Sources of NADPH and Expression of Mammalian NADP$^+$-specific Isocitrate Dehydrogenases in *Saccharomyces cerevisiae*  

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To compare roles of specific enzymes in supply of NADPH for cellular biosynthesis, collections of yeast mutants were constructed by gene disruptions and matings. These mutants include haploid strains containing all possible combinations of deletions in yeast genes encoding three differentially compartmentalized isozymes of NADP$^+$-specific isocitrate dehydrogenase and in the gene encoding glucose-6-phosphate dehydrogenase (Zwf1p). Growth phenotype analyses of the mutants indicate that either cytosolic NADP$^+$-specific isocitrate dehydrogenase (Idp2p) or the hexas monophosphate shunt is essential for growth with fatty acids as carbon sources and for sporulation of diploid strains, a condition associated with high levels of fatty acid synthesis. No new biosynthetic roles were identified for mitochondrial (Idp1p) or peroxisomal (Idp3p) NADP$^+$-specific isocitrate dehydrogenase isozymes. These and other results suggest that several major presumed sources of biosynthetic reducing equivalents are non-essential in yeast cells grown under many cultivation conditions. To develop an in vivo system for analysis of metabolic function, mammalian mitochondrial and cytosolic isozymes of NADP$^+$-specific isocitrate dehydrogenase were expressed in yeast using promoters from the cognate yeast genes. The mammalian mitochondrial isozyme was found to be imported efficiently into yeast mitochondria when fused to the Idp1p targeting sequence and to substitute functionally for Idp1p for production of α-ketoglutarate. The mammalian cytosolic isozyme was found to partition between cytosolic and organellar compartments and to replace functionally Idp2p for production of α-ketoglutarate or for growth on fatty acids in a mutant lacking Zwflp. The mammalian cytosolic isozyme also functionally substitutes for Idp3p allowing growth on petroselinic acid as a carbon source, suggesting partial localization to peroxisomes and provision of NADPH for β-oxidation of that fatty acid.

Reducing equivalents in the form of NADPH are required by many enzymes in central biosynthetic pathways, whereas the enzymatic sources of biosynthetic reducing equivalents are believed to be limited in number. The oxidative branch of the hexose monophosphate shunt or pentose phosphate pathway is generally accepted to be the major cellular source of NADPH. However, disruption of the *ZWF1* gene encoding glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme in the oxidative branch, was not found to produce significant defects in biosynthetic capacity in haploid strains of *Saccharomyces cerevisiae*. Instead, this disruption produces discrete phenotypes including a requirement for organic sulfur, e.g. methionine or cysteine (1), and an enhanced sensitivity to oxidizing agents including hydrogen peroxide and diamide (2). Since a yeast disruption mutant lacking cytosolic superoxide dismutase (Sod1p) is also a methionine auxotroph, these phenotypes have been interpreted as an impairment of redox status during aerobic stress (3). In addition, an earlier study of a zwf1 homozygous diploid mutant indicated that this enzyme is not essential for the significant increase in fatty acid biosynthesis that occurs during sporulation (4). Thus the contribution of Zwflp to the supply of NADPH for biosynthetic pathways is unclear.

A second major source of biosynthetic reducing equivalents is proposed to be NADP$^+$-specific isocitrate dehydrogenase (5, 6). In yeast, there are three highly homologous (>70% residue identity) but differentially compartmentalized isozymes as follows: mitochondrial Idp1p (7), cytosolic Idp2p (8), and peroxisomal Idp3p (9, 10). These isozymes are structurally and functionally distinct from the mitochondrial NAD$^+$-specific isocitrate dehydrogenase that functions in the tricarboxylic acid cycle (11). Gene disruption analyses have shown that loss of either or both of the major NADP$^+$-specific isozymes, Idp1p and Idp2p, produces no observable growth phenotype (12), whereas loss of Idp3p reduces or eliminates growth on unsaturated fatty acids indicative of a role for this enzyme as a compartmental source of NADPH for specific steps of β-oxidation (9, 10).

Another study has shown that either Idp1p or Idp2p is essential and sufficient for production of α-ketoglutarate to support cellular synthesis of glutamate in the absence of the mitochondrial NAD$^+$-specific enzyme (12). The latter study was conducted following construction of a collection of yeast mutants containing all possible combinations of disruptions of genes for the isozymes in question. We initiate the current analysis of major potential sources of NADPH with a similar approach to compare contributions of Zwflp and the NADP$^+$-specific isozymes of isocitrate dehydrogenase.

