Physiological Consequences of Loss of Allosteric Activation of Yeast NAD\(^+\)-specific Isocitrate Dehydrogenase*

Received for publication, November 15, 2005, and in revised form, March 17, 2006 Published, JBC Papers in Press, April 18, 2006, DOI 10.1074/jbc.M512281200

Gang Hu, An-Ping Lin, and Lee McAlister-Henn

From the Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229-3900

Based on allosteric regulatory properties, NAD\(^+\)-specific isocitrate dehydrogenase (IDH) is believed to control flux through the tricarboxylic acid cycle in vivo. To distinguish growth phenotypes associated with regulatory dysfunction of this enzyme in *Saccharomyces cerevisiae*, we analyzed strains expressing well defined mutant forms of IDH or a non-allosteric bacterial NAD\(^+\)-specific isocitrate dehydrogenase (IDHa). As previously reported, expression of mutant forms of IDH with severe catalytic defects but intact regulatory properties produced an inability to grow with acetate as the carbon source and a dramatic increase in the frequency of generation of petite colonies, phenotypes also exhibited by a strain (idh1Δidh2Δ) lacking IDH. Reduced growth rates on acetate medium were also observed with expression of enzymes with severe regulatory defects or of the bacterial IDHa enzyme, suggesting that allosteric regulation is also important for optimal growth on this carbon source. However, expression of IDHa produced no effect on petite frequency, suggesting that the intermediate petite frequencies observed for strains expressing regulatory mutant forms of IDH are likely to correlate with the slight reductions in catalytic efficiency observed for these enzymes. Finally, rates of increase in oxygen consumption were measured during culture shifts from medium with glucose to medium with ethanol as the carbon source. Strains expressing wild-type or cataclytically deficient mutant forms of IDH exhibited rapid respiratory transitions, whereas strains expressing regulatory mutant forms of IDH or the bacterial IDHa enzyme exhibited much slower respiratory transitions. This suggests an important physiological role for allosteric activation of IDH during changes in environmental conditions.

Mitochondrial NAD\(^+\)-specific isocitrate dehydrogenase (IDH)\(^2\) catalyzes an essentially irreversible, rate-limiting step in the tricarboxylic acid cycle (1). IDH is a complex oligomeric enzyme and is subject to kinetic control by multiple allosteric effectors. For example, allosteric activation of yeast IDH by AMP (2) or of mammalian IDH by ADP (3) dramatically increases affinity of the enzyme for isocitrate. This kinetic response is believed to accelerate flux through the tricarboxylic acid cycle in vivo in response to low energy charge, i.e. in response to concomitantly low levels of ATP (2). However, despite extensive analyses of kinetic behavior and structural/functional studies, a direct correlation between allosteric control of IDH and cellular responses has not been demonstrated.

Yeast IDH is an octamer composed of four IDH1 (\(M_r = 38,001\)) and four IDH2 (\(M_r = 37,755\)) subunits (4). IDH1 and IDH2 share 42% primary sequence identity (5, 6). The functions of each subunit have been analyzed by mutagenesis of analogous residues based on the well defined three-dimensional structure of the homodimeric *Escherichia coli* isocitrate dehydrogenase (ICD, 7, 8), which shares \(~32\)% sequence identity with both IDH subunits. Results suggest that the basic functional unit of the yeast enzyme is a heterodimer of a catalytic IDH2 subunit and a regulatory IDH1 subunit (9, 10). IDH2 contributes most of the residues in the catalytic substrate (isocitrate/Mg\(^2+\)) and cofactor (NAD\(^+\)) binding sites, whereas the analogous sites in IDH1 have apparently evolved for cooperative binding of isocitrate and for allosteric binding of AMP (9–12). We have further demonstrated that binding of isocitrate at the catalytic site of IDH2 is necessary for subsequent binding of NAD\(^+\) and, analogously, binding of isocitrate at the cooperative site of IDH1 is necessary for subsequent binding of allosteric activation by AMP (12). In addition, while each of the major ligand-binding sites is primarily composed of residues from one subunit, each site also contains a few residues contributed by the other subunit, providing a mechanism for communication between regulatory and catalytic ligand-binding sites (13, 14).

These studies produced well characterized mutant forms of IDH, and expression of these mutant enzymes in a yeast strain lacking endogenous IDH provides an opportunity for correlating cellular growth phenotypes with specific defects in catalytic or regulatory functions. To date, loss or severe catalytic dysfunction of IDH has been shown to correlate with an inability to grow with acetate as a carbon source (6, 15), a phenotype also observed with loss of several other tricarboxylic acid cycle enzymes in yeast (16, 17), and with an increase in the rate of production of respiratory-deficient (petite) segregants (10, 18). Specific phenotypes associated with regulatory dysfunction of IDH under these other steady-state growth conditions have not been elucidated. Therefore, in the current study we sought to identify transient phenotypes associated with regulatory kinetic dysfunction using shifts between different permissive growth conditions.

We also wished to express a heterologous non-allosteric enzyme in lieu of IDH in yeast. Most bacterial isocitrate dehydrogenases, including *E. coli* ICD (19), are non-allosteric, homodimeric, NADP\(^+\)-specific enzymes. We have previously shown that, when ICD is expressed in a yeast mutant lacking IDH as well as the mitochondrial and cytosolic NADP\(^+\)-specific isocitrate dehydrogenases, the bacterial enzyme can complement the glutamate auxotrophy of this strain (20). However, while ICD can provide \(\alpha\)-ketoglutarate in yeast, the difference in cofactor specificity limits complementation for other tricarboxylic acid cycle functions, including growth on acetate as a carbon source.

