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Suppression of metabolic defects of yeast isocitrate dehydrogenase and aconitase mutants by loss of citrate synthase

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Abstract

Yeast mutants lacking mitochondrial NAD⁺-specific isocitrate dehydrogenase (*idhΔ*) or aconitase (*aco1 Δ*) were found to share several growth phenotypes as well as patterns of specific protein expression that differed from the parental strain. These shared properties of *idhΔ* and *aco1 Δ* strains were eliminated or moderated by co-disruption of the *CIT1* gene encoding mitochondrial citrate synthase. Gas chromatography/mass spectrometry analyses indicated a particularly dramatic increase in cellular citrate levels in *idhΔ* and *aco1 Δ* strains, whereas citrate levels were substantially lower in *idhΔcit1 Δ* and *aco1 Δcit1 Δ* strains. Exogenous addition of citrate to parental strain cultures partially recapitulated effects of high endogenous levels of citrate in *idh Δ* and *aco1 Δ* strains. Finally, effects of elevated cellular citrate in *idhΔ* and *aco1 Δ* mutant strains were partially alleviated by addition of iron or by an increase in pH of the growth medium, suggesting that detrimental effects of citrate are due to elevated levels of the ionized form of this metabolite.

Keywords

isocitrate dehydrogenase; aconitase; citrate synthase; tricarboxylic acid cycle; citrate

Introduction

Mitochondrial NAD⁺-specific isocitrate dehydrogenase (IDH¹) catalyzes a rate-limiting reaction in the tricarboxylic acid (TCA) cycle. The enzyme is subject to extensive allosteric control, and the reaction is essentially irreversible under physiological conditions [1,2]. Yeast IDH is an octamer composed of four catalytic IDH2 subunits and four homologous regulatory IDH1 subunits [3,4].

Growth phenotypes associated with disruption of yeast *IDH1* and/or *IDH2* genes include an inability to grow with acetate as a carbon source, a phenotype shared with several other yeast TCA cycle mutants [5,6], and slow growth with glycerol as a carbon source. The latter phenotype is ameliorated by concomitant disruption of the *CIT1* gene encoding the TCA cycle enzyme citrate synthase [7]. Similar suppression effects have also been observed for other IDH mutant phenotypes. For example, yeast strains lacking IDH or expressing catalytically defective forms of IDH exhibit elevated frequencies of generation of respiratory-deficient

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¹Abbreviations used: IDH, NAD-specific isocitrate dehydrogenase; IDP, NADP-specific isocitrate dehydrogenase; CIT, citrate synthase; ACO, aconitase; COX, cytochrome c oxidase; GC/MS, gas chromatography/mass spectrometry.

petite colonies [8]. Concomitant loss of CIT1 reduces these frequencies to levels observed for the parental strain [9]. In addition, a global analysis of gene expression in yeast TCA cycle mutants [10] revealed a set of ~20 genes that exhibited elevated expression in *idh*Δ gene disruption mutants, depressed expression in *cit1* Δ mutants, and intermediate expression in *idh*Δ *cit1* Δ double mutant strains. Finally, a yeast mutant lacking IDH was found to exhibit reduced levels of mitochondrially-encoded proteins [11], which are hydrophobic protein components of the electron transport chain. In more recent studies using strains expressing catalytically defective forms of IDH [12], we found that reduced levels of IDH activity correlated with reduced levels of mitochondrially-encoded proteins. Furthermore, in mutants lacking both IDH and CIT1, levels of these proteins were similar to levels in parental strains. Thus, effects of IDH on mitochondrial gene expression are indirect and correlate with some undetermined function that is suppressed by concomitant loss of CIT1.

In the current study, we examined the possibility that the suppression by loss of CIT1 of phenotypes exhibited by IDH mutants might be due to reduction in levels of some metabolite (e.g. citrate or isocitrate) that otherwise accumulates in IDH mutants. As a corollary, we predicted that a yeast mutant lacking aconitase (ACO1) might exhibit similar phenotypes and metabolite accumulation as an IDH mutant, and that loss of CIT1 in an ACO1 mutant would similarly suppress these phenotypes. To test these hypotheses, it was necessary to implement two novel approaches. We developed a method for rapid extraction and reproducible analysis of TCA cycle metabolites in yeast cells. We also found it essential to initiate all experiments with newly constructed yeast IDH and ACO1 mutant strains to preclude genetic and phenotypic changes that otherwise occur with long-term storage of these strains.

Materials and methods

Strains and growth conditions

The parental yeast strain was MMY011 (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ole⁺*) [13]. Construction of an *idh*Δ mutant strain with *LEU2* and *HIS3* deletion/disruptions in *IDH1* and *IDH2* loci, respectively, and of the *idh*Δ*cit1* Δ mutant strain were previously described [9,14]. Mutant *aco1* Δ and *aco1* Δ*cit1* Δ strains were newly constructed by deletion/disruption mutagenesis using the *kanMX4* cassette [15] to replace the *ACO1* coding regions in parental and *cit1* Δ strains, respectively. Gene disruptions were confirmed by polymerase chain reaction using genomic DNA isolated from kanamycin-resistant transformants. Yeast strains were cultivated using rich YP medium (1% yeast extract, 2% Bactopeptone) or minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate) containing nutrients for supplementation or for selection of auxotrophic requirements of the strains. Carbon sources (galactose or glucose) were added to 2%. Cells were inoculated to a starting OD_{600nm} ≅ 0.1 and cultivated at 30°C.

