Metabolism of [3-^{13}C]Pyruvate in TCA Cycle Mutants of Yeast†

Balazs Sumegi,‡,§ Mark T. McCammon,‖ A. Dean Sherry,‡ Daniel A. Keys,⊥ Lee McAlister-Henn,⊥ and Paul A. Srere*,▽

Department of Chemistry, University of Texas at Dallas, Dallas, Texas 75083, Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235, Pre-Clinical Science Unit, Department of Veterans Affairs Medical Center, 4500 South Lancaster Road, Dallas, Texas 75216, and Department of Biological Chemistry, College of Medicine, University of California, Irvine, California 92717

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ABSTRACT: The utilization of pyruvate and acetate by *Saccharomyces cerevisiae* was examined using ^{13}C and ^{1}H NMR methodology in intact wild-type yeast cells and mutant yeast cells lacking Krebs tricarboxylic acid (TCA) cycle enzymes. These mutant cells lacked either mitochondrial (NAD) isocitrate dehydrogenase (NAD-ICDH1), α-ketoglutarate dehydrogenase complex (αKGDC), or mitochondrial malate dehydrogenase (MDH1). These mutant strains have the common phenotype of being unable to grow on acetate. [3-^{13}C]-Pyruvate was utilized efficiently by wild-type yeast with the major intermediates being [^{13}C]glutamate, [^{13}C]acetate, and [^{13}C]alanine. Deletion of any one of these Krebs TCA cycle enzymes changed the metabolic pattern such that the major synthetic product was [^{13}C]galactose instead of [^{13}C]glutamate, with some formation of [^{13}C]acetate and [^{13}C]alanine. The fact that glutamate formation did not occur readily in these mutants despite the metabolic capacity to synthesize glutamate from pyruvate is difficult to explain. We discuss the possibility that these data support the metabolon hypothesis of Krebs TCA cycle enzyme organization.

The ability to produce specific enzyme deficiencies in yeast by either point mutations or deletion of the gene encoding the enzyme offers an opportunity to study both the role of an enzyme's activity in metabolism as well as the possible role of the protein moiety of the enzyme. In addition, if mutants are defective in enzymes that have isoforms with a distinct cellular location or with altered substrate specificity (e.g., NADP instead of NAD), then additional metabolic insights may be gained concerning the interrelation of several metabolic pathways.

We have previously reported metabolic studies on yeast mutants in which (1) mitochondrial citrate synthase (CS1)[1] was deleted (CS1$^-$); (2) peroxisomal citrate synthase (CS2) was deleted (CS2$^-$); (3) both CS1 and CS2 were deleted (CS1$^-$CS2$^-$); and (4) CS1 was replaced with an inactive CS1 (CS1*) (Kispal et al., 1988, 1989). The growth of these mutants on various substrates, the change in various enzyme levels, mitochondrial oxidation of various substrates, and [^{13}C]-acetate metabolism have been examined (Kispal et al., 1988, 1989). In addition, we have reported on the growth constraints of yeast mutant cells lacking either mitochondrial malate dehydrogenase (MDH1) (Steffan & McAlister-Henn, 1991) or mitochondrial (NAD)–isocitrate dehydrogenase (NAD-ICDH1) (Keys & McAlister-Henn, 1988). These mutants (except CS2$^-$ and CS1*) were unable to grow on acetate. Since there are isozymes for each of these enzymes in the yeast cell, one would have expected the isozymes to provide a shunt reaction for the missing enzyme activity. It is puzzling that such was not the case, and explanations for this metabolic conundrum are being sought. (We offer in an explanation for this conundrum in the results.)

We and others have presented evidence for the organization of mitochondrial Krebs TCA cycle enzymes into a metabolic complex [see Srere (1987) for review]. The evidence includes data that show this organization increases the reaction rates through portions of the Krebs TCA cycle (Robinson et al., 1987; Sumegi et al., 1991b,c). We have reported ^{13}C NMR studies on *Saccharomyces cerevisiae* (Sumegi et al., 1990) and a mammalian system (Sumegi et al., unpublished) that indicated substrate channeling in the succinyl CoA synthetase–malate dehydrogenase span of the TCA cycle in vivo. It has also been shown that mutation of mitochondrial enzymes can change mitochondrial metabolism even if the mutant enzymes are not directly involved in the reactions (Sumegi & Porpaczy, 1990; Sumegi et al., 1990, 1992). We have hypothesized that the deletion of certain enzymes disrupts mitochondrial organization and in this way alters mitochondrial metabolism (Kispal et al., 1989).