Specific growth phenotypes associated with loss of each yeast isozyme of NADP$^+$-specific isocitrate dehydrogenase are defined in this study. Mutant strains displaying these phenotypes provide tests for in vivo function of altered or heterologous forms of the isozymes. We have used these tests to evaluate expression and function of highly homologous mitochondrial and cytosolic isozymes of mammalian NADP$^+$-specific isocitrate dehydrogenase in yeast cells.

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Sources of NADPH in Yeast

1) EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Yeast strains used in this study were the parental haploid strain S173-6B (MATa leu2-3, 112 his3-1 ura3-52 trp1-289, 13) and previously constructed mutants of this strain containing deletions and URA3 insertions in the IDP1 and/or IDP2 loci (7, 8). An isogenic haploid strain (MATa leu2-3, 112 ura3-52 trp1-289 ade2-101) was used for matings. Matings, sporulation, and tetrad analysis were conducted as described by Rose et al. (24). Yeast strains were cultivated in rich YP medium (1% yeast extract, 2% Bactopeptone) or in minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.0) with appropriate supplements of 20 μg/ml to satisfy auxotrophic requirements for growth. Carbon sources were glucose, glycerol plus lactate, ethanol, or acetate added to 2%. Fatty acids used as carbon sources were added to 0.1% with 0.25% ethanol. Growth rate analyses were conducted by monitoring colony growth on agar plates and by spectrophotometric measurements (Aoo_{600}) of doubling times in liquid medium.

Recombinant DNA Methods—Chromosome and locus designations for S. cerevisiae genes analyzed in this study are as follows: IDP1 (IV, YDL066w), IDP2 (XII, YLR174w), IDP3 (XIV, YNL009w), and ZWF1 (XIV, YNL241c). DNA manipulations including ligation, amplification, purification, and sequence analysis followed methods described by Sambrook et al. (15). Oligonucleotide-directed mutagenesis was conducted using the Transformer Site-directed Mutagenesis system of CLONTech.

Isolation of yeast genomic DNA and RNA for Southern and Northern blot analyses followed procedures described in Rose et al. (14). DNA restriction fragments used as probes were radiolabeled using the random primer method (16).

The yeast ZWF1 gene was disrupted as described by Nogae and Johnston (2) using a DNA fragment containing 0.58 kb of the coding region generated by polymerase chain reaction (PCR) and a URA3 gene insertion within the coding region. Plasmids containing the ZWF1 gene and the disruption construct were provided by Dr. Mark Johnston. For construction of diploid strains, the ZWF1 gene was also disrupted by transformation with a DNA fragment containing the selectable kanMX4 gene flanked by 5' and 3'-coding sequences of ZWF1. The fragment was constructed by PCR using primers homologous to ZWF1/kanMX4 sequences (5'-CTTCAATCCCTTTGGACCTCTTGATCCGTAGGATCGATGAATTCG and 3'-TTCCTCCGTCAGCCGATTCGAGCGCGGTGGAAC-3') and plasmid pFA6a-kanMX4 (17) as the template. The 5'-specific isocitrate dehydrogenase and for glucose-6-phosphate dehydrogenase, was conducted using a PCR fragment containing a URA3 insertion in the coding region as described by Nogae and Johnston (2) (Fig. 1A). A haploid strain containing IDP2 and ZWF1 disruptions was obtained by mating of strains containing each disruption and sporulation. This strain was subsequently mated with a haploid strain containing an IDP1 gene disruption. Following sporulation and tetrad dissection, the segregation of gene disruptions in haploid strains from this cross was analyzed to identify a collection of strains containing the desired combinations of disruptions. As illustrated in Fig. 1B, strains lacking Idp1p and/or Idp2p were identified by Western blot analysis and NADP+-specific isocitrate dehydrogenase enzyme assays of whole cell protein extracts. Strains lacking Zwf1p were identified by glucose-6-phosphate dehydrogenase assays (Fig. 1B), and the ZWF1 gene disruption was confirmed by Southern blot analysis (Fig. 1C). The resulting yeast mutant collection is comprised of a parental segregant (lanes 1), three strains containing single gene disruptions (lanes 2–4), three strains containing all possible combinations of two gene disruptions (lanes 5–7), and a strain containing all three gene disruptions (lanes 8).

