitochondrial NADP-specific enzyme cannot compensate for this function (Haselbeck & McAlister-Henn, 1993). Disruption of either or both genes for NADP-specific isozymes produced no dramatic growth phenotypes (Loftus et al., 1994), thus failing to clarify metabolic functions of these enzymes. In the current study, a complete collection of disruption mutants containing all possible combinations of the three isozymes has been constructed. New phenotypes contribute to an understanding of the contributions of each activity to production of α-ketoglutarate and glutamate.

In contrast to the eucaryotic isozyme family, a single isocitrate dehydrogenase (ICD, polypeptide M_r = 45 764) is present in *E. coli*. This enzyme is NADP-specific and a homodimer (Burke et al., 1974). While regulated by reversible phosphorylation (Garnak & Reeves, 1979), it is not subject to allosteric control. Thus, the procaryotic and yeast IDP1 and IDP2 enzymes share cofactor specificity and multimeric composition. However, their primary sequences show little relatedness. Instead, the procaryotic enzyme,

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which functions in the tricarboxylic acid cycle, shares approximately 32% primary sequence identity with the yeast IDH subunits, suggesting a possible evolutionary conservation of structure for this metabolic function. In the current study, functional relatedness is tested by expression of the procaryotic enzyme in mitochondrial and cytosolic compartments of yeast isocitrate dehydrogenase disruption mutants.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions. The parental haploid yeast strains were S173-6B (*MATa, leu2*-3,*112, his3-1, ura3-57, trp1-289*; Botstein et al., 1979) and an isogenic strain of opposite mating type. Previously constructed disruption mutants of these strains (Δ*IDH2::HIS3*, Cupp & McAlister-Henn, 1991; and Δ*IDP1::URA3*/Δ*IDP2::URA3*, Loftus et al.,1994) were used for mating, sporulation, and tetrad dissection according to standard techniques (Rose et al., 1990). Yeast strains were cultivated on 2% agar plates or in liquid cultures containing rich YP medium (1% yeast extract, 2% Bacto-peptone) or minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.0) with carbon sources added to 2%. Supplements of 0.1 mg/mL were added to minimal medium to satisfy strain auxotrophies. For growth rate studies, disruption mutants were precultivated in YP glucose medium and transformed strains in YNB glucose medium to maintain plasmid selection. Precultures were diluted into YP or YNB media containing various carbon sources and logarithmic growth rates measured spectrophotometrically at A_{600nm}. Yeast transformations were conducted using the lithium acetate protocol of Ito et al. (1983).

Protein Assays. Whole cell protein extracts were prepared by glass bead lysis of cell pellets as previously described (McAlister-Henn & Thompson, 1987). Organellar and cytosolic cellular fractions were obtained as described by Daum et al. (1982).

NAD- and NADP-specific isocitrate dehydrogenase activities were measured spectrophotometrically as previously described (Keys & McAlister-Henn, 1990; Haselbeck & McAlister-Henn, 1992). The Bradford method (Bradford, 1976) with bovine serum albumin as the standard was used to quantitate protein concentrations. For immunoblot analysis, protein samples were electrophoresed on 10% polyacrylamide−sodium dodecyl sulfate gels and transferred to poly(vinylidine difluoride) filters (Immobilon-P, Millipore). Antisera were used in dilutions of 1:500 to 1:1000 as described by Burnette (1981). Detection was performed using the enhanced chemiluminescent method and autoradiography. Antiserum prepared against native yeast IDH reacts with both subunits of the enzyme (Keys & McAlister-Henn, 1990). Antiserum prepared against purified IDP1 is equally reactive with IDP2 (Loftus et al., 1994). Isozyme-specific antisera against mitochondrial and cytosolic malate dehydrogenases were previously described (McAlister-Henn & Thompson, 1987; Minard & McAlister-Henn, 1991). Antiserum against *E. coli* isocitrate dehydrogenase was obtained from Dr. D. E. Koshland, Jr., Berkeley, CA.