In the studies reported in this paper, we have examined the metabolism of [3-^{13}C]pyruvate and [2-^{13}C]acetate in the following yeast mutants: (1) deletion mutation of α-ketoglutarate dehydrogenase ($\Delta kgd1^-$), (2) deletion of subunit 1 of NAD-ICHDH1 (*idh1*$^-$) or of subunit 2 of NAD-ICDH1 (*idh2*$^-$), (3) deletion of malate dehydrogenase (*mdh1*$^-$), and

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* Address correspondence to this author at the Department of Veterans Affairs Medical Center, 4500 S. Lancaster Rd., Dallas, TX 75216.

‡ Department of Chemistry, UT at Dallas.

§ Present address: University Medical School, Institute of Biochemistry, Szigeti ut 12, 7624 Pecs, Hungary.

‖ Department of Pharmacology, UT Southwestern Medical Center at Dallas.

⊥ University of California.

▽ Department of Veterans Affairs Medical Center and Department of Biochemistry, UT Southwestern Medical Center at Dallas.

[1] Abbreviations: TCA, tricarboxylic acid; PDC, pyruvate dehydrogenase complex; CS1, mitochondrial citrate synthase; CS1$^-$, mutant lacking CS1; CS2$^-$, mutant lacking CS2; CS1*, inactive site-directed mutant of CS1; CS2, peroxisomal citrate synthase; MDH1, mitochondrial malate dehydrogenase; MDH1$^-$, deletion mutation of MDH; MDH2, peroxisomal malate dehydrogenase; MDH2$^-$, mutant lacking MDH2; NAD-ICDH1, mitochondrial NADP–isocitrate dehydrogenase; NAD-ICDH1$^-$, deletion mutation of mitochondrial (NAD)–isocitrate dehydrogenase; αKGDC, α-ketoglutarate dehydrogenase complex; αKGDC1$^-$, αKGDC deletion mutation; IDH1, isocitrate dehydrogenase subunit 1; IDH2, isocitrate dehydrogenase subunit 2.

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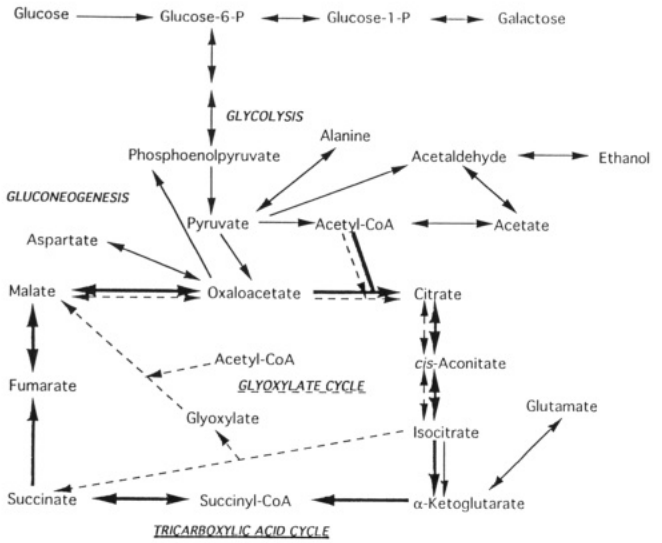

FIGURE 1: Pyruvate metabolism in *Saccharomyces cerevisiae*, modified from Gancedo and Serrano (1989).