Growth of strains in the IDP1/IDP2/ZWF1 mutant collection was examined using a variety of common cultivation conditions and conditions previously reported to be diagnostic of ZWF1 disruption (1, 2). No significant differences in growth on agar plates or in liquid cultures were observed using rich YP medium with glucose, glycerol/lactate, acetate, or ethanol as carbon sources (data not shown). Growth of the triple ΔIDP1/ΔIDP2ΔZWF1 mutant strain under these conditions did not satisfy the absolute requirement for any of the three enzyme activities. Growth phenotypes previously attributed to disruption of ZWF1 are reproducible in this collection (Table 1). Specifically, all strains containing ΔZWF1 were found to be unable to grow with glucose as the carbon source on minimal medium in the absence of methionine as described by Thomas et al. (1) or on rich medium in the presence of 0.5 mM H2O2 as described by Nogae and Johnston (2). In these initial tests of the mutant collection, the only novel growth phenotype identified, a defi-
Fig. 1. Construction of a collection of yeast mutants with disruptions in IDP1, IDP2, and ZWF1 genes. A, the yeast ZWF1 gene was disrupted by transformation with a 581-bp PCR fragment of the coding region (solid line) containing a 1.1-kbp URA3 gene insertion in the XmnI restriction site as described by Nogae and Johnston (2). Gene fragments used for kanMX4 disruption and as a radiolabeled probe are also indicated. B, a collection of yeast haploid mutants containing all possible combinations of IDP1, IDP2, and ZWF1 gene disruptions was constructed by mating, sporulation, and tetrad dissection. Strains lacking Idp1p and/or Idp2p were identified as described under “Experimental Procedures” by immunoblot analysis (100 µg of protein/lane) and enzyme activity assays of protein extracts prepared from cells grown on YP medium with glycerol/lactate as the carbon source. Lanes a and b were loaded with 0.5 µg of purified Idp1p and Idp2p, respectively. Strains lacking Zwf1p were identified by enzyme assays (B), and the ZWF1 gene disruption was confirmed by Southern hybridization (C). Lanes and strain designations in C correspond to those in B. Disruption of the ZWF1 locus results in an increase in size of a 1.1-kbp BglII genomic DNA fragment (C, lanes 1–3 and 5) to 2.2 kbp (lanes 4 and 6–8) due to insertion of the URA3 gene as detected with a radiolabeled BglII fragment from the cloned ZWF1 gene.

In addition to vegetative growth, it is speculated that cytosolic sources of NADPH are essential for extensive fatty acid synthesis that occurs during sporulation (30). We therefore constructed a series of homozygous diploid strains with disruptions in IDP2 and/or ZWF1. To simplify selection, PCR was used to generate a DNA fragment containing a selectable kanMX4 gene (17) with flanking regions homologous to 5′ and 3′ regions of the ZWF1-coding region (Fig. 1A). The fragment was used for transformation and ZWF1 disruption in α and α haploid parental strains and in α and α haploid strains containing disruptions of IDP2. Matings were conducted as indicated in Table II, and diploids were selected on plates with minimal medium containing glutamate and methionine to ensure growth of ΔIDP2 and ΔZWF1 homozygous diploids, respectively. Numbers of diploid colonies obtained from the parental cross, the ΔIDP2 cross, the ΔZWF1 cross, and the ΔIDP2ΔZWF1 cross were approximately equivalent. However, in both crosses containing ΔZWF1 haploids, growth of diploids was slow, with colonies achieving the size of parental diploid colonies after a 2–3-day lag.

Sporulation of the homozygous diploid strains reveals an additional phenotype associated with loss of both Idp2p and Zwf1p (Table II). Sporulation of the single disruption strains (ΔIDP2/ΔIDP2 or ΔZWF1/ΔZWF1) was found to occur with an efficiency equivalent to the parental diploid (20–25% after 3 days), but no evidence of sporulation is detected for the double disruption diploid (ΔIDP2ΔZWF1/ΔIDP2ΔZWF1) after extended periods of incubation. This phenotype suggests that Idp2p and Zwf1p may have overlapping or complementary...
function in the process of sporulation, perhaps in provision of reducing equivalents for fatty acid synthesis. Other possible biochemical defects are discussed below.

**Peroxisomal Idp3**—Co-disruption of **IDP1** and **IDP2** genes in a haploid yeast strain eliminates NADPH-specific isocitrate dehydrogenase activity in cells cultivated with a variety of carbon sources (8). Thus, the existence of an additional isozyme was missed until completion of the genome sequence analysis, which revealed an open reading frame (YNL009w, designated **IDP3**) with extensive homology to **IDP1**- and **IDP2**-coding regions (31). We and others (9, 10) have found that **IDP3** encodes a peroxisomal isozyme and that organelar localization is dependent on a peroxisomal type I targeting signal (32), in this case a carboxyl-terminal Cys-Lys-Leu tripeptide. Expression of **IDP3** appears to be limited to growth with fatty acids as a carbon source (9, 10), a condition that induces peroxisomal proliferation and metabolism in yeast (33). Henke et al. (9) have estimated that Idp3p contributes approximately 15% of the total cellular NADPH-specific isocitrate dehydrogenase activity in cells grown with oleate as the carbon source.