Prior to plating for growth phenotype analyses, yeast strains were grown in YNB glucose medium supplemented with 0.05% yeast extract to OD_{600nm} ≅ 2.0. Cells were harvested by centrifugation, washed, and resuspended in water to OD_{600nm} ≅ 2.0. The cell suspensions were serially diluted (10-fold dilutions), and 10 μl aliquots of each dilution were spotted onto YP or YNB plates (plus or minus 0.01% glutamate) containing galactose or glucose as the carbon source. Colony growth was assessed after 4–5 days of incubation at 30°C. To determine petite frequencies, cells were cultivated in YP galactose medium for 18–20 h (to OD_{600nm} ≅ 2.0). Diluted cell suspensions were plated onto YP glucose plates, and numbers of petite/total colonies were determined after 3–4 days of growth at 30°C. As previously described [9,10], the red pigment produced as a result of the *ade2* mutation in these strains permits tabulation of the percentage of smaller white (petite) colonies relative to larger red (grande) colonies.

Western blot analyses

Cell cultures (50 ml) were grown to $OD_{600nm} = 1.8\text{--}2.0$. Growth was stopped by addition of cycloheximide to 100 $\mu\text{g/ml}$ and sodium azide to 40 mM, and the cultures were placed on ice for 5 min. Cells were harvested by centrifugation (3 min at $1000 \times g$), and the pellets were washed once prior to storage at -70°C . For immunoblot analyses, cell pellets were resuspended in 8.0 M urea buffer (8.0 M urea; 50 mM sodium phosphate, pH 7.4; 10 mM Tris-HCl, pH 7.4). Following addition of phenylmethylsulfonyl fluoride to 10 mM, the pellets were lysed by vortexing with glass beads. The lysate was centrifuged (14,000 rpm) for 15 min at 4°C using a microfuge. Supernatants were removed, and aliquots were used for protein assays [16] or diluted 1:2 with 2 $\times$ Laemmli sample buffer [17] and boiled for 5 min prior to storage at -70°C .

Electrophoresis of protein samples was conducted using 10% and 14% polyacrylamide/sodium dodecyl sulfate gels. Immunoblot analyses for yeast proteins were conducted using antisera specific for IDH (1:2000 dilution) [3]; for subunits of cytochrome *c* oxidase including COX2 (1:2000 dilution), COX3 (1:1000 dilution), and COX4 (1:1000 dilution) (Molecular Probes, Inc.); for β -actin (1:2000 dilution, Abcam), and for CIT2 (1:2000 dilution, kindly provided by Drs. Mark T. McCammon and Gyula Kispal). The antiserum initially prepared using yeast IDP1 also recognizes the IDP2 protein (1:2000 dilution) [18]. Secondary antisera were used at dilutions of 1:5000. Immunoreactive polypeptides were detected using the enhanced chemiluminescent method (Amersham Biosciences) and autoradiography. Densitometric analyses were performed, and band intensities were expressed relative to that for β -actin, the internal standard for each cellular protein extract. The immunoblots presented in this study are representative results of experiments repeated two or three times.

Sample preparation for metabolite analyses

To prepare extracts for analysis by gas chromatography/mass spectrometry (GC/MS), cell cultures (50 ml) were grown and growth stopped as described above. Immediately after placing cultures on ice, samples (1.5 ml) were removed for determination of dry weight as described below. Remaining cells were rapidly harvested by centrifugation for 3 min at $1000 \times g$, and the pellets were washed twice by resuspension in water and centrifugation. Cell pellets were stored at -70°C . For lysis and metabolite extraction, cell pellets were resuspended in water (0.1 ml per original culture OD_{600nm}). The suspensions were immediately boiled for 15 min using a block heater, cooled on ice, and vortexed for 2 min. Duplicate samples (7.5 μl ea) were removed for determination of protein concentrations as described below. The remainder of each suspension was centrifuged for 15 min at high speed using a microcentrifuge. The clarified supernatants were stored at -70°C . Metabolite recoveries were tested by addition of standard metabolites (TCA cycle intermediates and related amino acids) to cell suspensions prior to harvesting or prior to boiling and extraction. Recoveries after subtracting cellular metabolite contributions ranged from 43–118% and were consistent for each metabolite. We also tested other cell harvesting procedures including transfer to cold buffered methanol as reported by others [19,20], but we found the most important variable in terms of quenching to prevent changes in levels of metabolites of interest was the addition of cycloheximide and sodium azide prior to harvesting cells. Other extraction procedures, e.g. with chloroform/methanol/water (1:3:1) [21,22], were also tested. As previously reported [20], recoveries of standard metabolites of interest were not substantially different from those obtained by extraction in water.

For derivatization, 10 μl samples of clarified supernatants or of standard metabolite mixtures (including TCA cycle intermediates plus aspartate and glutamate at concentrations ranging from 0–2.0 mM) were diluted with 10 μl of water containing labeled internal standards (1.0 mM [$1,5\text{-}^{13}\text{C}_2$]citrate and [$1\text{-}^{13}\text{C}$]L-glutamate, from Sigma-Aldrich and Cambridge Isotope

Laboratories, respectively) and dried in a microfuge for 3 h using a Speed-Vac. The dry residues were dissolved in 50 μ l of 20 mg/ml *O*-ethylhydroxylamine hydrochloride (Sigma-Aldrich) in pyridine, and reacted for 90 min at 30°C. For subsequent trimethylsilylation, an equal volume of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane (Pierce) was added, and the samples were incubated overnight at room temperature followed by 30 min at 37°C. The samples were transferred to glass inserts fitted into 2 ml clear screw cap vials (Supelco) and tightly capped prior to GC/MS analyses. This derivatization procedure, relative to others tested, provided detection of a broader range of metabolites with fewer derivatization byproducts.