Inoue et al. (21) recently reported purification, kinetic characterization, and the gene sequence of a homodimeric NAD\(^+\)-specific isocitrate dehydrogenase (designated IDHa, subunit \(M_r = 46,218\)) from the...
chemolithotrophic bacterium Acidithiobacillus thiooxidans. This enzyme exhibits a 78-fold preference for NAD\(^+\) over NADP\(^+\), suggesting it might be an appropriate heterologous enzyme for provision of tricarboxylic acid cycle functions of IDH. We expressed and purified IDHa to ascertain the absence of pertinent allosteric regulatory properties prior to tests for complementation of IDH catalytic and regulatory functions.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Cultivation Conditions** — The parental yeast strain was MMY011 (MATa ade2-1 his3-11,15 leu2-3,112, trp1-1 can1-100 (22)). Previously derived from this strain were an idh1\textDelta idh2\textDelta mutant strain containing deletions/insertions of LEU2 and HIS3 genes in the chromosomal IDH1 and IDH2 loci, respectively (23), and an idp1\textDelta idp2\textDelta idh2\textDelta mutant strain containing disruptions in genes encoding the major NADP\(^+\)-specific isocitrate dehydrogenases and the IDH2 subunit (18). Strains were routinely maintained on rich YP medium (1% yeast extract, 2% Bacto-peptone) with 2% glucose. Transformants were selected on defined minimal YNB medium (0.67% yeast nitrogen base) containing 2% glucose and nutrients to satisfy auxotrophic requirements of the strain or to maintain selection for plasmids. Other carbon sources included 2% ethanol or 2% sodium acetate.

**Growth Phenotype Analyses** — To measure steady-state growth rates, strains were grown overnight in YP medium to an optical density of \(A_{600 \text{nm}} = 0.6 - 0.8\), then diluted into YP medium with the same carbon source. Growth rates in liquid cultures were measured with \(A_{600} = 0.1\).

To analyze respiratory deficient petite \(rho^−\) mutations, strains were precultivated in yeast synthetic drop-out medium lacking uracil (Sigma) for plasmid selection, and dilutions of the cultures were plated on YP glucose plates. The petite frequency was the number of small white (petite) colonies relative to large red colonies counted after growth for 3 days at 30 °C. This assay is based on the red pigment produced as a result of the ade2 mutation in these strains and yields results comparable to the tetrazolium red overlay technique (24).

For plating assays, cells were pelleted from liquid cultures grown overnight in YP glucose medium, washed, and resuspended to 2 \(A_{600}\) per ml of H\(_2\)O. Cell samples were serially diluted in 10-fold steps for plating onto YP acetate plates or 100-fold for plating onto YNB glucose plates lacking or containing 100 \(\mu\)g/ml glutamate. 5-\(\mu\)l samples of each dilution were spotted onto the agar plates.

Respiratory rates were measured using a Clarke-type oxygen electrode (Yellow Springs Instrument Co., Inc.). Yeast strains were cultivated overnight in YP glucose medium. Cells were harvested, washed and diluted into H\(_2\)O, and used to inoculate a YP ethanol culture \((A_{600} \approx 0.1)\) and a YP glucose culture \((A_{600} \approx 0.2)\). After 4 h, the YP ethanol culture was used to measure steady-state ethanol respiratory rates, and the YP glucose culture was harvested. Cells from the glucose culture were washed, resuspended in H\(_2\)O, and used to inoculate a YP glucose culture \((A_{600} \approx 0.15)\) for determination of steady-state glucose respiratory rates and a YP ethanol culture \((A_{600} \approx 0.15)\) for determination of transitional respiratory rates. Rates of oxygen consumption and culture \(A_{600}\) values were measured every 30 min for 3 h.

**Expression of IDH\(a\) in Yeast** — To express IDH\(a\) in yeast, the IDH2 coding region was fused in-frame with the yeast IDH2 promoter and mitochondrially targeting sequence. The IDH\(a\) gene was obtained using PCR with A. thiooxidans genomic DNA (obtained from Dr. Cathrin Spro¨er, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) as the template. The 5′-oligonucleotide \((5′-\text{ATCTTAGGATC-}
C\text{ACCATATTCAAGAAAACCGCGAAG})\) contained a BamHI site (underlined) for subsequent in-frame ligation with IDH2 sequences, and the 3′-oligonucleotide \((5′-\text{TAATTGAGCTTTTATGGTGATGCTGAGAAACGCGATCAGTCGCTGCC})\) contained codons for six histidine residues (italics) prior to the stop codon and a HindIII site (underlined). To facilitate subcloning, a single-copy plasmid \((\text{pRS316/IDH2})\) containing the promoter, coding region, and 3′-noncoding sequences of the yeast IDH2 gene on a 1.8-kbp SstII/HindIII DNA fragment (13) was used for site-directed mutagenesis (QuikChange, Stratagene) to insert a BamHI site following codons for the 15-residue mitochondrial targeting sequence (25). The BamHI/HindIII IDHa PCR product was used to replace the BamHI/HindIII fragment in the pRS316/IDH2 plasmid, placing the IDHa gene 3′ of the IDH2 promoter and in-frame with the IDH2 mitochondrial targeting codons. The resulting plasmid was designated pRS316/IDHa. The sequence of the IDHa gene (21) and the fusion with IDH2 sequences were verified by nucleotide sequence analysis. Subcloning was subsequently used to transfer the 1.8-kbp SstII/HindIII fragment from this plasmid to a 2 μ pRS426 plasmid, generating a multicopy plasmid designated pRS426/IDHa. The pRS316/IDHa and pRS426/IDHa plasmids were transformed into the idh1\textDelta idh2\textDelta yeast strain for complementation analyses and for enzyme purification, respectively.