Recombinant DNA Techniques. To construct a gene fusion between yeast *IDP1* and *E. coli icd*, the *E. coli* gene on a 1.9 kbp DNA fragment (Thorsness & Koshland, 1987) was used to replace a 1.1 kbp *Bam*HI/*Sac*I fragment containing

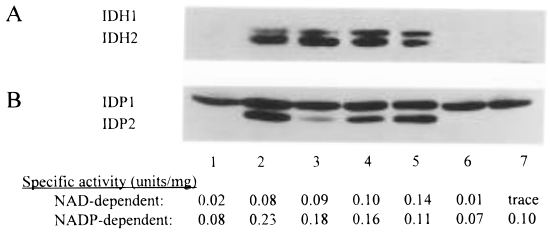

FIGURE 1: Cultivation-dependent expression of yeast isocitrate dehydrogenases. The parental yeast strain was cultivated in rich YP medium with glucose (lane 1), acetate (lane 2), ethanol (lane 3), glycerol (lane 4), or oleate (lane 5) as the carbon source and in minimal YNB medium with glucose in the absence (lane 6) or presence of 0.1 mg/ml glutamate (lane 7). Cells were harvested in logarithmic growth at $A_{600nm} = 0.8-1.0$. Protein extracts were prepared as described under Experimental Procedures for enzyme and protein assays, and 100 μg samples were electrophoresed for immunoblot analysis using antisera against IDH (panel A) or IDP1/IDP2 (panel B). The specific activities shown for each extract represent averages of three independent determinations.

3′ coding and noncoding regions of the cloned *IDP1* gene. A fragment of approximately 750 bp between the translational initiation codons was removed; this deletion also removed several codons from the *IDP1* mitochondrial targeting presequence. The presequence codons were restored, and a remaining region of 350 bp containing the *icd* promoter was simultaneously deleted by oligonucleotide-directed mutagenesis using an 80 bp primer. This aligned the terminal codon for the *IDP1* mitochondrial targeting sequence with the first codon in the *icd* open reading frame. The *IDP1/icd* gene fusion, which contains 340 bp from the 5′ noncoding region of *IDP1*, was confirmed by nucleotide sequence analysis and subcloned into pRS415 (Sikorsky & Hieter, 1989) and YEp351 (Hill et al., 1986), single and multicopy plasmids, respectively, carrying the yeast *LEU2* gene for selection.

To construct an *IDP2/icd* fusion, the 1.9 kbp fragment carrying *icd* was used to replace a 190 bp *Bcl*I/*Eco*RI fragment in the cloned *IDP2* gene. A region of 450 bp was deleted by oligonucleotide-directed mutagenesis using a 54 bp primer to fuse the *IDP2* promoter with the AUG initiation codon of *icd*. The fusion was confirmed by sequence analysis. The *IDP2/icd* fusion, which contains 2.5 kbp of the 5′ noncoding region of *IDP2*, was subcloned into pRS415 and YEp351.

RESULTS

Expression of Isocitrate Dehydrogenase Isozymes. To directly compare relative levels of the three yeast isocitrate dehydrogenases, extracts were prepared from the parental yeast strain grown under various conditions. As shown in Figure 1A, specific activity and immunochemical levels of the IDH1 and IDH2 subunits of the mitochondrial NAD-specific isozyme are approximately equivalent in extracts from cells cultivated with a variety of nonfermentable carbon sources (lanes 2−5). Activity is reduced 5−10-fold by growth on glucose as a carbon source (lanes 1 and 6) and an additional 5-fold on minimal glucose medium supplemented with glutamate (lane 7). Immunochemical levels are similarly reduced and detectable only with exposure times longer than those shown. These and previous experiments (Haselbeck & McAlister-Henn, 1993) suggest that expression of the IDH1 and IDH2 subunits is coordinately regulated

and subject to repression by glucose and by glutamate. Previous work also demonstrated that glucose repression is due to reduced levels of corresponding mRNAs.

Immunochemical levels of the cytosolic NADP-specific isozyme (IDP2) are similarly responsive to carbon source; i.e., the polypeptide is present at similar levels with growth on nonfermentable carbon sources (Figure 1B, lanes 2−5) but is undetectable in extracts from cells grown on rich or minimal medium with glucose (lanes 1, 6, and 7); unlike the IDH subunits, IDP2 is not immunochemically detectable with longer exposure times. In contrast, levels of the mitochondrial NADP-specific polypeptide (IDP1) are similar with all carbon sources and are unresponsive to the presence or absence of glutamate (lanes 1−7). The similar immunochemical levels of IDP1 and IDP2 under nonfermentable conditions and the reduction in cellular activity by approximately 50% when IDP2 is not expressed indicate that cellular levels of IDP1 and IDP2 are approximately equivalent under nonfermentable growth conditions. This is consistent with analysis of disruption mutants described below.