Dry weights were determined using cells pelleted from 1.5 ml culture samples in tared microfuge tubes. The pellets were washed twice with water, then dried for 16–20 h in open tubes placed in a block heater set at 60°C. Dry weights were measured after cooling the tubes for 1–2 h at room temperature; we found that a constant weight was reached using this procedure.

Concentrations of proteins in 7.5 μ l samples taken as described above were determined following solubilization of denatured proteins by diluting samples 1:5 in 8.0 M urea containing 5% β -mercaptoethanol and boiling for 2 min. Following centrifugation using a microfuge for 5 min, samples of the clarified supernatant were used for protein determinations using a modified Bradford assay [23]. Cellular protein concentrations determined in this manner were essentially identical to those obtained following direct lysis of cell pellets for immunoblot analyses as described above, indicating that similar recoveries were obtained with both methods.

GC/MS Analyses

GC/MS analyses were performed on a TRACE DSQ single quadrupole mass spectrometer (Thermo Fisher; San Jose, CA). GC conditions were as follows: column, DB17MS (J&W/Agilent; Santa Clara, CA), 15 m $\times$ 0.25 mm, 0.25 μ m film thickness; carrier gas, helium; linear velocity, 1 ml/min (constant flow); injection, split, 50 ml/min split flow; injector temperature, 220°C; column temperature program, initial temperature of 125°C held for 1 min followed by an increase to 250°C at 20°C/min. MS conditions were: ionization, electron impact (EI, 70 eV); detection, positive ion; full scan analyses, m/z 50 – m/z 650 at two scans/sec. Standard curves were generated from analyses of derivatized samples containing increasing quantities of standard metabolites of interest in the presence of a fixed amount of stable isotope, as described above. Quantitative values were obtained by comparison of experimental peak area ratios with the standard curves through use of the QuantBrowser component of the GC/MS Xcalibur software. Metabolite levels were determined as μ mol/mg dry weight; a value of 2.5 μ l/mg dry weight [20,24] was used to convert the values to cellular concentrations. Similar metabolite levels could be obtained using μ mol/mg protein, since a ratio of ~2.5 mg dry weight/mg protein was observed here and previously [24]. Metabolite concentration profiles for each strain were found to be reproducible for cultures grown under similar conditions and harvested at similar OD_{600nm} values. The latter was important since, as previously reported [20], changes in concentrations of metabolites of interest can vary as a function of growth phase.

Results

Growth phenotypes and protein expression in *idh* Δ *cit1* Δ and *aco1* Δ *cit1* Δ mutants

Yeast mutants lacking IDH or ACO1 have been reported to share some growth phenotypes, including an inability to grow with acetate as a carbon source [14] and a propensity for generation of petite segregants [10]. To better understand the phenotypes exhibited by IDH mutants and their suppression by CIT1 defects, we wished to first compare the properties of

idhΔ and *idhΔ cit1 Δ* mutant strains with those of *aco1 Δ* and *aco1 Δcit1 Δ* mutant strains. In initial experiments with previously constructed *aco1 Δ* gene disruption strains [10,14], we could not retrieve any respiratory-competent colonies capable of growth with non-fermentable carbon sources, i.e. these strains had become entirely petite during storage. Therefore, we disrupted the *ACO1* gene anew in our parental strain and in the *cit1 Δ* disruption strain. For each experiment in the current study, strains recovered from storage at -70°C were streaked onto YP glucose plates, and large red colonies were used to inoculate cultures. This size and color indicate respiratory competence with this strain's genetic background [9]. This procedure was necessary for analyses of *idhΔ* and *aco1 Δ* strains because of their tendency to produce respiratory-deficient petite segregants. We note that results presented for *aco1 Δ* and *aco1 Δcit1 Δ* strains are novel, because these precautions were not taken in previous studies [10, 14]

In initial tests of growth on plates with galactose as the carbon source (Fig. 1), all mutant strains were found to grow well on rich medium (YP) plates, although differences were noted in doubling times for YP galactose cultures: 3.3–3.4 h for parental, *cit1 Δ*, and *idhΔcit1 Δ* strains; ~5.1 h for *idhΔ* and *aco1 Δcit1 Δ* strains; and ~9.1 h for the *aco1 Δ* strain. Thus, *idhΔ* and *aco1 Δ* strains grew more slowly than the strains also containing the *CIT1* gene disruption. On minimal medium (YNB) plates (plus or minus 0.01% glutamate), the *cit1 Δ* mutant strain was found to grow as well as the parental strain (Fig. 1). The *idhΔ* mutant strain exhibited impaired growth on the minimal medium plates (plus or minus glutamate), whereas colony sizes for the *idhΔcit1 Δ* strain were similar to parental colony sizes, again indicating that co-disruption of the *CIT1* gene suppresses the slow growth of the *idhΔ* strain. In contrast, the *aco1 Δ* and *aco1 Δcit1 Δ* mutant strains exhibited no growth in the absence of glutamate on minimal medium plates. Even with glutamate supplementation, the *aco1 Δ* strain exhibited very poor growth, whereas colony sizes for the *aco1 Δcit1 Δ* strain were similar to those for the *idhΔ* strain. Thus, co-disruption of the *CIT1* gene enhances growth of both the *idhΔ* strain and the *aco1 Δ* strain in these growth tests. Similar results were obtained using plates with glucose as the carbon source (data not shown).