**Protein Analyses** — For affinity purification, transformants of the idh1\textDelta idh2\textDelta strain carrying multicopy plasmids for expression of IDHa with a carboxyl-terminal hexa-histidine tag (pRS426/IDHa) or of IDH with a carboxyl-terminal penta-histidine tag (pRS426/IDH) were precultivated overnight in 150 ml of YNB glucose medium lacking uracil to select for plasmid retention. These cultures were used to inoculate 2.0-liter cultures in YP ethanol medium, a condition that maximizes expression of IDH gene promoters (20). Cells were harvested after growth for 36 h at 30 °C and lysed by glass-bead breakage. Affinity purification using Ni\(^2+\)-nitrotetracycl acid resin (Qiagen) was conducted as previously described (13). Concentrations of purified enzymes were determined at \(A_{280 \text{nm}}\) using molar extinction coefficients of 77,280 M\(^{-1}\)cm\(^{-1}\) for IDHa and 168,820 M\(^{-1}\)cm\(^{-1}\) for IDH (26).

Kinetic properties of IDHa and IDH with respect to isocitrate were compared using assays containing 40 mM Tris-HCl (pH 7.4), 4 mM MgCl\(_2\), 0.5 mM NAD\(^+\), and DL-isocitrate concentrations ranging from 0 to 300 \(\mu\)M for IDHa and from 0 to 5.0 mM for IDH. Assays were conducted in the absence or presence of 100 \(\mu\)M AMP. To compare properties with respect to NAD\(^+\), assays were conducted with 40 mM Tris-HCl (pH 7.4), 4 mM MgCl\(_2\), a concentration of d-isocitrate (measured as 50% of the total concentration of dL-isocitrate) equal to 5-fold the determined \(S_{0.5}\) value, and NAD\(^+\) concentrations ranging from 0 to 3.0 mM. Kinetic properties of IDHa were also examined using a Tris-HCl buffer at pH 9.0 as previously described (21). A unit of activity is defined as production of 1 \(\mu\)mol of NADH/min at 24 °C. Apparent \(V_{\text{max}}\) values are the maximal rates per milligram of purified enzyme.

For cellular fractionation, transformants of the idh1\textDelta idh2\textDelta strain carrying single-copy plasmids for expression of IDHa or of IDH were precultivated overnight in 150 ml of YNB glucose medium lacking uracil to select for plasmid retention. These cultures (and control strains pre-cultivated in YP glucose) were used to inoculate 2.0-liter cultures in YP ethanol medium to increase expression of IDH gene promoters (20). Cells were harvested for fractionation to obtain mitochondrial pellets and post-mitochondrial supernatants as described by Daum et al. (27). Enzymatic assays for IDH or IDHa in cellular fractions were conducted as previously described for IDH (20). Protein concentrations were determined using the Bradford method (28) with bovine serum albumin as the standard.
Protein samples were electrophoresed on 10% polyacrylamide/sodium dodecyl sulfate gels. Immunoblot analyses were conducted using an antiserum against yeast IDH as previously described (4). Based on a primary sequence identity of 59% (21), we tested an antiserum against E. coli ICD previously obtained from Dr. D. E. Koshland, Jr. (Berkeley, CA), and found it to interact well with IDH in immunoblot analyses at a dilution of 1:300. An antiserum against yeast porin (Molecular Probes, Inc.) was used at a dilution of 1:5000. The enhanced chemiluminescence system (ECL, Amersham, Arlington Heights, IL, USA) was used at a dilution of 1:300. An antiserum against yeast porin (Molecular Probes, Inc.) was used at a dilution of 1:5000. The enhanced chemiluminescence method and autoradiography were used for detection.

RESULTS

Effects of Residue Substitutions on Growth Phenotypes—To examine effects of specific defects in catalytic and regulatory properties of IDH on steady-state and transitional growth phenotypes, we chose well-characterized mutant forms of IDH for expression in a yeast strain (idh1Δidh2Δ) containing chromosomal disruptions in both IDH1 and IDH2 loci. Mutant enzymes were expressed using a single-copy plasmid carrying one wild-type subunit gene and the other mutant subunit gene, each under control of its authentic promoter (13). As summarized in Table 1, these enzymes included a pair of mutant enzymes with an alanine residue replacement for a critical serine residue in the isocitrate/Mg2+-binding site of IDH2 (IDH2S98A) or in the isocitrate-binding site of IDH1 (IDH1S92A). These replacements essentially eliminate isocitrate binding by each type of subunit (11) but have very different kinetic consequences. The IDH2S98A enzyme exhibits a dramatic 160-fold decrease in apparent V_max but no effect on cooperative binding of isocitrate or on AMP activation, whereas the IDH1S92A enzyme exhibits loss of cooperativity with respect to isocitrate and loss of AMP activation with a lesser effect on V_max (9, 10). The other pair of mutant enzymes (Table 1) contains alanine replacements for adjacent aspartate/isoleucine pairs in the respective adenine nucleotide-biding sites of each subunit. The primary kinetic defect exhibited by the IDH2D286A,E287A mutant enzyme is a ~300-fold decrease in apparent V_max due to a dramatic decrease in affinity for the cofactor NAD+, whereas the primary kinetic defect exhibited by the IDH1D279A,I280A mutant enzyme is a loss of allosteric regulation by AMP (12, 13).

As previously reported, an idh1Δidh2Δ strain is unable to grow with acetate as the carbon source. We compared culture doubling times on acetate and other carbon sources for an idh1Δidh2Δ strain expressing each of the mutant forms of IDH listed in Table 1. Control strains were the parental strain or the idh1Δidh2Δ strain expressing wild-type IDH using a single-copy plasmid. With acetate as the carbon source, no culture doubling was observed over a 36-h period for the idh1Δidh2Δ strain (data not shown) or for strains expressing the catalytically deficient IDH2S98A or IDH2D286A,E287A enzyme (Table 1). Strains expressing the regulatory mutant IDH1S92A or IDH1D279A,I280A enzyme exhibited doubling times of 12 and 8 h, respectively, relative to 4–5 h for control strains. Thus, YP acetate medium is non-permissive for growth of strains expressing IDH enzymes with severe catalytic defects. For strains expressing regulatory deficient forms of IDH, it is difficult to distinguish if reduced growth rates on acetate medium are due to the regulatory defects or to the relatively slight reductions in catalytic capacity for these mutant enzymes.