The glucose repression of NAD-specific isocitrate dehydrogenase expression is similar to effects on many mitochondrial functions in yeast. Similarly, repression of cytosolic IDP2 expression suggests that this enzyme's physiological function may also support or require respiratory activity. The absence of glucose repression of IDP1 expression is unusual for a mitochondrial enzyme and suggests a physiological function independent of specific growth conditions.

Construction and Phenotype Analysis of Isocitrate Dehydrogenase Mutants. To assess the metabolic functions of each isozyme of isocitrate dehydrogenase, a complete collection of mutants was constructed by a genetic cross of a haploid strain containing a *HIS3* insertion/disruption of *IDH2* with a strain containing *URA3* insertion/disruptions of both *IDP1* and *IDP2* genomic loci. Since both subunits of NAD-dependent isocitrate dehydrogenase are required for enzymatic activity, the single disruption of *IDH2* was used to simplify genetic analysis. Resulting tetrad progeny were analyzed for His^+ and Ura^+ segregation patterns and by immunoblot analysis to distinguish *IDP1* and *IDP2* disruption strains. The three loci were found to segregate independently in the cross. Haploid strains chosen as representatives of the total mutant collection were cultivated in permissive rich medium with glycerol plus ethanol as carbon sources for immunoblot analysis and enzyme assays. As shown in Figure 2, the collection includes a strain lacking any gene disruption (lane 1), strains containing each of the single gene disruptions (lanes 2−4), strains containing the different pairwise gene disruptions (lanes 5−7), and a strain containing all three gene disruptions (lane 8). Enzyme assays confirm the loss of NAD-dependent isocitrate dehydrogenase activity in strains containing the *IDH2* gene disruption and of NADP-dependent isocitrate dehydrogenase activity in strains containing both *IDP1* and *IDP2* disruptions. Disruption of either *IDP1* or *IDP2* reduces cellular activity approximately 50% under these conditions, again suggesting similar levels of expression of mitochondrial and cytosolic isozymes. These results also show that various disruptions and combinations of disruptions have no dramatic effects on the expression of residual isocitrate dehydrogenase isozymes.

Growth of strains in the isocitrate dehydrogenase mutant collection was assessed under a variety of conditions, using

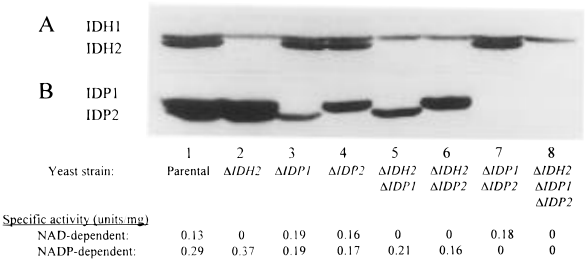

FIGURE 2: Immunoblot analysis of yeast isocitrate dehydrogenase gene disruption mutants. Haploid strains containing different combinations of *IDH2*, *IDP1*, and *IDP2* gene disruptions, as indicated for each lane, were grown in YP medium with glycerol plus ethanol as carbon sources. Cellular protein extracts were prepared, and 100 μg samples were used for immunoblot analysis with antisera against IDH (panel A) or IDP1/IDP2 (panel B). The specific activities shown for each extract represent averages of two or three independent determinations.

Table 1: Growth Phenotypes of Yeast Isocitrate Dehydrogenase Disruption Mutants[a]

	rich (YP) medium			minimal (YNB) medium	
yeast strain	glucose[b]	acetate[c]	ethanol[d]	glucose (with/without glutamate)[e]	ethanol (with/without glutamate)[f]
parental	+	+	+	+/+	+/+
Δ*IDH2*	+	−	+	+/+	+/+
Δ*IDP1*	+	+	+	+/+	+/+
Δ*IDP2*	+	+	+	+/+	+/+
Δ*IDH2*Δ*IDP1*	+	−	+	+/−	+/+
Δ*IDH2*Δ*IDP2*	+	−	+	+/s	+/s
Δ*IDP1*Δ*IDP2*	+	+	+	+/+	+/+
Δ*IDH2*Δ*IDP-1*Δ*IDP2*	+	−	+	+/−	+/−