To compare directly relative expression of the three genes encoding yeast **IDP** isozymes, Northern blot analysis was conducted using RNA isolated from the parental strain grown in rich medium with a variety of carbon sources. As summarized in Table I and as previously reported (2, 34), expression of **IDP1** and **ZWF1** mRNAs is essentially constitutive, whereas **IDP2** expression is subject to glucose repression. Under the conditions tested, **IDP3** mRNA is detected only in cells grown with oleate as a carbon source, a condition also producing an increase in **IDP2** mRNA to levels approximately 3.5-fold higher than those detected with other non-fermentable carbon sources.

To examine metabolic contributions of Idp3p, we disrupted the **IDP3** gene (Fig. 2) in each of the strains in the collection listed in Table I. This was accomplished and verified as described under “Experimental Procedures.” Growth of the mutant strains was examined using conditions described for the original collection. Disruption of **IDP3** alone or in combination with other disruptions was found to have no effect on growth of the transformed strains under conditions including use of glucose, glycerol, lactate, ethanol, or acetate as the carbon source. These results are consistent with the absence of expression of **IDP3** under these conditions (Table III).

Disruption of **IDP3** was found by Henke et al. (9) and van Roermund et al. (10) to have no effect with growth on stearate and little effect on growth with oleate but to dramatically reduce growth with petroselinate as the carbon source. Reduc-

### Table II

| Table II | Effects of **IDP2** and **ZWF1** gene disruptions on mating and sporulation |
|----------------|----------------------------------------------------------------------------------|
| Mating | Diplod genotype | Sporulation |
| **ΔZWF1** x **ΔZWF1** | Parental/parental | + |
| **ΔIDP2** x **ΔIDP2** | **ΔIDP2**/**ΔIDP2** | + |
| **ΔZWF1**IDP2 x **ΔZWF1**IDP2 | **ΔZWF1**IDP2 | + |

* Mating of a x a haploid strains was monitored as colony growth on minimal medium agar plates lacking auxotrophic markers for each strain (adenine and histidine) but containing glutamate and methionine to supplement auxotrophies due to **IDP2** or **ZWF1** gene disruption.

### Table III

| Table III | Relative levels of gene expression |
|----------------|----------------------------------------------------------------------------------|
| RNA | Carbon source |
| **IDP1** | Glucose | 0.54 | 0.33 | 0.43 | 0.45 | 0.43 |
| **IDP2** | Glyceraldehyde | 1.33 | 1.63 | 1.69 | 1.66 | 5.52 |
| **IDP3** | Acetate | 0.54 | 0.33 | 0.43 | 0.45 | 0.43 |
| **ZWF1** | Ethanol | 0.67 | 1.07 | 1.19 | 1.35 | 0.70 |

* RNAs for corresponding genes were detected using radiolabeled gene fragments as follows: a 1.2-kbp XbaI/BglII fragment from **IDP1** (7), a 1.8-kbp SalI/PstI fragment from **IDP2** (8), a 1.5-kbp PetI/EcoRV fragment from **IDP3** (Fig. 2), and a 1.1-kbp BglII fragment from **ZWF1** (Fig. 1A).

**FIG. 2. Partial restriction map of the S. cerevisiae **IDP3** gene.** A yeast DNA fragment containing the **IDP3**-coding region (solid line) and promoter was subcloned by PCR using genomic DNA as a template. Also indicated are boundaries of a DNA fragment used for gene disruption as described under “Experimental Procedures,” and the gene fragment was used as a radiolabeled probe for blot analyses.

Expression of **Mammalian NADPH**-specific Isocitrate Dehydrogenase in Yeast—In the course of analysis of these and other yeast isocitrate dehydrogenase mutants, distinctive phenotypes associated with the absence of each **IDP**-specific isozyme have been identified. Growth on petroselinate requires the peroxisomal enzyme; growth on oleate in a **ΔZWF1** mutant requires the cytosolic enzyme, and growth on minimal medium with glucose in a mutant lacking the mitochondrial **NADP**-specific enzyme has been reported (12). Thus, restoration of growth under these specific conditions should be an appropriate assay for in vivo function of altered or heterologous forms of the isozymes. We have used these mutants and conditions to express and test function of mammalian mitochondrial and cytosolic **NADP**-specific isozymes, respectively, designated mamIdp1p and mamIdp2p, in yeast.