Similar results were previously obtained in analyses of another phenotype, the frequency of generation of respiratory-deficient petite segregants (10, 18). This frequency for the idh1Δidh2Δ strain was >10-fold that for control strains and was similarly elevated for strains expressing the IDH2S98A or IDH2D286A,E287A catalytic mutant enzyme (Table 1). This frequency was considerably lower for strains expressing the IDH1S92A or IDH1D279A,I280A regulatory mutant enzyme but was still 2–3-fold higher than frequencies for control strains. Thus, these steady-state phenotypes do not clearly distinguish growth defects due to dramatic regulatory defects or to moderate catalytic defects of the allosteric mutant enzymes.

As shown in Table 1, all control and IDH mutant strains exhibited comparable doubling times in YP medium with either glucose or ethanol as the carbon source, suggesting that these carbon sources are permissive for growth of IDH mutants. We therefore attempted to identify phenotypes attributed to regulatory IDH defects by analyzing rates of culture adaptation during shifts from permissive YP glucose medium, a condition associated with rapid fermentation and slower respiratory rates, to YP ethanol medium, a condition associated with rapid respiratory metabolism. Culture growth rates were measured following a shift of cells from logarithmically growing YP glucose cultures (A600 = 0.6–0.8) to YP ethanol medium (starting A600 = 0.1–0.2). All control strains and strains expressing the mutant forms of IDH exhibited similar lags of 7–8 h prior to attaining logarithmic culture doubling times (5–7 h) in YP ethanol medium. Thus, there were no apparent differences in adaptation to growth in ethanol medium. We have also observed no differences in protein expression among these strains. For example, Fig. 1 illustrates immunochromel levels of IDH and of porin, a mitochondrial membrane protein, in these strains growing logarithmically in YP eth-

---

**TABLE 1**

| Ligand defect | Residue change(s) | Major kinetic effect | Growth (wt = + + +) | % Petite (wt = + + + + + +) |
|---------------|------------------|----------------------|---------------------|---------------------------|
| Substrate (isocitrate/Mg2+) | IDH2S98A | ↓ 160× | + | - | ↓ 9× |
| Cofactor (NAD+) | IDH2D286A,E287A | ↓ 300× | + | + | ↑ 12× |
| Regulator (isocitrate) | IDH1S92A | ↓ 14× | - | + | ↑ 3× |
| Regulator (AMP) | IDH1D279A,I280A | ↓ 2× | + | + | + | + | + | 2× |

* *Culture doubling times (indicated as + + + + + +) were 2–3 h on YP glucose and 5–7 h on YP ethanol. Doubling times on YP acetate were: + + = 4–5 h; + = 8–12 h; – = no doubling in a 36-h period.*

*Petite frequencies were previously reported (10, 18).*
Loss of Allosteric Activation of IDH

![Graph](image)

**FIGURE 2. Rates of oxygen consumption for idh1Δidh2Δ transformants expressing wild-type and mutant forms of IDH.** Rates of oxygen consumption were determined as described under “Experimental Procedures” using the idh1Δidh2Δ strain (no IDH) or the idh1Δidh2Δ strain transformed with single-copy plasmids for expression of wild-type IDH (wt IDH) or of the indicated mutant forms of IDH. Rates are expressed relative to oxygen saturation of the medium, with 0% consumption representing full oxygen saturation. Average steady-state rates of oxygen consumption for each culture on YP ethanol medium or on YP glucose medium are indicated by graphs. Strains exhibiting rapid rates of transition are grouped under A, and strains exhibiting slower rates of transition are grouped under B.

![Graph](image)

**FIGURE 3. Relative rates of transitional oxygen consumption.** Data representing two or three independent determinations for strains exhibiting rapid (A) or slower (B) rates of transitional respiration following a shift from YP glucose to YP ethanol medium were plotted relative to glucose steady-state rates (set as 0) and to ethanol steady-state rates (set as 100) measured for control cultures. Graphic symbols for strains are the same as those shown in Fig. 2.

anol medium. IDH is absent in extracts from the idh1Δidh2Δ strain (lane 1), but levels are comparable in strains expressing wild-type IDH (lane 2) or mutant forms of IDH (lanes 3–6). (Note: this gel did not electrophoretically resolve IDH1 and IDH2 subunits, but some differences in mobility due to altered residues are apparent.)

To examine more immediate responses during culture shifts from YP glucose to YP ethanol medium, we monitored oxygen consumption rates. During these shift experiments, steady-state rates of oxygen consumption in YP glucose and YP ethanol media were measured using parallel cultures of each strain. Representative results illustrated in Fig. 2 show that steady-state rates of oxygen consumption for each strain were higher on YP ethanol medium (upper dashed line) than on YP glucose medium (lower dashed line). These steady-state rates were found to vary among the strains and between different experiments for the same strain. Consistently, however, following a shift from YP glucose to YP ethanol medium, the idh1Δidh2Δ strain expressing wild-type IDH (Fig. 2, wt IDH) attained the higher steady-state rate of oxygen consumption within 2 h. For the idh1Δidh2Δ disruption mutant strain (Fig. 2, no IDH), adaptation to more rapid respiration occurred more slowly with rates of oxygen consumption reaching only ~60% of the steady-state ethanol rate 3 h after the medium shift. Patterns for strains expressing the catalytic IDH2D296A, IDH2D286A, D287A mutant enzymes were similar to those for wild-type IDH (group A), i.e. with rapid initial increases in oxygen consumption following the shift, whereas patterns for the allosteric IDH1S92A and IDH1D279A, D280A mutant enzymes were similar to those for the strain lacking IDH (group B). To compare changes in respiratory patterns among experimental replicates and among different strains, the changes in oxygen consumption rates were expressed relative to the maximum change in each case (Fig. 3). As shown in Fig. 3A, strains expressing IDH or the catalytic mutant enzymes attained ~60% of the ethanol steady-state rates in 1 h and ~100% of the ethanol steady-state rates in 2 h following the shift. In contrast, the strain lacking IDH or strains expressing regulatory mutant enzymes (Fig. 3B) attained only ~50% of the ethanol rates after 2 h and 60–70% of the ethanol rates after 3 h. Slopes (47–49%/h) for the rapidly adapting strains (Fig. 3A) were ~2-fold those (20–23%/h) for the slowly adapting strains (Fig. 3B). The latter strains did attain steady-state rates of ethanol respiration after 4–6 h (data not shown).