[a] Culture doubling times during logarithmic growth were determined spectrophotometrically. [b] Culture doubling times of 2−2.5 h are indicated by "+". [c] Culture doubling times of 4−6 h are indicated by "+"; "−" indicates no doubling over a 24 h period. [d] Culture doubling times of 4−5 h are indicated by "+". [e] Culture doubling times of 4−5 h are indicated by "+" and of 7.5−8 h are indicated by "s"; "−" indicates no doubling over a 24 h period. [f] Culture doubling times of 7−7.5 h are indicated by "+" and of 9−9.5 h are indicated by "s"; "−" indicates no doubling over a 24 h period.

plates and cultures with rich medium containing different carbon sources (glucose, glycerol, ethanol, oleate, or acetate) and with minimal medium containing different carbon sources with or without supplements of glutamate. Table 1 shows a summary of growth conditions which produce the most diagnostic phenotypes for the mutant strains. All eight strains in the collection demonstrate similar growth rates on rich medium containing a variety of carbon sources including glucose or ethanol. However, every strain containing the *IDH2* gene disruption fails to grow on rich medium with acetate as the carbon source. This phenotype was previously reported for strains with disruptions of *IDH1* and/or *IDH2* (Cupp & McAlister-Henn, 1991, 1992) and is also characteristic of strains with disruptions in other genes encoding tricarboxylic acid cycle enzymes including malate dehydrogenase (McAlister-Henn & Thompson, 1987) and citrate synthase (Kim et al., 1986). Because this growth defect is displayed on rich medium, it is believed to indicate an energetic rather than an auxotrophic deficiency. Also, disruption of *IDH2* alone does not result in glutamate auxotrophy in minimal medium, suggesting that, under these conditions, production of α-ketoglutarate by the NADP-dependent isozymes is sufficient for normal cellular growth.

Disruption of either or both *IDP1* and *IDP2* genes produces no measurable growth rate reduction (Table 1). However, new growth phenotypes are obtained by various combinations of these disruptions with that of *IDH2*. Codisruption of *IDH2* and *IDP1*, resulting in loss of both mitochondrial activities, produces an inability to grow on minimal glucose medium without glutamate supplements. This double disruption mutant does grow on unsupplemented minimal medium with nonfermentable carbon sources, suggesting that residual IDP2 is sufficient for normal cellular growth under conditions permissive for IDP2 expression (cf. Figure 1). This conclusion is supported by codisruption of all three isocitrate dehydrogenase loci which produces a strain exhibiting glutamate auxotrophy on minimal medium with any carbon source (e.g., glucose and ethanol in Table 1). Codisruption of *IDH2* and *IDP2* results in reduced but measurable growth on all carbon sources in the absence of glutamate, suggesting that the function of residual IDP1 or that mitochondrial localization of this constitutively expressed enzyme results in less than optimal cellular levels of α-ketoglutarate.

The diagnostic growth phenotypes obtained for the collection of isocitrate dehydrogenase disruption mutants should allow definitive tests for *in vivo* function of altered or heterologous enzymes expressed in these strains. Replacement of NAD−isocitrate dehydrogenase activity should restore growth of an Δ*IDH2* (and/or ΔIDH1) disruption strain on rich medium with acetate as the carbon source. Replacement of IDP2 function, assuming similar expression patterns, should restore growth of the triple gene disruption mutant on minimal medium with ethanol but not with glucose as the carbon source. Finally, replacement of IDP1 function, assuming mitochondrial localization and constitutive expression patterns, should partially restore glutamate prototrophy of the triple disruption mutant on minimal medium with any carbon source.

Expression and Function of E. coli ICD in Yeast. We have utilized the yeast mutant collection to test expression and function of *E. coli* isocitrate dehydrogenase (ICD). As described above, the bacterial enzyme is more similar to yeast IDH at the level of primary structure but shares the cofactor specificity of IDP1 and IDP2. To determine the extent of structural/functional relatedness, we constructed gene fusions designed to obtain both mitochondrial and cytosolic localization of ICD in yeast. For mitochondrial localization, the *icd* coding region was fused in-frame with the yeast *IDP1* promoter and mitochondrial targeting sequence as described under Experimental Procedures. The 16-residue presequence was previously shown to be essential for mitochondrial localization of IDP1 (Haselbeck, 1993). For cytosolic localization and appropriate expression, the *icd* coding region retaining its translational initiator methionine codon was fused with the *IDP2* promoter region.