Constructs for expression of the mammalian isozymes illus-

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*Sources of NADPH in Yeast* 31489
treated in Fig. 3 were designed to utilize promoters from corresponding yeast genes. As described under “Experimental Procedures,” oligonucleotide-directed mutagenesis was used to fuse the yeast IDP1 promoter, initiator methionine codon, and mitochondrial targeting sequence (16 codons) in frame with the codon for the first residue of the mature pig mamIdp1p enzyme (20). For expression of the cytosolic enzyme, the yeast IDP2 promoter and initiator methionine codon were fused in frame with the codon for the first residue of the mature rat mamIdp2p enzyme (23).

The two fusion constructs were subcloned into centromere-based plasmids for single copy expression and into 2-μm plasmids for multicopy expression, and the resulting set of four plasmids was transformed into a yeast strain containing chromosomal disruptions of IDP1 and IDP2. Very low levels of activity and immunoreactivity were detectable in yeast cells transformed with the centromere plasmids (data not shown).

### Table IV

| Relevant genotype | Carbon source<sup>a</sup> | Spermid acid | Oleic acid | Petroselinic acid |
|------------------|--------------------------|--------------|------------|-----------------|
| Parental         | +                        | +            | +          |                 |
| ΔIDP2            | +                        | +            | +          |                 |
| ΔIDP3            | +                        | +            | +          |                 |
| ΔZWF1            | +                        | +            | +          |                 |
| ΔIDP2ΔIDP3       | +                        | +            | +          |                 |
| ΔIDP3ΔZWF1       | +                        | +            | +          |                 |
| ΔIDP2ΔIDP3ΔZWF1  | -                        | +            | +          | +               |

<sup>a</sup> Yeast strains were cultivated on YP agar plates or in liquid medium at 30 °C. + indicates significant colony growth on plates within 7 days and culture doubling times of 6–9 h; − indicates little or no growth on plates after 14 days of incubation or after 3 days in liquid medium.

suggesting either low levels of expression or instability of the expressed proteins. Thus, all subsequent experiments were conducted with various yeast mutant strains transformed with the 2-μm plasmids, designated pmamIDP1 or pmamIDP2. Immunoblot analyses indicate that expression of pmamIDP1 in yeast is constitutive, whereas expression of pmamIDP2 is glucose-repressed (data not shown), the expected patterns of expression with use of corresponding yeast IDP1 and IDP2 promoters (34) (Table III).

Levels of expression, activity, and localization were examined using subcellular fractions from a ΔIDP1 strain transformed with pmamIDP1 and a ΔIDP2 strain transformed with pmamIDP2 (Fig. 4). Expression of mamIdp1p was found to increase cellular specific activity ~2.5-fold due to an increase in activity associated with organellar pellets (mitochondria and peroxisomes). Immunoblot analysis also indicates organellar localization and, since the size of the expressed protein correlates with that of the pure enzyme, suggests efficient mitochondrial localization and processing. Localization was also verified by immunoblot analysis of mitochondrial fractions following Nycodein gradient centrifugation of organellar pellets (data not shown).

Expression of mamIdp2p (Fig. 4) in a ΔIDP2 strain was found to increase cellular specific activity ~3.5-fold with most of the activity associated with the soluble "cytosolic" fraction. However, immunoreactive mamIdp2p and a slight increase in specific activity were detected in organellar pellet fractions. The mammalian enzyme contains an Ala-Lys-Leu tripeptide at its carboxyl terminus (23). This is potentially a type I peroxisomal targeting sequence (32), and there is at least one report of low levels of NADP⁺-specific isocitrate dehydrogenase activity associated with peroxisomes in mammalian cells (35). We

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**Fig. 3.** Gene fusions for expression of mammalian NADP⁺-specific isocitrate dehydrogenases in yeast. The coding region for pig mitochondrial NADP⁺-specific isocitrate dehydrogenase (A, solid line) contained on a 1.6-kbp cDNA fragment was fused in frame with codons for the mitochondrial targeting sequence in the yeast IDP1 gene. The coding region for rat cytosolic NADP⁺-specific isocitrate dehydrogenase (B, solid line) contained on a 1.7-kbp cDNA fragment was fused in frame with the initiator methionine codon of the yeast IDP2 gene.