We also found that supplementation of YNB galactose plates with higher concentrations of glutamate (to 1%) only slightly improved the growth of the *aco1 Δ* strain, suggesting that other components present in rich YP medium are needed for optimum growth. The *idh1 Δ* mutant is not a glutamate auxotroph due to the presence of NADP-specific isocitrate dehydrogenases (IDPs), which are alternate cellular sources of α -ketoglutarate [25]. Mitochondrial IDP1 is constitutively expressed, and expression of cytosolic IDP2 is induced by growth with non-fermentable carbon sources and with galactose ([10,18] and as described below).

In other assays, the parental and *cit1 Δ* strains exhibited similar low frequencies of generation of respiratory-deficient petite colonies (Fig. 2), whereas the *idhΔ* and *aco1 Δ* strains respectively exhibited frequencies ~12-fold and 18-fold higher, respectively, than that of the parental strain. These frequencies were reduced to parental strain levels in the *idhΔcit1 Δ* strain and to a level ~4-fold that of the parental strain in the *aco1 Δcit1 Δ* strain. Thus, co-disruption of *CIT1* dramatically reduces the petite frequencies observed for both *idhΔ* and *aco1 Δ* strains.

Previous global analyses of gene expression in yeast TCA cycle mutants indicated that changes observed in an *idhΔ* mutant strain were less dramatic in an *idhΔcit1 Δ* strain, which exhibited expression patterns more similar to those of the parental strain [10]. We therefore used immunoblots to compare expression of some representative proteins in these strains and in the *aco1 Δ* and *aco1 Δcit1 Δ* strains in our collection. For these and subsequent experiments, strains were grown with YP galactose medium, since this medium is non-repressing for mitochondrial function and is permissible for growth of these (Fig. 1) and other yeast TCA cycle mutants [10]. As shown by representative results in Fig. 3A, expression levels of all proteins analyzed

(relative to control levels of β -actin) were similar in parental and *cit1* Δ strains. In contrast, cellular levels of the IDH subunits (IDH1 and IDH2) were substantially elevated (~three-fold relative to parental strain levels, as determined by densitometry) in extracts from the *aco1* Δ strain, but were not elevated in extracts from the *aco1* Δ *cit1* Δ strain. Using an antiserum that recognizes both cellular isozymes of NADP⁺-isocitrate dehydrogenase [18], expression of mitochondrial IDP1 was found to be constitutive in all strains (Fig. 3A), whereas cytosolic IDP2 expression was induced in the *idh* Δ strain, as mentioned above. IDP2 levels were similarly elevated in extracts from the *aco1* Δ strain.² Expression of IDP2 was reduced in the *idh* Δ *cit1* Δ strain (to ~40% of the level in the *idh* Δ strain), and IDP2 was not observed in the *aco1* Δ *cit1* Δ strain. We also examined levels of the mitochondrially-encoded COX2 and COX3 proteins (subunits 2 and 3 of cytochrome *c* oxidase), since these were reported to be reduced in an *idh* Δ strain [10,12]. Reductions to <2% of the parental strain levels of COX2 and COX3 were observed for both *idh* Δ and *aco1* Δ strains (Fig. 3A), but not for the *idh* Δ *cit1* Δ or *aco1* Δ *cit1* Δ strains which exhibited parental levels of these proteins. In contrast, levels of the nuclearly-encoded COX4 protein (subunit 4 of cytochrome *c* oxidase) were generally similar in all parental and mutant strains.

Thus, in these examples of expression of various key proteins, effects observed due to disruption of *IDH* or *ACO1* genes are mitigated by co-disruption of *CIT1*. Interestingly, within the group of proteins exhibiting altered levels in *idh* Δ and *aco1* Δ strains, levels of nuclearly-encoded proteins (IDP2, and IDH1 and IDH2 in the *aco1* Δ strain) are elevated, whereas levels of mitochondrially-encoded proteins (COX2 and COX3) are reduced.

Analyses of metabolite levels

Among possible explanations for the suppression effect of *CIT1* gene disruption on various phenotypes and properties of *idh* Δ and *aco1* Δ mutant strains is restoration of levels of some metabolite(s) that may be detrimentally altered in these strains relative to the parental strain. To examine this possibility, we used GC/MS to quantify relevant metabolites. Reconstructed total ion current chromatograms obtained for cellular extracts were quite complex, indicating recovery of many different metabolites (data not shown). For the current study, we focused quantitative analyses on measurements of metabolites that demonstrated significant changes in mutant strains. Levels were quantified as described in Materials and Methods using standard curves generated for TCA cycle intermediates and key amino acids.

As shown in Table 1, citrate and succinate were the most abundant TCA cycle intermediates in extracts from the parental strain grown with YP galactose medium. The level of glutamate was the highest among metabolites analyzed, consistent with previous reports that this is an abundant free amino acid in yeast cells [26,27]. In contrast, the level of aspartate was more similar to those of abundant TCA cycle metabolites. Levels of some metabolites in the *cit1* Δ strain differed relative to the parental strain. Surprisingly, citrate and aconitate levels were elevated ~two-fold in this strain; this finding is discussed in more detail below. Isocitrate and α -ketoglutarate levels were reduced to ~25–30%. Notably, malate was elevated ~four-fold and oxaloacetate was elevated ~three-fold, as might be expected for a defect at this point in the TCA cycle. Aspartate levels were also elevated three-fold, suggesting that excess malate may be transaminated to produce aspartate. The *cit1* Δ strain also exhibited a substantial decrease in levels of the most abundant metabolite glutamate (to ~50% of the parental strain level).

The most dramatic changes in metabolite levels for the *idh* Δ mutant strain were substantial increases relative to the parental strain in levels of the tricarboxylic acids (~12-fold for citrate,

²We note that expression of IDP2 in an *aco1* Δ mutant strain was not observed in a previous report [10], presumably due to use of a petite derivative of the strain in that study.