The gene fusions were subcloned into pRS415, a centromere-based yeast expression plasmid containing a *LEU2* gene for selection. The resulting plasmids, designated p*IDP1/icd* and p*IDP2/icd*, were transformed into the yeast triple gene disruption mutant (ΔIDH2ΔIDP1ΔIDP2) lacking all endogenous isocitrate dehydrogenase activity. The transformants were grown with glucose and ethanol as carbon sources to examine levels and patterns of ICD expression (Figure 3). Immunochemical levels of the ICD polypeptide in extracts from p*IDP1/icd* and p*IDP2/icd* transformants

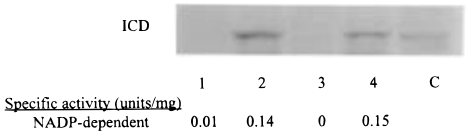

FIGURE 3: Expression of *E. coli* ICD in yeast transformants. Protein extracts were prepared from the yeast Δ*IDH2*Δ*IDP1*Δ*IDP2* disruption mutant following transformation with p*IDP1/icd* (lanes 1 and 2) or p*IDP2/icd* (lanes 3 and 4). Transformants were cultivated in YP medium with glucose (lanes 1 and 3) or ethanol (lanes 2 and 4) as the carbon source. The extracts were used for enzyme and protein assays, and 100 μg samples were electrophoresed for immunoblot analysis using an antiserum against ICD. Specific activities represent averages of two independent determinations. Lane C contained 50 μg of a protein extract from *E. coli* strain DH5αF′ (Raleigh et al., 1988).

grown on ethanol are similar as are cellular levels of activity (lanes 2 and 4). The activities measured for ICD in both extracts are approximately 50% of the activities measured for residual yeast IDP isozymes in corresponding single *IDP1* or *IDP2* disruption mutants grown under similar conditions. Thus, *E. coli* ICD is stable and active when expressed in yeast cells. Similar expression studies conducted with gene fusions on multicopy plasmids gave activities 8−10-fold higher than authentic IDP levels. Therefore, complementation tests described below were conducted with the centromere-based gene fusion plasmids.

In terms of regulated expression, no polypeptide or activity is detected in p*IDP2/icd* transformants grown on glucose (Figure 3, lane 3), indicating expected patterns of repression of the *IDP2* promoter. However, an unexpected difference in expression is the low level of ICD polypeptide, which requires long exposure times for immunodetection, and of activity in extracts from p*IDP1/icd* transformants grown on glucose (lane 1). To determine the basis for this deviation from expected constitutive expression patterns, we tested expression in the triple disruption mutant of authentic yeast *IDP1* using the same pRS plasmid and *IDP1* promoter region. Expression of IDP1 from this construct (p*IDP1*) was found to be equivalent to chromosomal expression with growth on nonfermentable carbon sources but repressed by growth on glucose with activity levels approximately 5−10% of that obtained with chromosomal expression of IDP1. Thus, either chromosomal localization or an additional regulatory element(s) appear(s) to be necessary for constitutive expression. However, the reduced level of activity in the p*IDP1* transformant was found to be sufficient to restore glutamate prototrophy on glucose. Since the levels of activity obtained with p*IDP1* and p*IDP1/icd* in glucose-grown transformants are similar, we assumed that tests of the physiological function of the bacterial enzyme would be valid.

To examine localization of ICD in p*IDP1/icd* and p*IDP2/icd* transformants, the strains were grown with ethanol as the carbon source, and cellular fractionation was conducted to obtain organellar pellets (mitochondria) and soluble cytosolic fractions. As shown in Figure 4 (lanes 1 and 2), ICD in p*IDP1/icd* transformants is distributed in both cytosolic and mitochondrial fractions. The presence of ICD in the cytosolic fraction is not due to excessive mitochondrial lysis during fractionation as seen by comparison with immunochemical levels of the control, mitochondrial malate dehydrogenase (MDH1). Although mitochondrial localization is apparently incomplete, the sizes of ICD polypeptides in both fractions are similar, suggesting that the cytosolic