**Fig. 4.** Expression of mammalian NADP⁺-specific isocitrate dehydrogenases in yeast transformants. Protein extracts following cellular fractionation of a yeast ΔIDP1 strain transformed with pmamIDP1 and a ΔIDP2 strain transformed with pmamIDP2 were used for enzyme assays and immunoblot analysis. The pmamIDP1 transformant was grown with glucose as the carbon source to repress expression of Idp1p and Idp3p; immunoblot analysis (100 μg of whole cell and supernatant fractions, 50 μg of pellet fraction, and 2.5 μg of pure mamIdp1p) was conducted with an antiserum prepared against yeast Idp1p which is cross-reactive with the mammalian enzyme. Specific activities are expressed relative to values measured for the parental strain (0.08 units/mg for whole cell, 0.01 units/mg for supernatant, and 0.84 units/mg for organellar fractions) grown under the same condition. The pmamIDP2 transformant was grown with glycerol/ethanol as the carbon source, and immunoblot analysis (50 μg of cellular fractions and 1 μg of pure mamIdp2p) was conducted with an antiserum specific for mamIdp2p (29). Specific activities are expressed relative to those measured for cellular fractions from the parental strain (0.16 units/mg for whole cell, 0.08 units/mg for supernatant, and 1.47 units/mg for organellar fractions) grown under the same conditions.
abolish glutamate rates obtained with yeast Idp1p, sug-


gest that the mammalian enzyme functions efficiently in production of α-ketoglutarate in yeast mitochondria. In the same ΔIDP1ΔIDP2ΔIDH2 mutant, restoration of yeast Idp2p, a glucose-repressed enzyme, permits growth with ethanol but not with glucose in the absence of glutamate (12). Expression of mamIdp2p (with or without the carboxyl-terminal Ala-Lys-Leu tripeptide), which is also glucose-repressed, in the triple dis-

ruptant mutant, restores growth in the absence of glutamate with ethanol but not with glucose as the carbon source (Table V, top). Thus, partial cytosolic distribution of mamIdp2p can compensate for function of the cytosolic yeast enzyme in production of α-ketoglutarate.

Another test for a functional cytosolic NADP⁺-specific isozyme is restoration of growth with fatty acids as a carbon source in a strain containing chromosomal disruptions of IDP2 and ZWF1 genes. As illustrated in Table V, bottom, expression of mamIdp2p (Δ-Ala-Lys-Leu carboxyl terminus) in this mutant restores growth on plates with oleate, stearate, or petroselinate as the carbon source. Finally, since mamIdp2p appears to be partially distributed to organelar pellets in yeast transformants, the ability of this enzyme to restore growth of a ΔIDP3 strain with petroselinate was tested. The positive result shown in Table V, bottom, suggests that mamIdp2p, due to localization in both cytosolic and peroxisomal compartments in yeast transformants, can functionally replace both Idp2p and Idp3p. Thus, various yeast mutant strains appear to provide a reliable in vivo test system for metabolic function of these heterologous enzymes.

**DISCUSSION**

Gene disruption analyses were used to investigate relative contributions of the hexose monophosphate pathway and reactions catalyzed by NADP⁺-specific isocitrate dehydrogenases to vegetative growth and sporulation in *S. cerevisiae*. Phenotype analyses indicate that either cytosolic glucose-6-phosphate dehydrogenase (Zwf1p) or cytosolic NADP⁺-specific isocitrate dehydrogenase (Idp2p) is essential for vegetative growth with a fatty acid as carbon source and for sporulation. Since these analyses were conducted in the presence of potential auxotrophic requirements (methionine and glutamate) that might result from corresponding gene disruptions, the assumption is that the fatty acid and asporulation phenotypes of ΔIDP2ΔZWF1 mutants are results of deficits in cytosolic pools of NADPH. This assumption regarding the asporulation phenotype is supported by 13C NMR studies by Dickinson et al. (30) that indicated significant conversion of [2-13C]acetate to glutamate during the first 4 h of sporulation, followed by significant cycling of glucose through the hexose monophosphate pathway and large scale synthesis of saturated fatty acids. These events would be facilitated by both Idp2p and Zwf1p activities. Dickinson and Hewlins (4) also used 13C NMR to show that fatty acid synthesis during sporulation is reduced in a zwf1 homoz-


gous diploid to approximately 25% that in a parental strain. They suggested that the residual fatty acid synthesis, which does permit sporulation, might be dependent on production of NADPH by an NADP⁺-specific isocitrate dehydrogenase. Our results indicate that cytosolic Idp2p may be the source of these biosynthetic reducing equivalents.