Table I: Genotypes and Sources of *Saccharomyces cerevisiae* Strains

strain	relevant genotype[a]	source or reference
W303ΔKGD1	*MATα ade2-1 his3-11.15 leu2-3.112 trp1-1 ura3-1 can1-100 kgd1::URA3*	Repetto & Tzagoloff (1989)
MMYO11	*MATα ade2-1 his3-11.15 leu2-3.112 trp1-1 ura3-1 can1-100* Ole$^+$	McCammon et al. (1990)
A10-4	*idh1-1*	MMY011
G33-3	*idhl-2*	MMY011
T14-3	*idhl-3*	MMYO30, McCammon et al. (1990)
A14-3	*idh2-1*	MMY011
G30-3	*idh2-2*	MMY011
T12-3	*idh2-3*	MMYO30
A21-3	*mdh1-3*	MMY011
G36-4	*mdh1-5*	MMY011
G39-1	*mdh1-6*	MMY011
G36-5	*mdh2-3*	MMY011

[a] Only genetic changes from parental strain are noted.

(4) mutation in peroxisomal malate dehydrogenase (*mdh2*$^-$). Pyruvate metabolism in yeast is illustrated in Figure 1. The use of ^{13}C-labeled substrates and subsequent or real-time NMR analyses of progressively-labeled intermediates has proven to be a powerful analytic method for following rates of interacting pathways. Especially useful are various ^{13}C isotopomer analyses that yield important quantitative data unobtainable from ^{14}C tracer studies.

EXPERIMENTAL PROCEDURES

Strains. The genotypes and sources of yeast strains used in this study are listed in Table I. The α-ketoglutarate dehydrogenase complex (KGDC1) W303ΔKGD1 deletion mutant was generated and characterized as described (Repetto & Tzagoloff, 1989). Isolation and characterization of acetate$^-$ mutants from strain MMYO11 are described by M. T. McCammon and J. M. Goodman (unpublished data). Isocitrate dehydrogenase subunit 1 (*IDH1*) and isocitrate dehydrogenase subunit 2 (*IDH2*) mutants were identified from this collection as described earlier (Keys & McAlister-Henn, 1988). Western blots for the IDH and MDH mutants are shown in Figure 2.

Yeast cells were grown in standard YP medium (1% yeast extract and 2% peptone) with 0.25% glucose and 2% galactose as carbon sources. Cells were harvested in the late log phase by centrifugation at 4000 rpm for 10 min.

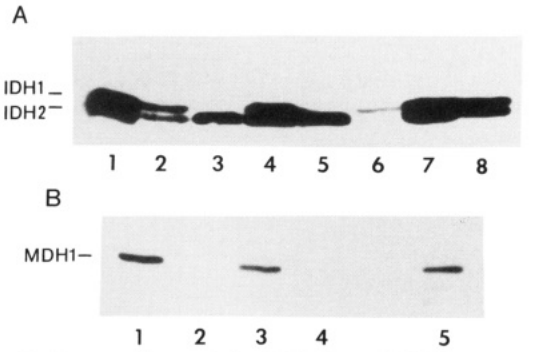

FIGURE 2: Immunoblot analysis of ICDH$^-$ and MDH$^-$ mutants. (A) Whole-cell protein extracts (100 μg) from yeast mutants in two complementation groups defective in mitochondrial NAD-dependent isocitrate dehydrogenase activity were probed with antiserum specific for yeast NAD-ICDH1 (Keys & McAlister-Henn, 1988). Wild-type strain MMY011 (lane 2), *idh1-1* strain A10-4 (lane 3), *idh1-2* strain G33-3 (lane 4), *idh1-3* strain T14-3 (lane 5), *idh2-1* strain A14-3 (lane 6), *idh2-2* strain G30-3 (lane 7), and *idh2-3* strain T12-3 (lane 8) are shown. Lane 1 contained 0.2 μg of purified yeast NAD-ICDH1. Transformation of the *idh2-1* strain with the cloned *IDH2* gene has been shown to restore expression of levels of IDH2 equivalent to wild type (Cupp & McAlister-Henn, 1991). (B) Whole-cell protein extracts (100 μg) from yeast mutants in a complementation group defective in mitochondria malate dehydrogenase activity were probed with antiserum specific for yeast MDH1 (Steffan & McAlister-Henn, 1991). Wild-type strain MMY011 (lane 1), *mdh1-3* strain A21-3 (lane 2), *mdh1-5* strain G38-4 (lane 3), and *mdh1-6* strain G39-1 (lane 4) are shown. Lane 5 contained 0.5 μg of purified yeast MDH1.