65-fold for aconitate, and ~seven-fold for isocitrate) and a decrease in glutamate (to ~50% of the parental strain level). For the *idhΔcit1* Δ mutant strain, levels of citrate and of aconitate were elevated (five- and 16-fold, respectively, relative to the parental strain) but much more moderately than observed for the *idh*Δ strain. Isocitrate in the *idhΔcit1* Δ strain was comparable to the parental level, but the glutamate level was reduced to ~38%. For the *aco1* Δ mutant, citrate was also substantially elevated (~12-fold relative to the parental strain level), aconitate and isocitrate were below limits of detection, and the level of glutamate was reduced (to ~40% of the parental strain level). For the *aco1* Δ*cit1* Δ mutant, citrate was comparable to the parental strain level, while glutamate was reduced to ~24%. Aspartate was elevated relative to the parental level in extracts from the *aco1* Δ and *aco1* Δ*cit1* Δ strains (~eight-fold and 12-fold, respectively), again suggesting that these lesions in the TCA cycle result in increased transamination of malate to form aspartate. Among other changes in these strains, the level of succinate was slightly elevated in the *idh*Δ strain and reduced to ~16% in the *aco1* Δ strain, whereas levels of succinate were more similar to the parental level in the *aco1* Δ*cit1* Δ strain. In all mutant strains, oxaloacetate levels were elevated three- to seven-fold, and α-ketoglutarate was essentially undetectable.

Metabolite concentrations were also determined using extracts from these strains grown in rich YP medium with glucose as the carbon source (data not shown). In general, levels of tricarboxylic acid cycle intermediates were slightly lower (~50%) in glucose-grown cells as compared with galactose-grown cells; for the parental strain, metabolite levels were comparable to those reported in other analyses of glucose-grown yeast strains [20,27,28]. However, compared to the parental strain, relative levels of metabolites for mutant strains grown with glucose were very similar to those observed with galactose.

These measurements suggest that the most dramatic and consistent correlation between phenotypes observed for *idh*Δ and *aco1* Δ mutants (e.g., increased petite frequencies and changes in protein expression) is an increase in cellular citrate to dramatic levels in excess of 70 mM. These levels are reduced upon co-disruption of the *CIT1* gene, which also suppresses various phenotypes of these strains. There is no strong correlation with levels of other metabolites. For example, while glutamate levels were low in *idh*Δ and *aco1* Δ mutants, levels remained low in the *idhΔcit1* Δ and *aco1* Δ*cit1* Δ mutants. Thus, glutamate levels exhibit little correlation with suppression of growth phenotypes.

It is interesting that the cellular level of citrate in the *cit1* Δ mutant strain is ~two-fold higher than the parental strain level (Table 1), suggesting that the non-mitochondrial CIT2 enzyme, which functions in the glyoxylate cycle, might compensate for synthesis of this metabolite. However, as shown in Fig. 3B, we found that immunochemical levels of CIT2 were approximately equivalent in parental and *cit1* Δ strains, and reduced ~50% in *aco1* Δ mutant cell extracts. Levels of CIT2 were elevated ~four-fold in the *idh*Δ mutant, reduced to near parental strain levels in the *idhΔcit1* Δ mutant, and undetectable in extracts from the *aco1* Δ*cit1* Δ strain. Thus, expression of CIT2 is quite different in *idh*Δ and *aco1* Δ mutants but appears to be suppressed by co-disruption of the *CIT1* gene in both strains. For the *cit1* Δ strain, it may be that basal levels of expression of CIT2 are sufficient for maintaining cellular levels of citrate. Some citrate may also accumulate as a result of the reversible reaction catalyzed by ACO1.

Effects of exogenous citrate

Since results presented above suggest that accumulation of endogenous citrate might result in the elevated petite frequencies of *idh*Δ and *aco1* Δ strains and in altered patterns of expression of certain proteins in these strains, we tested if similar results could be obtained by adding citrate exogenously to a culture of the parental strain. We found no changes in terms of petite frequencies of the parental strain upon addition of citrate to 5 mM in the medium. However,

these petite frequencies (2–3%) were increased with higher concentrations, two-fold with 10 mM citrate, ~six-fold increase with 50 mM citrate, and ~eight-fold with 100 mM citrate. Thus, exogenous citrate at levels approximating endogenous levels in *idhΔ* and *aco1Δ* strains does increase the petite frequency of the parental strain. We also examined changes in key protein expression in the parental strain in response to increased citrate levels in the medium. As shown in Fig. 4, extracts from parental cells grown with citrate levels as high as 100 mM exhibited little change in levels of IDH1 and IDH2, while IDP2 was only barely detectable at the 50 mM and 100 mM concentrations. Levels of COX2 and COX3, however, were noticeably reduced (to 5–20% of control levels) in extracts of the parental strain grown with 50 mM or 100 mM citrate. Collectively, these results suggest that elevated citrate levels are directly connected to an increase in petite frequency and to reduced expression of the mitochondrially-encoded proteins.

As a correlate, we predicted that addition of exogenous citrate might also exacerbate the petite frequencies of mutant strains. As shown in Fig. 5, relative to petite frequencies measured in the absence of exogenous citrate (A), the addition of 10 mM citrate to the growth medium (B) had little effect on petite frequencies for parental and *cit1Δ* strains, but dramatically increased these frequencies for the *idhΔ* and *aco1Δ* strains to >90%. The *idhΔcit1Δ* and *aco1Δcit1Δ* strains also exhibited increased petite frequencies (~seven-fold and five-fold, respectively) in the presence of 10 mM citrate, but these frequencies were still considerably lower than those of the *idhΔ* and *aco1Δ* strains.