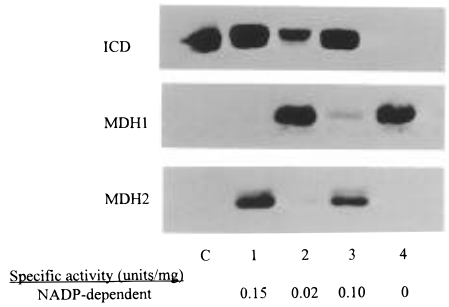

FIGURE 4: Compartmental localization of *E. coli* ICD in yeast transformants. Organellar (lanes 2 and 4, 25 μg) and cytosolic protein fractions (lanes 1 and 3, 100 μg) were prepared as described under Experimental Procedures from yeast disruption mutants transformed with p*IDP1/icd* (lanes 1 and 2) or with p*IDP2/icd* (lanes 3 and 4). Immunoblot analysis was conducted using antisera against ICD and, as fractionation standards, against yeast mitochondrial (MDH1) and cytosolic (MDH2) isozymes of malate dehydrogenase. Specific activities represent averages of two independent determinations. Lane C contained 50 μg of a cellular protein extract from *E. coli* strain DH5αF′.

Table 2: Growth Rates of Yeast *icd* Transformants[a]

yeast strain	doubling time (h)[b] glucose	ethanol
parental	3.9	7.5
Δ*IDH2*Δ*IDP1*Δ*IDP2*	NG[c]	NG
Δ*IDH2*Δ*IDP1*Δ*IDP2* (p*IDP1/icd*)[d]	6.9	10.1
Δ*IDH2*Δ*IDP1*Δ*IDP2* (p*IDP2/icd*)	NG	14.6

[a] Yeast strains were cultivated in minimal YNB medium lacking glutamate with the indicated carbon sources. Culture doubling times were determined spectrophotometrically. [b] Values represent averages of two independent determinations. [c] NG indicates no culture doubling over a 24 h period. [d] Plasmids used for transformation are indicated in parentheses.

form has been accurately processed but is inefficiently imported. Mitochondrial levels of activity in these transformants are approximately 5-fold lower than authentic IDP1 levels, and the rate of conversion of isocitrate to α-ketoglutarate is approximately 2-fold lower than that obtained with authentic IDH. In p*IDP2/icd* transformants, ICD is localized exclusively in the soluble cytosolic fraction (lanes 3 and 4), and levels of activity are approximately 50% of authentic IDP2 levels. Thus, in the absence of a targeting presequence, ICD does not associate with mitochondria.

Complementation of physiological function by *E. coli* ICD was assessed by analyzing growth phenotypes of appropriate disruption mutants transformed with p*IDP1/icd* or p*IDP2/icd*. The results of plate and culture growth tests indicate that transformation of strains containing a genomic disruption of *IDH2* with either plasmid is insufficient to restore growth on rich medium with acetate as the carbon source (data not shown). Thus, despite significant mitochondrial levels of ICD in p*IDP1/icd* transformants, the structurally similar bacterial enzyme does not compensate for loss of IDH. To test for restoration of IDP function, ΔIDH2ΔIDP1ΔIDP2 disruption mutants transformed with p*IDP1/icd* or p*IDP2/icd* were tested on plates and in liquid cultures for glutamate prototrophy with glucose or ethanol as the carbon source. As shown in Table 2, significant restoration of growth with either glucose or ethanol as the carbon source is obtained by transformation with p*IDP1/icd*, whereas transformation with p*IDP2/icd* restores slow growth only on ethanol. These results are compatible with patterns of ICD expression described above and suggest that the bacterial enzyme is a functionally competent NADP-specific isocitrate dehydrogenase in yeast. Similar effects on growth were obtained using strains transformed with multicopy *icd* expression vectors, suggesting that catalytic differences between the yeast and bacterial enzymes, and not differences in levels of expression, may account for the incomplete restoration of glutamate prototrophy.

DISCUSSION

The growth phenotypes associated with various combinations of disruptions of three yeast genes encoding isocitrate dehydrogenases clarify metabolic functions of each isozyme. This activity is essential for production of adequate cellular levels of α-ketoglutarate, since disruption of all three genes produces glutamate auxotrophy. Any one of the isozymes can fulfill this catalytic function, however, since strains containing all pairwise combinations of disruptions are glutamate prototrophs. One caveat is that the growth condition must be conducive for expression of the residual isozyme, explaining the glutamate auxotrophy of a ΔIDH2ΔIDP1 mutant on glucose, a condition which dramatically represses expression of residual cytosolic IDP2. In addition to α-ketoglutarate production, the NAD-specific IDH isozyme has an additional function in delivery of reducing equivalents to the respiratory chain. The absence of a mitochondrial transhydrogenase in yeast (Rydstrom et al., 1976) disallows equilibration of reducing equivalents between NADH and NADPH, providing convincing evidence that mitochondrial NADP-dependent IDP1 cannot compensate for this respiratory function of IDH.