Pyruvate Oxidation. Packed yeast cells (2 g) were resuspended in 10 mL of minimal medium [yeast nitrogen base (Difco)] containing 5 mg of [3-^{13}C]pyruvate (sodium salt) (4.5 mM). Experiments in which cells were washed to remove residual substrate before incubation with [^{13}C]pyruvate showed no differences in the results reported. The resuspended cells were incubated at 30 °C for 30 min with vigorous shaking to supply the required amount of oxygen. The reaction was stopped by adding perchloric acid (4% final concentration). The neutralized supernatant was freeze-dried, and the resulting powder was redissolved in 0.65 mL of D_2O. When necessary, paramagnetic ions were removed by chromatography on a chelating resin.

Acetate Oxidation. Packed yeast cells (2 g) were resuspended in 10 mL of minimal medium containing 5 mg of [2-^{13}C]acetate (sodium salt) (6.1 mM). In some cases, 0.5 mg of aspartate and 0.5 mg of malate were also added as indicated. Yeast cells were incubated at 30 °C for 30 min under the same conditions as described under Pyruvate Oxidation. We supplied the yeast cells with a small quantity of aspartate to ensure oxalacetate availability in the yeast mitochondria. All following steps were the same as described under the Pyruvate Oxidation experiments.

NMR Spectroscopy. High-resolution ^{13}C and ^{1}H NMR spectra were recorded on a GN-500 spectrometer at 11.75 T. The number of scans used for each sample varied between 6000 and 8000. All spectra reported in this work were acquired by using a 45° carbon pulse and 6-s delay between pulses to ensure nonsaturating conditions. All samples were maintained at 25 °C during data acquisition. Proton spectra of the extracts were run by using a 5-s presaturation pulse on the HOD resonance followed by a 90° observe pulse and a 5-s delay. The areas of the ^{13}C satellite resonances centered around the alanine methyl resonance at 1.47 ppm and about the acetate methyl resonance at 1.9 ppm were quantitated using peak

areas or peak heights to determine the fractional enrichments in these two carbon pools. In addition, integration of the glutamate C4 proton signal provided information about the relative quantity of unlabeled glutamate in the mutant cells. Determination of the quantity of labeled glutamate from the proton spectra was not possible because of overlap of the glutamate satellite resonances with other resonances. Qualitatively one can conclude from the spectra that there was not a significant quantity of ^{13}C-labeled glutamate (C4) in any of the mutants (see below).

RESULTS

The isolation of yeast mutants blocked at various stages of the TCA cycle has allowed for the initial characterization of the importance of both the catalytic function and structural interactions of these proteins. The yeast mutants were isolated from the genetic background of W303 or its direct descendants, MMY011 or MMYO30 (McCammon et al., 1990). The strain W303ΔKGD1 was constructed by Repetto and Tzagoloff (1989) by deleting the αKGD1 gene. Hence, this strain lacks the 114-kDa α-ketoglutarate dehydrogenase protein (*Δkgd1*$^-$) of the αKGDC. The remaining mutants were selected from a collection of mutants unable to grow on acetate (McCammon and Goodman, unpublished observations). While the mutations have not been defined at the nucleotide level, their effects on the encoded proteins were characterized by probing cell extracts using antisera prepared against the purified enzymes. In order to study the most severe defect in a particular enzyme, only those mutants lacking the protein as well as the enzyme activity were used subsequently. Strains A10-4 and A14-3 lacked subunit 1 (40 kDa, *idh1*$^-$) and subunit 2 (39 kDa, *idh2*$^-$) of the NAD-ICDH1, respectively; despite the absence of one subunit, the other subunit of this complex is still present in these strains (Figure 2A). The *IDH2* gene was recently cloned by complementation (Cupp & McAlister-Henn, 1991). Strain A21-3 lacked the 33.5-kDa MDH1 (*mdh1*$^-$; Figure 2B). Finally, a mutant expressing a detectable but nonfunctional 40-kDa peroxisomal malate dehydrogenase (MDH2) (*mdh2*$^-$; Minard & McAlister-Henn, 1991) was used to compare a block in the peroxisomal glyoxylate cycle portion of gluconeogenesis (McCammon et al., 1990) with the blocks in Krebs cycle function described above. While none of these mutants can grow with acetate as a sole carbon and energy source, only the αKGDC mutant was unable to grow on glycerol as a sole carbon and energy source. The difference between the two classes of mutants is probably due to the presence of other genes encoding functional isozymes for MDH and ICDH that can bypass the genetic blocks to allow for some growth on the C3 compound. Transformation of mutant strains with *MDH2* (Minard & McAlister-Henn, 1991) or *IDH2* (Cupp & McAlister-Henn, 1991) genes restores growth on acetate media.