Effects of exogenous iron and medium pH

Reasoning that some of the effects of elevated citrate levels in *idhΔ* and *aco1Δ* mutant strains might be due to the abundant ionized form of the metabolite, we tested (a) the effect of addition of iron (as FeSO₄) as a potential chelating agent to the medium and (b) the effect of an increase in medium pH from 6.8 to 7.8. Since elevated iron can itself have a toxic effect, we first established that addition of 1–2 mM FeSO₄ had little effect on growth rates of parental and mutant strains in culture, whereas concentrations greater than 2 mM resulted in slower doubling times. The elevated pH of the medium was similarly found to have little effect on growth rates. The effects on petite frequencies of the addition of 1 mM FeSO₄ or of an increase in the pH of the medium are shown in Figs. 5C and 5D, respectively. Both conditions had similar effects. There was no substantial difference in petite frequencies for parental, *cit1Δ*, *idhΔcit1Δ*, and *aco1Δcit1Δ* strains under either condition relative to those obtained under normal growth conditions (Fig. 5A). In contrast, petite frequencies of *idhΔ* and *aco1Δ* strains obtained under normal growth conditions (Fig. 5A) were reduced ~50% by either the addition of iron (Fig. 5C) or by an increase in medium pH (Fig. 5D).

We also examined the expression profiles for key reporter proteins in strains grown with exogenous iron or at an elevated medium pH. We observed little change in profiles for the parental strain under these conditions (Figs. 6A and 6B), or for the *cit1Δ*, *idhΔcit1Δ*, and *aco1Δcit1Δ* mutant strains (data not shown), as might be predicted by the absence of effects on petite frequencies (Fig. 5). With 1 mM FeSO₄ in the medium (relative to no addition, Fig. 6A), extracts from both *idhΔ* and *aco1Δ* strains exhibited increases in COX2 levels (~ten-fold and four-fold, respectively), and in COX3 levels (~ten-fold and three-fold, respectively). With an increase in medium pH to 7.8 (relative to pH 6.8, Fig. 6B), extracts from both *idhΔ* and *aco1Δ* strains exhibited increases in levels of COX2 (~ten-fold and three-fold, respectively) and in COX3 levels (~four-fold in both strains). Thus, both conditions expected to neutralize the abundant ionized form of intracellular citrate in *idhΔ* and *aco1Δ* strains, by chelation or by increasing the pH of the medium, correlated with not only a reduction in petite frequencies of these strains but also with an increase in expression of mitochondrially-encoded proteins.

Interestingly, however, levels of IDH1 and IDH2 subunits in the *aco1* Δ strain remained elevated in cells cultivated with added iron or at pH 7.8 (Figs. 6A and 6B). Also, levels of IDP2 levels increased slightly (~two-fold) in the *idh* Δ and *aco1* Δ strains grown with iron or in pH 7.5 medium. There was also a trace of IDP2 detected in the parental strain grown with added iron. These results suggest that the elevated level of total intracellular citrate, irrespective of chelation or neutralization, is the determinant for high levels of IDH and/or IDP2 expression in the *idh* Δ and *aco1* Δ strains.

Discussion

In this report, yeast *aco1* Δ and *idh* Δ mutant strains were shown to exhibit similar elevations in petite frequencies, as well as similarities in TCA cycle metabolite profiles and patterns of expression of several cellular proteins, that were distinct from the parental strain (and from other TCA cycle mutants that we have examined, data not shown). Many of these properties in both *aco1* Δ and *idh* Δ strains were moderated toward parental patterns by concomitant disruption of the *CIT1* gene. In order to conduct these studies, it was necessary to reconstruct mutant strains with *aco1* Δ and *aco1* Δ *cit1* Δ gene disruptions to obtain respiratory-competent colonies. We also note that it was necessary to make the *aco1* Δ *cit1* Δ strain by disruption of the *ACO1* gene in a *cit1* Δ strain that exhibits a low petite frequency, because the opposite approach of disrupting the *CIT1* gene in an *aco1* Δ strain produced only respiratory-deficient transformants.

In analyses of metabolite levels, the most consistent correlation with properties exhibited by the *idh* Δ and *aco1* Δ strains was an elevation in cellular levels of citrate, and suppression of these properties in *idh* Δ *cit1* Δ and *aco1* Δ *cit1* Δ strains coincided with a decrease in citrate levels. In support of these conclusions, increasing exogenous citrate concentrations in the medium used for cultivation of the parental strain was found to produce some of the effects observed in *idh* Δ and *aco1* Δ strains. Also, the petite frequencies of the mutant strains were substantially exacerbated by addition of 10 mM citrate to the medium.

Citrate is already a relatively abundant metabolite in parental cells, and the dramatic elevation in concentrations of this tricarboxylic acid in *idh* Δ and *aco1* Δ strains might be expected to have pleiotropic effects. The elevated intracellular levels of citrate in these mutants (and elevated exogenous citrate levels for the parental strain) appear to be detrimental and result, for example, in higher petite frequencies as well as in reduced expression of mitochondrially-encoded proteins. That these effects are due to ionized form of citrate (i.e. citric acid) is suggested by the mitigation of these properties when the *idh* Δ and *aco1* Δ strains are cultivated with 1 mM FeSO₄ or in medium with elevated pH. The iron is presumably acting as a chelator for the citric acid. In a related study, Matsuno *et al.* [29] reported that sporulation defects observed in *Bacillus subtilis* due to deletion of the gene encoding IDH could be partially bypassed by deletion of the gene encoding citrate synthase or by addition of some divalent cations (including Fe²⁺) to the medium. The former resulted in reduction of cellular citrate levels, similar to observations in the current study, and the latter effect was attributed to chelation and neutralization of excess citrate produced by the *B. subtilis* IDH mutant. Our results showing a reduction in the petite frequencies of *idh* Δ and *aco1* Δ mutants by iron (Fig. 6) are consistent with this hypothesis. Also, results showing similar effects by increasing the pH of the medium further suggest that the intracellular citric acid in these mutants can be titrated to reduce the detrimental effects.