One interesting and inexplicable observation is the absence of a classical "petite" or respiratory-deficient phenotype for the yeast mutant containing disruptions in all three isocitrate dehydrogenase genes. This phenotype, classically manifest as an inability to grow on rich medium with nonfermentable carbon sources, is obtained with defects or disruption of yeast genes encoding many mitochondrial proteins. These include genes for subunits of α-ketoglutarate dehydrogenase (Repetto & Tzagoloff, 1989) or succinate dehydrogenase (Lombardo & Scheffler, 1989), i.e., of unique tricarboxylic acid cycle enzymes which have no counterparts in other cellular compartments. Since strains with disruptions in *IDH1* and/or *IDH2* grow with nonfermentable carbon sources other than acetate, we assumed that the NADP-specific isozymes allow bypass of the IDH reaction under energetic conditions less stringent than growth on acetate and that codisruption of all isocitrate dehydrogenase loci would eliminate this bypass. This assumption is shown to be incorrect in the current study and in similar studies of related isozyme families in yeast. For example, the acetate$^-$ growth phenotype is also obtained by disruption of the gene for the tricarboxylic acid cycle isozyme of malate dehydrogenase (McAlister-Henn & Thompson, 1987), and codisruption of genes for two other compartmentalized isozymes does not result in generalized respiratory deficiency (Steffan & McAlister-Henn, 1992). An understanding of catalytic alternatives will likely require construction of synthetic lethal mutants by additional disruption of genes for metabolically related enzymes.

In addition to providing insight about metabolic function, the yeast mutant collection provides a system for testing the function *in vivo* of altered yeast or of heterologous isocitrate

dehydrogenases. We are using these mutant strains to express altered forms of yeast and mammalian enzymes to test the roles of compartmentation and of conserved residues in catalytic function. The current study demonstrates that *E. coli* isocitrate dehydrogenase can be stably expressed in yeast and that the bacterial enzyme can partially compensate for loss of IDP1 and IDP2 function but not for loss of IDH. Our results do not rule out some evolutionary conservation of structure in ICD and IDH for optimized function in the tricarboxylic acid cycle, but they do indicate that the cofactor specificity of the bacterial enzyme may prevent function in the yeast tricarboxylic acid cycle. Our results also show that mitochondrial localization of the procaryotic enzyme requires addition of a targeting presequence. The observed incomplete localization of an apparently mature bacterial polypeptide in p*IDP1/icd* transformants suggests that processing of the precursor is efficient but that translocation is not. A possible explanation for this observation is suggested by recent studies of yeast fumarase (Stein et al., 1994). Partitioning of cytosolic and mitochondrial forms of this enzyme reportedly involves partial translocation and precursor cleavage followed by import of some molecules and cytosolic retention and folding of others.

The glucose repression observed with p*IDP1* and p*IDP1/icd* fusion plasmids in these experiments is of significant interest because the chromosomal *IDP1* gene is refractory to this type of control (cf. Figure 1). A *Cla*I site 340 bp upstream of the translational initiation codon of *IDP1* was used as the boundary for plasmid constructs because of the proximity of a second gene, *COX9*, which encodes subunit VIIa of cytochrome *c* oxidase (Wright et al., 1986). The AUG initiation codon for the divergently transcribed *COX9* gene is located 440 bp upstream of the *IDP1* AUG codon. The *Cla*I site was a convenient choice to eliminate the *COX9* open reading frame but to retain sufficient intragenic region presumed to contain the *IDP1* promoter. Loss of constitutive expression patterns with this subclone of *IDP1* suggests that normal promoter elements may extend into the remaining 100 bp intragenic region or even into the *COX9* coding sequence. Alternatively, transcription of *COX9*, which is subject to glucose repression, may affect chromosomal *IDP1* expression. These possibilities will be examined with various truncations of the *COX9* gene. Interestingly, even with glucose-repressed levels of IDP1 or of ICD expressed from this plasmid construct, reversion of glutamate auxotrophy was achieved, suggesting that normal levels of IDP1 are in excess for this metabolic function.

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