When wild-type yeast cells were incubated with [3-^{13}C]-pyruvate, the major products labeled with ^{13}C were glutamate, acetate, and alanine (Figure 3A). The ratio of ^{13}C enrichment in the glutamate protonated carbons (C2/C4) was near unity, indicating that either the system was near isotopic steady state or the flux of [3-^{13}C]pyruvate into the OAA pool via pyruvate carboxylase was relatively high compared to the conversion of pyruvate to [2-^{13}C]acetyl-CoA via the pyruvate dehydrogenase complex (PDC).

MDH Mutants. The metabolism of [3-^{13}C]pyruvate in a yeast mutant lacking MDH1 protein showed little formation of glutamate from pyruvate (Figure 3B). There was formation of acetate from pyruvate, but most of the pyruvate was converted to alanine. Since pyruvate can be carboxylated to oxalacetate and can also be converted to acetyl-CoA in these mutants, one might expect many of the Krebs cycle intermediates to accumulate (for example, citrate). However, only small amounts of glutamate was detected (Figure 3B). In separate proton NMR studies (see below), the MDH1 mutant contained a significant quantity of aspartate which could generate oxalacetate by transamination; this indicated that lack of an acceptor for acetyl-CoA was not the reason for the lack of TCA cycle intermediates.

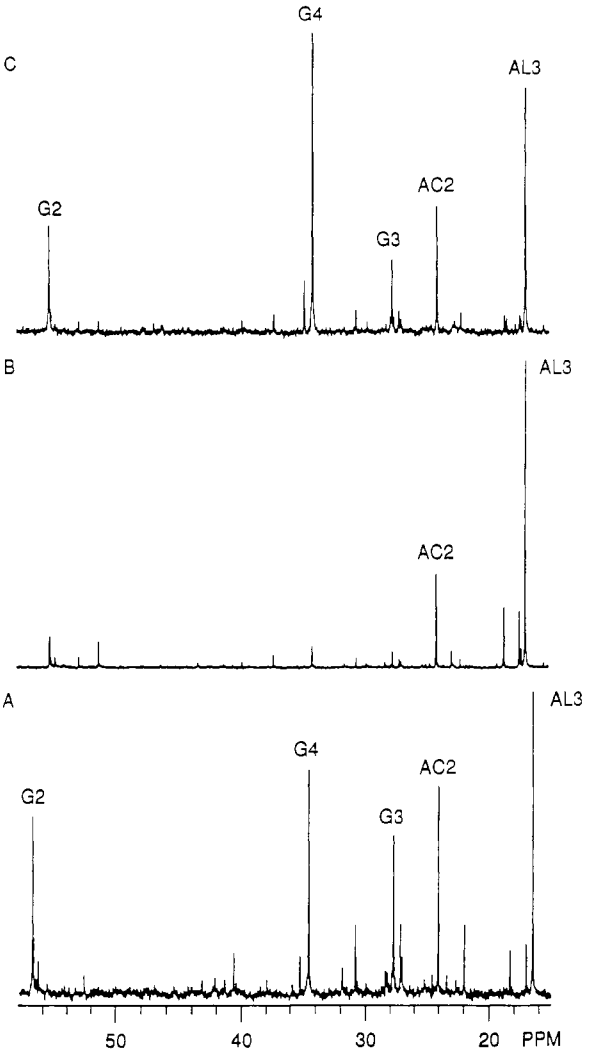

FIGURE 3: ^{13}C NMR spectra showing the metabolic effect of mutation of mitochondrial and cytoplasmic malate dehydrogenase on oxidation of [3-^{13}C]pyruvate. (A) Parental strain MMY011; (B) mitochondrial malate dehydrogenase mutant; (C) cytoplasmic malate dehydrogenase mutant. Resonances labeled G2, G3, and G4 correspond to glutamate C2, C3, and C4. AC2 corresponds to acetate C2, and AL3 corresponds to alanine C3.