This relatively slow rate of adaptation to rapid respiratory rates following a shift from glucose to ethanol medium is the first growth phenotype that we have found to correlate with defects in allosteric regulation of IDH. Apparently, low levels of allosterically regulated catalytic IDH activity are sufficient for rapid respiratory transitions, whereas the absence either of IDH activity or of allosterically regulated IDH activity is not.

**Purification and Kinetic Characterization of IDHa—** As an alternative for examining the role of allosteric control of IDH in vivo, we wished to express a non-allosteric bacterial NAD"-specific isocitrate dehydrogenase in yeast mitochondria. The enzyme (designated IDHa) from A. thiooxidans was previously reported to be a NAD"-specific homodimeric enzyme (21), but allosteric properties were not assessed. Therefore, we tested whether IDHa with phenotypes, we expressed this bacterial enzyme for purification and kinetic characterization.

The IDHa gene was amplified using PCR with oligonucleotides that added six histidine codons onto the 3'-end of the coding region. The PCR fragment was subcloned to place the IDHa coding region 3' of the IDH2 gene promoter and in-frame with codons for the 15-residue IDH2 mitochondrial targeting sequence (25). This gene fusion
was constructed in both a single-copy centromere-based plasmid (pRS316/IDHa) and a multicopy 2μ-based plasmid (pRS426/IDHa).

For enzyme purification, the pRS426/IDHa plasmid was transformed into the idh1Δidh2Δ yeast strain, and transformants were grown on YP ethanol medium, a condition conducive for maximum enzyme activity (20). IDHa was purified from cell extracts using Ni²⁺-nitrilotriacetic acid resin column chromatography as described under “Experimental Procedures.” The purified enzyme was essentially homogenous (Fig. 4, lane 3), with the major IDHa polypeptide migrating as expected under denaturing electrophoretic conditions as a band of ~45 kDa (21) relative to IDH1 (~38 kDa) and IDH2 (~37.5 kDa) subunits of the histidine-tagged form of yeast IDH (Fig. 4, lane 4) that was similarly purified. Mass spectrophotometric analysis confirmed that the IDHa polypeptide amino terminus was that expected following processing of the mitochondrial presequence in vivo. However, a faint larger band in the IDHa preparation suggests the presence of a small amount of the unprocessed mitochondrial precursor.

Inoue et al. (21) previously analyzed the catalytic activity of IDHα using a pH 9.0 buffer. We compared activity using the pH 9.0 buffer and the pH 7.4 buffer normally employed for assays of IDHα. We found that apparent V max values for the IDHa enzyme were comparable at both pH values but that affinities for both isocitrate and NAD⁺ were greater at pH 7.4. Therefore, kinetic comparisons of IDHα and IDHa (Table 2) were conducted using the same buffer at pH 7.4. IDHα and IDHa were found to have similar apparent V max values under most conditions, with the value for IDHα exceeding that of IDHα by ~40%. The enzymes also had similar affinities for NAD⁺. A major significant difference was the absence of AMP activation of IDHα, i.e., the apparent affinity for isocitrate was similar in the absence or presence of AMP as compared with a ~5-fold increase in affinity of IDHα for isocitrate in the presence of AMP. In addition, the IDHa enzyme exhibited a much greater affinity for isocitrate relative to IDHα. The apparent affinity of IDHα for isocitrate exceeded that of IDHα by 83-fold in the absence of AMP and by 17-fold in the presence of AMP. IDHα exhibited modest cooperativity with respect to isocitrate (Hill coefficient of ~1.5), whereas IDHα was highly cooperative (Hill coefficients of 3.4 and 3.9) as previously reported (10, 29). Thus, in general, because IDHα and IDHa have similar apparent V max values, but because IDHα is not activated by AMP, IDHα appeared to be more efficient at stimulating the activity of IDHα relative to IDHα.

Expression of IDHα in Yeast Mitochondria—To examine cellular localization of IDHα in yeast transformants, cells were grown in YP ethanol medium, and cellular extracts were used for preparation of mitochondrial pellet and post-mitochondrial supernatant fractions as described by Daum et al. (27). Enzyme assays were performed and immunoblot analyses were conducted with cellular fractions obtained from each strain. As illustrated in Fig. 5, using an antiserum against E. coli ICD (upper panel), IDHα was detected in mitochondrial fractions (P) from the single-copy (lanes 3) and multicopy (lanes 4) transformants but not in post-mitochondrial supernatant fractions (S) from either strain. (Note: this antiserum also interacts weakly with unrelated bands seen in mitochondrial fractions from the idh1Δidh2Δ and parental strains (lanes 1P and 2P, respectively.) As expected, with an antiserum against IDHα (lower panel), IDHα was detected only in the mitochondrial fraction from the parental strain (lane 2P).