The metabolic basis for the vegetative fatty acid growth phenotype of ΔIDP2ΔZWF1 mutants is less clear since these mutants grow well with acetate as a carbon source. Among several possibilities is that the absence of Idp2p and Zwf1p is detrimental to β-oxidation, although there is no obvious requirement for NADPH for utilization of a saturated fatty acid, and peroxisomal Idp3p is available to provide NADPH for utilization of petroselinate or similar unsaturated fatty acids. The ability of ΔIDP2ΔZWF1 mutants to grow with acetate

| Relevant genotype (plasmid) | YP medium | YNB glucose | YNB ethanol |
|-----------------------------|------------|-------------|-------------|
| Parental                    | +          | +           | +           |
| ΔIDP1ΔIDP2ΔIDH2             | +          | +           | +           |
| ΔIDP1ΔIDP2ΔIDH2 (pnamIDP2)  | +          | +           | +           |
| ΔIDP1ΔIDP2ΔIDH2 (pnamIDP2 Δ ALK) | +       | +           | +           |

* Plasmids were used for transformation of the disruption mutant strain for expression of mamIdp1p or of mamIdp2p (with or without the carboxyl-terminal Ala-Lys-Leu tripeptide).

* Growth was monitored on minimal medium agar plates or in liquid medium. + indicates significant growth on plates after 5–6 days at 30 °C or 18 h culture doubling times; – indicates little or no growth after 10–12 days of incubation on plates or after 18 h in liquid medium.

* Growth with fatty acid carbon sources was monitored as described in Table IV.

Therefore used immunoblot analysis of cellular fractions and densitometry to compare distribution of yeast peroxisomal malate dehydrogenase (Mdh3p, 25). With the cellular fractions from the pnamIDP2 transformant shown in Fig. 4, approxi-

mately 50% of immunoreactive Mdh3p is associated with organe-

lärr fractions with the remainder of the polypeptide found in supernatant fractions (data not shown), presumably due to lysis of fragile peroxisomes. After correction for this breakage, it is estimated that approximately 20% of total immunoreactive mamIdp2p is found in organelar fractions. This distribution suggests an incomplete but significant association with the organelar pellet. Also, although the increase in organelar specific activity attributed to mamIdp2p is low relative to cyto-

solic levels, it is similar to organelar levels contributed by authentic yeast Idp3p under the same growth condition.

Attempts to isolate peroxisomes from strains expressing mamIdp2p using Nycodenz gradients have been unsuccessful presumably due instability of the protein during centrifuga-

tion. We also investigated the role of the Ala-Lys-Leu tripeptide by deletion of the corresponding codons in pnamIDP2. Expres-

sion of the truncated coding region was found to have no effect on levels of activity or on the distribution of immunoreactive mamIdp2p between soluble and organelar pellet fractions (data not shown). Thus, the carboxyl-terminal tripeptide of mamIdp2p does not appear to be a determinant for association of the protein with organelles in yeast.

The robust expression obtained by transformation with pnamIDP1 and pnamIDP2 permits assessment of metabolic function. The plasmids were transformed into various yeast mutant strains that demonstrate definitive growth phenotypes associated with loss of isocitrate dehydrogenase isozymes. As illustrated in Table V, top, a yeast strain containing disruptions of ΔIDP1, ΔIDP2, and ΔIDH2 (which eliminates mitochondri-

al NAD⁺-specific isocitrate dehydrogenase activity, see Ref. 11) is a glutamate auxotroph with both fermentable and non-

fermentable carbon sources. Restoration of yeast Idp1p, a con-

stitutively expressed enzyme, in this strain restores glutamate prototrophy with both types of carbon sources, e.g., glucose and ethanol, respectively (12). Expression of mamIdp1p in this strain (Table V, top) was found to restore rates of growth in the absence of glutamate to rates obtained with yeast Idp1p, sug-
suggests that fatty acid synthesis is not impaired and that alternative metabolic sources of NADPH are available to support vegetative growth with acetate but not with a fatty acid as the carbon source. The possibility of differential expression of a gene(s) encoding an alternative enzymatic source of NADPH under these conditions is considered below. Another discrepancy would appear to be the vegetative growth of ΔIDP2ΔZWF1 mutants with acetate versus the asporulation phenotype with acetate under conditions of nitrogen starvation. However, Dickinson et al. (30) have noted significant differences in [2,14C]acetate utilization under these conditions; in contrast to sporulation patterns described above, there is little flux through the hexose monophosphate pathway and minimal utilization of acetate for glutamate or fatty acid synthesis in cells growing vegetatively on rich medium with acetate as the carbon source.