Another study [30] suggested that elevated levels of citrate in yeast cells might be detrimental if this metabolite chelates endogenous cellular iron. This was hypothesized to result in removal of iron needed by some enzymes or even to result in formation of a citrate/Fe³⁺ chelate that could produce oxidative damage to cellular metabolites. However, we measured cellular

activity for aconitase, an iron/sulfur protein frequently used as a reporter for intracellular iron availability [31,32] and found no decrease in activity in *idhΔ* mutant cells. In addition, the hypothesis above would predict that the levels of an oxidatively damaging chelate would be increased in our experiments in which iron was added to cultures of *idhΔ* and *aco1Δ* mutants and, presumably, would result in an increase in petite frequencies. This was clearly not the case (Fig. 5), since addition of iron had the opposite effect, reducing petite frequencies of *idhΔ* and *aco1Δ* strains. Thus, our results do not support a detrimental effect of increased intracellular citrate upon chelation of endogenous iron.

Interestingly, the increase in IDH1 and IDH2 expression in the *aco1Δ* mutant and in IDP2 expression in both the *idhΔ* and *aco1Δ* mutants appears to be less dependent on the high levels of the ionized form of citrate than on the high levels of the metabolite itself. Levels of these proteins were found to be reduced to parental levels only upon co-disruption of the *CIT1* gene, which resulted in concomitant reduction of cellular citrate levels, and not by growth with iron in the medium or in medium with higher pH. These results suggest that citrate itself may be involved as a signal for elevated expression of these nuclearly-encoded genes. In addition, since IDH1/2 and COX4 (which is also nuclearly-encoded and which exhibits little change in expression levels) are mitochondrial proteins, elevated citrate levels in *idhΔ* and *aco1Δ* mutants do not have a general effect on the stability of all mitochondrial proteins. It will clearly be of interest in future studies to determine the relative compartmental concentrations of citrate in *idhΔ* and *aco1Δ* mutants, since our results suggest substantially different effects on mitochondrial functions (petite frequency and levels of mitochondrially-encoded proteins) and on nuclear gene expression.

We were particularly interested in tracking levels of glutamate in *aco1Δ* and *idhΔ* mutant strains, because a low level of this metabolite is postulated to trigger the retrograde (RTG) signaling pathway in yeast [33]. The RTG pathway results in elevated levels of *CIT2* mRNA in yeast cells lacking mitochondrial DNA [34]. Also, most mitochondrial protein expression is under global control of the HAP2-5 transcription system but, as mitochondrial function becomes compromised, expression of *CIT1*, *ACO1*, and *IDH1/2* genes was found to switch to control by the RTG pathway [35], suggesting a cellular need for regulation of glutamate synthesis under this condition. Another indication of a direct correlation is the shared phenotype of glutamate auxotrophy resulting from disruption of individual genes encoding RTG regulatory proteins [36,37], from disruption of *ACO1* [38], and from co-disruption of genes encoding major cellular isozymes of citrate synthase [5] or of isocitrate dehydrogenase [25].

Our results do show a significant decrease in cellular glutamate levels in *aco1Δ* and *idhΔ* mutant strains, but these levels were similarly low in *aco1Δcit1Δ* and *idhΔcit1Δ* mutant strains, suggesting there is no direct correlation between glutamate levels and the suppression of properties of *aco1Δ* and *idhΔ* strains by co-disruption of the *CIT1* gene. Also, we found that levels of the CIT2 protein in an *idhΔ* mutant strain grown on minimal medium were reduced by addition of exogenous glutamate (data not shown), but to a lesser extent than obtained by co-disruption of the *CIT1* gene (Fig. 3B). Thus, we find little correlation between levels of CIT2 and cellular levels of glutamate, suggesting either that CIT2 is not a reliable reporter for the RTG pathway or that the RTG pathway may be responsive to metabolites other than glutamate. However, since our studies have focused on CIT2 protein levels whereas previous analyses of the RTG response involved quantitative analyses of mRNA levels [33,35], it is important that future studies investigate different levels of the response.

ACO1 and IDH have both been described to have bifunctional roles in yeast mitochondria. In addition to TCA cycle functions, ACO1 is involved in mitochondrial DNA maintenance [39], and IDH has been shown to be a mitochondrial mRNA-binding protein [40]. The latter observation led to a hypothesis that IDH might be a regulator of mitochondrial translation

[41]. However, we previously showed that the reduction in levels of mitochondrial translation products (e.g. COX2 and COX3) in an *idhΔ* strain was not observed in an *idhΔ cit1Δ* strain, suggesting that any effect of loss of IDH on translation is indirect [12]. Since, in the current study, we saw similar relative effects on COX2 and COX3 levels in *aco1Δ* and in *aco1Δ cit1Δ* strains, it is possible that elevated cellular levels of citrate in the *idhΔ* and *aco1Δ* strains may detrimentally affect mitochondrial translation or stability of the translation products, and that this effect is reduced by co-disruption of the *CIT1* gene. For ACO1, a distinction between its dual functions was recently demonstrated using mutants expressing catalytically inactive forms of ACO1 [39]. These mutant strains were found to be glutamate auxotrophs (i.e. exhibited a loss of TCA cycle function) but they retained mitochondrial DNA stability, indicating a structural role for the inactive enzyme. ACO1 apparently binds and protects mitochondrial DNA from accumulation of point mutations and single strand breaks [42]. Our results indicated a lower petite frequency for the *aco1Δ cit1Δ* strain as compared to the *aco1Δ* strain, but this frequency was still elevated relative to that of the parental strain. Since citrate levels were approximately equivalent in *aco1Δ cit1Δ* and parental strains, our results also suggest that ACO1 may have a function independent of catalysis in the TCA cycle.