Enzyme assays (Fig. 5) indicated that multicopy expression of IDHα increased the mitochondrial specific activity 3-fold relative to single-copy expression. Levels of IDHα activity in the mitochondrial fraction from the parental strain were 1.8-fold higher than mitochondrial IDHα activity obtained with single-copy expression and 1.7-fold lower than mitochondrial IDHα activity obtained with multicopy expression. Only the multicopy IDHα transformant exhibited a level of measurable activity in the post-mitochondrial supernatant. Thus, IDHα appears to be efficiently localized to mitochondria in yeast transformants and is expressed at sufficient levels to provide a specific activity comparable to that of IDHα. In fact, the single-copy IDHα transformant strain exhibited higher levels of mitochondrial specific activity than any of the mutant IDH transformants. For many of the phenotype analyses described below, we chose to use this single-copy IDHα transformant strain because of the relative stability of the plasmid and expression under a variety of cultivation conditions.

Complementation for Functions of IDHα by IDHα—We tested the ability of IDHα to complement a number of phenotypes associated with loss or dysfunction of IDHα. As shown in Fig. 6A, the idh1Δidh2Δ disruption mutant strain was unable to grow on YP acetate plates. Transformation of this strain with a single-copy plasmid for expression of IDHα restored growth to wild-type levels.

**TABLE 2**

Kinetic properties of IDHα and IDHa

| Isocitrate | V max (units/mg) | S 0.5 (μM) | Hill coefficient |
|-----------|-----------------|------------|-----------------|
| IDH  | 32.9/32.7 (±1.3/1.1) | 475/87.5 (±15/25) | 3.9/3.4 (±0.2/0) |
| IDHa | 22.6/22.9 (±3.2/3.1) | 5.7/5.2 (±1.4/2.6) | 1.5/1.4 (±0.2/0.1) |

| NAD⁺ | V max (units/mg) | S 0.5 (μM) | Hill coefficient |
|------|-----------------|------------|-----------------|
| IDH  | 37.6 (±0.7)     | 0.22 (±0.01) | 1.2 (±0.1)      |
| IDHa | 27.7 (±0.7)     | 0.31 (±0.01) | 1.2 (±0.1)      |

**FIGURE 4**. Affinity purification of IDHα. Protein fractions taken during Ni²⁺-nitrilotriacetic acid resin chromatographic purification of IDHα were electrophoresed and stained with Coomassie Blue. Protein samples included the cellular extract from the idh1Δidh2Δ strain transformed with pRS426/IDHa plasmid (~300 μg, lane 1), the column flow-through fraction (~50 μg, lane 2), purified IDHα (~15 μg, lane 3) obtained following elution with imidazole, and IDHα (~30 μg, lane 4) obtained by affinity purification from the idh1Δidh2Δ strain transformed with pRS426/IDHa.
Loss of Allosteric Activation of IDH

parental levels of colony growth, and transformation with a single-copy plasmid for expression of IDHa restored growth to almost the same extent as did addition of glutamate to YNB glucose plates. Thus IDHa activity as well as two isozymes (mitochondrial Idp1p and cytosolic Idp2p) lacking (idh1Δidh2Δ) strain expressing IDH (plasmid pRS316IDH1/IDH2) or IDHa (plasmid pRS316IDHa). Each value represents the average of three independent determinations.

frequency to the parental strain level, and expression of IDHs in the idh1Δidh2Δ strain produced a petite frequency only ~15% higher than the parental strain level, suggesting that IDHa can complement this phenotype associated with tricarboxylic acid cycle function of IDH.

Finally, we examined the impact of IDHa expression in the idh1Δidh2Δ strain on the transition from slower rates of oxygen consumption inYP glucose medium to faster rates of oxygen consumption inYP ethanol medium. As illustrated in Fig. 8 (A and B), the idh1Δidh2Δ strain expressing IDHs (filled circles) exhibited a slow rate of transition, to ~50% of the ethanol rate by 2 h and to ~70% of the ethanol rate by 3 h after the shift from glucose to ethanol medium. This is quite similar to the patterns exhibited by idh1Δidh2Δ strains expressing the IDH1S92A and IDH1D279A,D280A allosteric mutant enzymes (Figs. 2B and 3B). Because this is the major phenotype associated with expression of IDHs, we also constructed single-copy and multicopy plasmids for expression of native (i.e. non-histidine tagged) IDHs using a 3′-oligonucleotide lacking histidine codons as otherwise described under “Experimental Procedures.” The respiratory transitions for idh1Δidh2Δ transformants expressing these forms of IDHs (Fig. 8 (A and B); single copy; multicopy) were very similar to those for the histidine-tagged enzyme, suggesting that the slow transition to ethanol respiratory rates is not due to the presence of the affinity tag or to lower levels of expression.

These analyses suggest that expression of IDHs in yeast mitochondria can complement major growth phenotypes associated with loss or severe catalytic dysfunction of IDH. The level of compensation function suggests that IDHa is indeed behaving like an allosterically defective form of IDH, because moderate levels of growth onYP acetate medium are achieved, and because the rates of transition from fermentative to active respiratory rates of oxygen consumption are relatively slow. These results also support the idea that the latter properties are characteristic for allosteric dysfunction of this regulated step in the tricarboxylic acid cycle.

**DISCUSSION**

In current and previous studies, we have used the yeast molecular genetic system to dissect the multiple physiological contributions of mitochondrial NAD⁺-specific isocitrate dehydrogenase, a key tricarboxylic acid cycle enzyme. It was clear from initial observations of an acetate⁻ phenotype (and slow growth on glycerol as the carbon source) for yeast mutants with disruptions in IDH1 and/or IDH2 loci (6, 15) that the residual mitochondrial NAD⁺-specific isocitrate dehydrogenase
Loss of Allosteric Activation of IDH

An acetate growth phenotype was also observed for a yeast strain expressing E. coli NADP⁺-specific ICD in lieu of IDH (20). Thus, the production of NADH appears to be an essential and specific catalytic function of IDH.