The limited number of cultivation conditions that result in reduced growth of strains containing disruptions of IDP2 and ZWF1 genes, and of IDP1 and IDP3 genes, suggests that other enzymatic sources or shuttle cycles must exist for provision of NADPH for growth on most carbon sources. We investigated two other possible sources, malic enzyme and the glutamate dehydrogenase isozyme system. Identification of an S. cerevisiae gene (MAE1) encoding malic enzyme was recently reported (36). We cloned, disrupted, and overexpressed this gene. Disruption was found to reduce total cellular activity, i.e. malate-dependent reduction of NADP+, to less than 30% that detected in extracts from the parental strain, and overexpression results in an approximate 9-fold increase in cellular malic enzyme activity. Expression of the corresponding mRNA was found to be similar with most carbon sources but reduced approximately 7-fold in cells grown with oleate as the carbon source. This is the pattern of expression that might be associated with an alternative enzymatic source of NADPH to explain the fatty acid phenotype of ΔIDP2ΔZWF1 disruption mutants. However, disruption of MAE1 alone was found to produce no discernible growth phenotype under any condition tested and, in combinations with other gene disruptions listed in Table I, produces no dramatic differences in growth phenotypes. In addition, cell fractionation studies indicate that malic enzyme activity is localized to mitochondria in S. cerevisiae. Collectively, these results suggest that yeast malic enzyme is unlikely to significantly contribute to cytosolic pools of NADPH.

To investigate possible contributions of NADH/NADPH interconversion catalyzed by glutamate dehydrogenases with different cofactor specificities, we disrupted the GDH1 gene (37, 38), encoding the major cytosolic NADP+-specific enzyme, in the strains listed in Table I. No distinctive new growth phenotypes were observed, suggesting that a major lesion in this isozyme system does not affect biosynthetic pathways in these mutants. Another alternative source of NADPH in many eucaryotic cells is direct transhydrogenation of NADH to NADPH. However, there is no evidence for such transhydrogenases in S. cerevisiae (39).

Thus, our data suggest that Zwf1p and Idp2p are important sources of NADPH under certain growth conditions. Otherwise, however, yeast cells appear to be remarkably flexible in terms of sources of biosynthetic reducing equivalents, and the sources speculated to be major contributors in eucaryotic cells are apparently not essential under many growth conditions.

We have used genes and mutants from this and other studies (12) to express mammalian NADP+-specific isocitrate dehydrogenases in yeast to examine the utility of the yeast system for future functional analyses of naturally occurring and of constructed mutant variants of the mammalian enzymes. Expression of both mitochondrial mammIdp1p and cytosolic mammIdp2 required use of multicopy vectors to obtain measurable levels of activity, presumably due to differences in codon bias or to some instability of the mammalian enzymes in yeast. Use of multicopy vectors with yeast IDP1 and IDP2 promoters, however, results in significant levels and appropriate patterns of expression of the mammalian isozymes. The mammIdp1p isozyme was found to be efficiently imported into yeast mitochondria using the yeast Idp1p targeting sequence and appears to be correctly processed to the mature form. That the mitochondrial form of the mammalian isozyme, which shares 63% residue identity with yeast Idp1p (20), can functionally replace its yeast homologue is shown by restoration of glutamate prototrophy with glucose as carbon source in a mutant strain lacking both mitochondrial NADP+– and NADP+–specific isocitrate dehydrogenases.

The cytosolic mammIdp2p isozyme is distributed between both cytosolic and organelar compartments in yeast transformants. This isozyme, which shares ~60% residue identity with the yeast enzymes (25), was found to be the functional counterpart of both yeast Idp2p and Idp3p isozymes. Restoration of growth with petroselineate as a carbon source in a ΔIDP3 strain strongly suggests peroxisomal localization of the fraction of mammIdp2p found in organelar fractions. However, this functional and apparent localization in yeast cells are independent of the carboxyl-terminal Ala-Lys-Leu tripeptide of mammIdp2p, suggesting the existence of an internal sequence determinant for peroxisomal localization. Although the mammalian enzyme is reported to be cytosolic (29, 40, 41), similar dual localization in mammalian cells is a possibility. There is a previous report of NADP+–specific isocitrate dehydrogenase activity in peroxisomes of mammalian cells (35). Also, there are several reports of enzymes encoded by the same gene that exhibit dual compartmental localization. Relevant examples are yeast cytosolic aspartate aminotransferase (42), which is targeted to peroxisomes during growth with oleate as a carbon source, and yeast carnitine acetyltransferase (43), which is targeted to peroxisomes or to mitochondria depending on whether mRNA transcripts contain a mitochondrial targeting sequence. Immunolocalization studies of mammIdp2p will clearly be of interest for future studies.

The studies presented here demonstrate the utility of yeast mutant collections for assessing metabolic function of mammalian NADP+–specific isocitrate dehydrogenases. In combination with Escherichia coli expression systems established for these enzymes (44, 45), it should be possible in future studies to directly correlate structural/kinetic results with in vivo function of mutant forms of these enzymes.

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