The yeast mutant strain G38-5 lacking MDH2 (Minard & McAlister-Henn, 1991) showed some differences in its metabolism of [3-^{13}C]pyruvate when compared to the wild-type cell (Figure 3C). Although glutamate once again becomes enriched, there was now a large difference in ^{13}C enrichment of glutamate-C4 versus glutamate-C2 (and C3) indicating that either isotopic steady state had not been reached in this system or that there was less flux of [3-^{13}C]pyruvate into the oxalacetate pool.

Isocitrate Dehydrogenase Mutant. NAD-ICDH1 is composed of two protein subunits. In the NAD-ICDH1$^-$ mutant strain A14-3 lacking subunit 2, pyruvate utilization was greatly diminished. There was relatively little alanine formation and

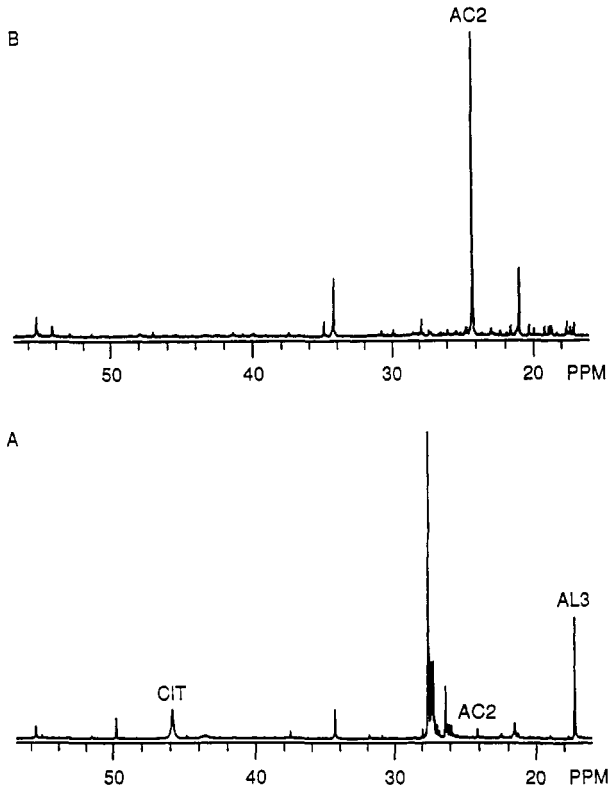

FIGURE 4: ^{13}C NMR spectra of extracts of cells incubated with [3-^{13}C]pyruvate. (A) α-Ketoglutarate dehydrogenase complex mutant. The major unlabeled peak at 27.5 ppm is [3-^{13}C]pyruvate. (B) NAD-dependent isocitrate dehydrogenase mutant (A14-3). AC2 corresponds to acetate C2, AL3 corresponds to alanine C3, CIT corresponds to citrate C2 and C4, and PY3 corresponds to pyruvate-C3.

a small amount of acetate formation (Figure 4A) which varied from experiment to experiment. Also, very little [^{13}C]-glutamate was observed although some [^{13}C]citrate was detected that was not observed in the MDH1$^-$ mutant or in the wild-type strain. Similar data were seen when the disruption was in component 2 of NAD-ICDH1 (data not shown). It has been shown previously that there are no changes in NADP-ICDH, MDH1, fumarase, aconitase, αKGDC, or CS activities in the mitochondria of this mutant when compared to the wild type (Keys & McAlister-Henn, 1988).