Another index for specific catalytic function of IDH is the stability of mitochondrial DNA, assessed by analysis of the frequency of generation of petite segregants (10, 18). Although the biological basis for this phenomenon is not clear, we have found a fairly close correlation between this frequency and the catalytic competence of IDH. Yeast strains expressing mutant forms of IDH with apparent Vₘₐₓ values of ≥150-fold less than the wild-type value exhibit elevated petite frequencies similar to that exhibited by a strain lacking IDH (~10-fold above parental strain frequencies). We have also found that the strain expressing E. coli ICD in lieu of IDH exhibits a petite frequency similar to those for catalytic mutant forms of IDH, again suggesting that ICD cannot functionally replace IDH because of differences in cofactor specificity.

It is interesting that the growth phenotypes (slow growth with glycerol as the carbon source and the increase in petite frequency) associated with the absence or catalytic dysfunction of IDH are eliminated or largely abrogated by simultaneous loss of citrate synthase (18, 23). This suggests that the loss of citrate synthase function may either reduce levels of some deleterious metabolite (e.g. citrate or isocitrate) that may accumulate in IDH mutants, or it may reverse or modulate some change in gene expression observed in IDH mutants (24). In any event, these observations suggest that the absence of the IDH protein is not the basis for these “catalytic” growth phenotypes. This is an important point because IDH has also been reported to physically associate with both mitochondrial mRNAs (30) and with mitochondrial DNA (31). Although these interactions have previously been invoked to explain a reduction in levels of mitochondrial translation products (32) and the increase in petite frequency in mutants lacking IDH (10), we and others (18, 24, 33) have shown that these defects are largely eliminated in strains lacking both IDH and citrate synthase. Thus, the importance of mitochondrial macromolecular interactions involving IDH remains unclear. Also, the demonstration in this study that expression of IDHa in lieu of IDH permits growth on acetate and produces a low petite frequency suggests that these interactions are not specific for the yeast enzyme.

The phenotypes described above contrast with the role of IDH in the cellular production of α-ketoglutarate, which can be assessed as glutamate auxotrophy. Yeast strains lacking IDH are glutamate prototrophs, and we have shown that expression of any of the yeast isocitrate dehydrogenases (NAD⁺- or NADP⁺-specific), or of bacterial ICD or IDHa enzymes, is sufficient for glutamate prototrophy (Fig. 6) (20). Thus, production of α-ketoglutarate is a generic physiological function of isocitrate dehydrogenase enzymes.

The growth phenotypes observed under similar steady-state conditions for yeast strains expressing primarily regulatory mutant forms of IDH are much less severe (10, 18). These strains exhibit intermediate growth rates with acetate as the carbon source and moderately increased petite frequencies (Table 1). However, because these regulatory mutant enzymes also exhibit relatively slight reductions in Vₘₐₓ values, it has been difficult to identify specific growth deficiencies due to regulatory defects. In the current study, we expressed the bacterial IDHa enzyme, which is refractory to activation by AMP but which provides similar levels of catalytic activity, in lieu of IDH in yeast mitochondria. Expression of this enzyme produced intermediate growth rates with acetate but had very little effect on petite frequency, suggesting that the former phenotype may be due to the absence of allosteric control, but that the latter phenotype may be associated with the reduced catalytic activity of regulatory mutant forms of IDH.

In the current study, we used regulatory mutant forms of IDH and the bacterial IDHa enzyme to identify other specific defects due to the loss of allosteric activation by AMP. Yeast strains expressing these enzymes were found to exhibit extended times of transition to more rapid rates of respiration following a shift from medium with glucose to medium with ethanol as the carbon source. Both types of carbon sources are permissive for growth of IDH mutant strains, and we have observed no significant differences in overall steady-state culture growth rates in these media, suggesting that the lag in achieving more rapid respiratory rates is specifically due to allosteric dysfunction.

A strain lacking IDH also exhibits a lag in respiratory transition from glucose to ethanol steady-state rates (Fig. 2B). In contrast, strains expressing catalytically deficient but regulatory competent forms of IDH exhibit rapid rates of respiratory transition similar to those observed for control strains. These observations suggest that the catalytic mutant enzymes support sufficient flux through the IDH reaction to facilitate this transition, i.e. that AMP activation may increase affinity for isocitrate to a level needed for increased rates of respiration. For the strain lacking IDH and for the strains expressing regulatory mutant enzymes, the slow rate of transition appears to be due, respectively, to the absence of flux and to low rates of flux presumably due to inadequate affinity for isocitrate.

Results obtained with expression of IDHa, an enzyme with high affinity for isocitrate but which fails to support the rapid respiratory transition, suggest that an allosteric increase in IDH affinity for isocitrate may not be the only mechanism involved in this transition. Normally, because yeast mitochondrial NADP⁺-specific Idp1p enzyme has a

G. Hu and L. McAlister-Henn, unpublished observations.
greater affinity for isocitrate than does IDH (an ~17-fold difference when measured in the absence of AMP (34)). It is thought that, under conditions of energy sufficiency, the relatively low affinity of IDH for isocitrate could regulate preferential delivery of this metabolite to biosynthetic reactions (NADPH production) versus oxidative metabolism. With a change to cultivation or environmental conditions requiring more rapid respiration, allosteric up-regulation of IDH would increase utilization of isocitrate by the tricarboxylic acid cycle and production of NADH for increased respiration. Our results suggest that allosteric activation of IDH by AMP in yeast does, in fact, contribute to such control of metabolic flux during transitions in cultivation conditions. However, results obtained for the high affinity IDHα enzyme suggest that factors other than simply increasing the affinity of the enzyme for isocitrate may be involved. One possibility is that allosteric activation of IDH in vivo may mediate interactions with other components of the tricarboxylic acid cycle to channel this intermediate into oxidative pathways. A model for structural/functional organization of the tricarboxylic acid cycle for substrate channeling has, in fact, previously been proposed (35, 36). Although highly speculative, this model would predict that the heterologous bacterial IDHα enzyme would be unlikely to participate in the specific macromolecular interactions necessary for rapid respiratory transitions. Another correlate supported by our results is that catalytic efficiency might be much less critical than regulatory competence for control of such channeling.

