Archives of Biochemistry and Biophysics 474 (2008) 205–212

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

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

An-Ping Lin, Kevin W. Hakala, Susan T. Weintraub, Lee McAlister-Henn *

Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900, USA

ARTICLE INFO

Article history:
Received 14 February 2008
and in revised form 5 March 2008
Available online 10 March 2008

Keywords:
Isocitrate dehydrogenase
Aconitase
Citrate synthase
Tricarboxylic acid cycle
Citrate

ABSTRACT

Yeast mutants lacking mitochondrial NAD^+-specific isocitrate dehydrogenase (*idh*Δ) 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 *idh*Δ 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 *idh*Δ and *aco1*Δ strains, whereas citrate levels were substantially lower in *idh*Δ*cit1*Δ and *aco1*Δ*cit1*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 *idh*Δ 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.

© 2008 Elsevier Inc. All rights reserved.

Mitochondrial NAD^+-specific isocitrate dehydrogenase (IDH[1]) 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 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.

* Corresponding author. Fax: +1 210 567 6595.
E-mail address: henn@uthscsa.edu (L. McAlister-Henn).

[1] *Abbreviations 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.

0003-9861/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2008.03.005

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 *idhΔ* mutant strain with *LEU2* and *HIS3* deletion/disruptions in *IDH1* and *IDH2* loci, respectively, and of the *idhΔcit1Δ* mutant strain were previously described [9,14]. Mutant *aco1Δ* and *aco1Δcit1Δ* strains were newly constructed by deletion/disruption mutagenesis using the *kan*MX4 cassette [15] to replace the *ACO1* coding regions in parental and *cit1Δ* 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% Bacto-peptone) 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 $OD_{600nm} \cong 0.1$ 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 $OD_{600nm} \cong 2.0$. Cells were harvested by centrifugation, washed, and resuspended in water to $OD_{600nm} \cong 2.0$. 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 $OD_{600nm} \cong 2.0$). 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} = 1.8–2.0. Growth was stopped by addition of cycloheximide to 100 μg/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*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× 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 reference 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*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% to 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 to 2.0 mM) were diluted with 10 μl of water containing labeled internal standards (1.0 mM [1,5–$^{13}C_2$]citrate and [1-^{13}C]L-glutamate, from Sigma–Aldrich and Cambridge Isotope Laboratories, respectively) and dried for 3 h using a Speed-Vac. The dry residues were dissolved in 50 μl 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 μl 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 microcentrifuge 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 × 0.25 mm, 0.25 μm 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 μmol/mg dry weight; a value of 2.5 μl/mg dry weight [20,24] was used to convert the values to cellular concentrations. Similar metabolite levels could be obtained using μmol/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 idhΔcit1Δ and aco1Δcit1Δ 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 *idhΔcit1Δ* mutant strains with those of *aco1Δ* and *aco1Δcit1Δ* mutant strains. In initial experiments with previously constructed *aco1Δ* 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

Download English Version:

https://daneshyari.com/en/article/8291993

Download Persian Version:

https://daneshyari.com/article/8291993

Daneshyari.com