Future studies will focus on developing methods for assessing compartmental distribution of metabolites as mentioned above. We also plan to extend our analyses to other yeast TCA cycle mutants to examine the relevance of changes in metabolite levels in various different phenotypes and alterations in protein expression associated with these strains.

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Fig. 1.

Growth with galactose as the carbon source. The indicated yeast strains were grown and dilutions of cells were plated as described under Materials and Methods on agar plates containing rich YP medium or minimal YNB medium (plus or minus 0.01% glutamate) with galactose as the carbon source.

Fig. 2. Petite frequencies of mutant strains. Petite frequencies for the indicated yeast strains were determined as described under Materials and Methods. Values represent averages ($\pm$ standard deviations) for four independent experiments.

Fig. 3.

Immunoblot analyses of extracts from mutant strains. Immunoblot analyses were conducted as described under Materials and Methods using 5 μ g protein samples prepared from the indicated yeast strains grown with rich YP medium containing galactose as the carbon source. (A) Levels of IDH1 and IDH2 subunits of IDH, of mitochondrial IDP1 and cytosolic IDP2, and of subunits of cytochrome *c* oxidase (mitochondrially-encoded COX2 and COX3, and of nuclearly-encoded COX4) were compared to that of β -actin in each sample using densitometry. (B) Immunochemical levels of CIT2 were examined using proteins samples from each strain in panel A.

Fig. 4.

Immunoblot analyses of extracts from the parental strain grown with various concentrations of citrate. Immunoblot analyses were conducted as described under Materials and Methods using 5 μ g protein samples prepared from the parental grown with rich YP medium containing galactose as the carbon source and with additions of sodium citrate at concentrations indicated. Densitometry was used to quantify levels of proteins relative to that of β -actin in each sample.

Fig. 5.

Effects of citrate, iron, and pH on petite frequencies. Petite frequencies for the indicated yeast strains were determined as described under Materials and Methods using cultures grown in (A) YP galactose medium (pH 6.8), (B) in YP galactose medium (pH 6.8) containing 10 mM sodium citrate, (C) in YP galactose medium (pH 6.8) containing 1 mM FeSO₄, or (D) in YP galactose medium (pH 7.8). Values represent averages ($\pm$ standard deviations) for 3–4 independent cultures. Note differences in scales for values of petite frequencies.

Fig. 6.

Immunoblot analyses of extracts from strains cultivated with iron or at elevated pH.

Immunoblot analyses were conducted as described under Materials and Methods using 5 μg protein samples prepared from the indicated yeast strains grown with rich YP medium containing galactose as the carbon source. (A) The medium used for cultivation lacked added iron (–) or contained 1 mM FeSO₄ (+). (B) The medium used for cultivation had an initial pH of 6.8 or of 7.8 as indicated.

Table 1
Metabolite concentrations in parental and mutant yeast strains^a

Metabolite	Yeast strains: Parental	Cellular concentration (mM)				<i>acil</i> Δ	<i>acil</i> Δ	<i>acil</i> Δ
		<i>cit</i> Δ	<i>idh</i> Δ	<i>idh</i> Δ <i>cit</i> Δ	<i>acil</i> Δ			
citrate	6.34^b (±1.10)	10.6 (±1.20)	74.2 (±6.25)	33.0 (±6.16)	75.7 (±10.1)	8.23 (±2.48)		
aconitate	0.05 (±0.01)	0.10 (±0.02)	3.25 (±0.80)	0.80 (±0.11)	0	0		
isocitrate	0.64 (±0.12)	0.16 (±0.04)	4.79 (±0.28)	0.76 (±0.20)	0	0		
α-ketoglutarate	0.06 (±0.01)	0.02 (±0)	0	0	0	0		
succinate	5.01 (±0.39)	3.71 (±0.14)	8.43 (±1.70)	3.41 (±0.74)	0.81 (±0.17)	2.11 (±0.48)		
fumarate	0.22 (±0.09)	0.69 (±0.21)	0.44 (±0.13)	0.37 (±0.10)	0.72 (±0.21)	0.29 (±0.03)		
malate	1.52 (±0.30)	5.90 (±1.25)	2.06 (±0.56)	4.87 (±0.74)	0.60 (±0.27)	1.63 (±0.42)		
oxaloacetate	0.14 (±0.03)	0.45 (±0.05)	0.92 (±0.11)	0.97 (±0.02)	1.01 (±0.23)	0.47 (±0.06)		
glutamate	130 (±33.8)	71.3 (±14.4)	64.2 (±10.5)	50.4 (±1.63)	51.8 (±0.85)	31.8 (±7.01)		
aspartate	2.10 (±0.79)	6.45 (±1.49)	2.26 (±0.24)	4.03 (±0.73)	17.5 (±6.43)	24.7 (±7.29)		

^aYeast strains were cultivated in YP galactose medium. Metabolite concentrations were determined using GC/MS, as described under Materials and Methods.

^bData represent averages from n = 2 to n = 4 independent measurements (± standard deviations).