Data presented here suggest that phenotypes associated with allosteric dysregulation may be more readily discerned during transitions in cultivation or environmental conditions. This seems reasonable, because loss of allosteric regulatory properties may have little effect on enzymatic function under steady-state growth conditions. We plan in the future to use similar transitions to assess changes in levels of cellular metabolites and NADH:NAD ratios as a function of the loss of allosteric regulation of IDH.

Acknowledgments—We thank Elizabeth Morris for technical assistance, Dr. Karyl I. Minard for advice and critiques of the manuscript, and Drs. Susan T. Weinstein and Christopher A. Carroll for mass spectrophotometric analyses.

REFERENCES
1. Barnes, L. D., McGuire, J. I., and Atkinson, D. E. (1972) Biochemistry 11, 4322–4329
2. Hathaway, J. A., and Atkinson, D. E. (1963) J. Biol. Chem. 238, 2875–2881
3. Chen, R. F., and Plaut, G. W. E. (1963) Biochemistry 2, 1023–1032
4. Keys, D. A., and McAlister-Henn, L. (1990) J. Bacteriol. 172, 4280–4287
5. Cupp, J. R., and McAlister-Henn, L. (1991) J. Biol. Chem. 266, 22199–22205
6. Cupp, J. R., and McAlister-Henn, L. (1992) J. Biol. Chem. 267, 16417–16423
7. Hurley, J. H., Thorness, P. E., Ramalingam, V., Helmers, N. H., Koshland, D. E., Jr., and Stroud, R. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8635–8639
8. Hurley, J. H., Dean, A. M., Koshland, D. E., Jr., and Stroud, R. M. (1991) Biochemistry 30, 8671–8678
9. Cupp, J. R., and McAlister-Henn, L. (1993) Biochemistry 32, 9323–9328
10. Lin, A.-P., McCammon, M. T., and McAlister-Henn, L. (2001) Biochemistry 40, 14291–14301
11. Lin, A.-P., and McAlister-Henn, L. (2002) J. Biol. Chem. 277, 22475–22483
12. Lin, A.-P., and McAlister-Henn, L. (2003) J. Biol. Chem. 278, 12864–12872
13. Zhao, W.-N., and McAlister-Henn, L. (1997) J. Biol. Chem. 272, 21811–21817
14. Panisko, E., and McAlister-Henn, L. (2001) J. Biol. Chem. 276, 1204–1210
15. McCammon, M. T. (1996) Genetics 144, 57–69
16. Kim, K., Rosenkrantz, M. S., and Giurente, L. (1986) Mol. Cell. Biol. 6, 1936–1942
17. McAlister-Henn, L., and Thompson, L. M. (1987) J. Bacteriol. 169, 5157–5166
18. McCammon, M. T., and McAlister-Henn, L. (2003) Arch. Biochem. Biophys. 419, 222–233
19. Reeves, H. C., Daumy, G. O., Lin, C. C., and Houston, M. (1972) Biochim. Biophys. Acta 258, 27–39
20. Zhao, W.-N., and McAlister-Henn, L. (1996) Biochemistry 35, 7873–7878
21. Inoue, H., Tamura, T., Ebara, N., Nishito, A., Nakayama, Y., Maekawa, M., Imada, K., Tanaka, H., and Inagaki, K. (2002) FEMS Microbiol. Lett. 214, 127–132
22. McCammon, M. T., Veenhuis, M., Trapp, S. B., and Goodman, J. M. (1990) J. Bacteriol. 172, 5816–5827
23. Przybyla-Zawislak, B., Gaddie, D. M., Ducharme, K., and McCammon, M. T. (1999) Genetics 152, 153–166
24. McCammon, M. T., Epstein, C. B., Przybyla-Zawislak, B., McAlister-Henn, L., and Butow, R. A. (2003) Mol. Biol. Cell 14, 958–972
25. Zhao, W.-N., and McAlister-Henn, L. (1996) J. Biol. Chem. 271, 10347–10352
26. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
27. Daum, G., Bohni, P. C., and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Barnes, L. D., Kuemmerle, G. D., and Atkinson, D. E. (1971) J. Bacteriol. 142, 401–409
30. Barnes, L. D., Kuehn, G. D., and Atkinson, D. E. (1971) J. Bacteriol. 141, 786–794
31. McAlister-Henn, L., and Thompson, L. M. (1987) J. Bacteriol. 169, 5157–5166
32. De Jong, L., Elzinga, S. D., McCammon, M. T., Grivell, L. A., and van der Spek, H. (1993) FEMS Microbiol. Lett. 118, 175–179
33. Anderson, S. L., Lin, A.-P., and McAlister-Henn, L. (2005) Biochemistry 44, 16776–16784
34. Contreras-Shannon, V., Lin, A.-P., McCammon, M. T., and McAlister-Henn, L. (2005) J. Biol. Chem. 280, 4469–4475
35. Sumegi, B., McCammon, M. T., Sherry, A. D., Keys, D. A., McAlister-Henn, L., and Sreer. P. A. (1992) Biochemistry 32, 8720–8725
36. Ovadi, J., and Sreer. P. A. (2000) Int. Rev. Cytol. 192, 255–280