αKGDC Mutants. In a yeast strain lacking the α-ketoglutarate dehydrogenase component of αKGDC, little conversion of pyruvate to alanine was observed while substantial oxidation to acetate was detected (Figure 4B). Again, little glutamate was formed in the mutant even though the block in the TCA cycle is located after steps involved in the formation of glutamate.

Pool Sizes. It was possible to estimate the relative quantities of acetate, alanine, and glutamate (γ protons) in the [^{13}C]-pyruvate experiments in the wild-type and mutant strains by using ^{1}H NMR. Also, the ^{13}C fractional enrichment in the acetate and alanine methyl carbons could be determined from the area of the ^{13}C satellite resonance. These data (Figure 5 and Table II) indicated that the pool size of acetate increased about 3-fold in the αKGDC mutant, while the pool size of alanine was about 3.5-fold higher in the MDH mutant. Qualitatively, the pool size changes for all other metabolic intermediates or products did not appear to be more than ±50%. The ^{13}C fractional enrichment in the acetate methyl carbon did not change dramatically in any of the mutants, while the alanine C3 enrichment was significantly less in all mutants, approximately 2–4-fold lower than the wild type. If

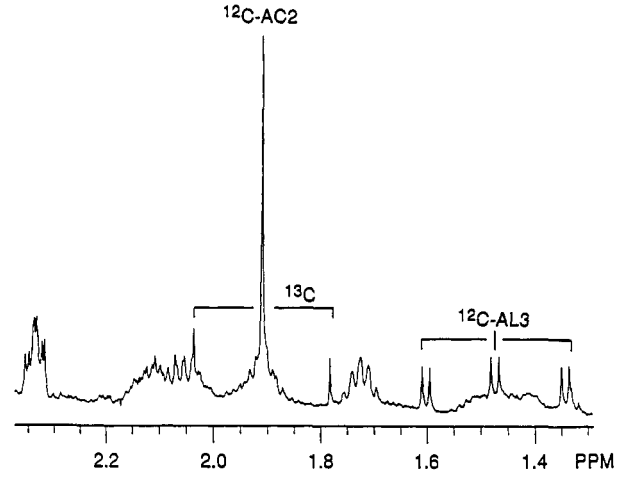

FIGURE 5: ^{1}H NMR spectrum of an extract of wild-type yeast cells during oxidation of [3-^{13}C]pyruvate. The singlet at 1.9 ppm corresponds to the methyl protons of [^{12}C]acetate and the doublet at 1.47 ppm corresponds to the methyl protons of [^{12}C]alanine. The ^{13}C satellite resonances equally spaced around the center acetate and alanine resonances reflect the ^{13}C enrichment in each of these pools.

Table II: Changes in the Pool Sizes in TCA Cycle Mutant Yeast Cells during the Oxidation of [3-^{13}C]Pyruvate[a]

	acetate C2		alanine C3		
cell lines	pool size (%)	enrich-ment (%)	pool size (%)	enrich-ment (%)	glutamate C4 pool size (%)
wild type	100	21.2 ± 2	100	68.3 ± 5	100
MDH1$^-$	126 ± 15	19 ± 1	353 ± 30	28.3 ± 3	142 ± 15
ICDH2$^-$	103 ± 12	18 ± 2	79 ± 10	23 ± 3	144 ± 18
KGDC1$^-$	296 ± 25	27 ± 4	93 ± 11	12.9 ± 2	148 ± 20

[a] Experimental conditions are the same as described under Experimental Procedures.

the alanine–pyruvate–acetyl-CoA–citrate conversion is significant under our experimental conditions, then the decrease in ^{13}C fractional enrichment in alanine could, at least partially, decrease the labeling in glutamate. On the other hand, if the pyruvate–acetaldehyde–acetate–acetyl-CoA–citrate pathway is dominant, then the decrease in the ^{13}C labeling of glutamate cannot be attributed to dilution by the unlabeled intermediates because the ^{13}C fractional enrichment of acetate did not change significantly in the mutant cells.

Gluconeogenesis. There was very limited gluconeogenic activity in the wild-type yeast cells under the conditions used here (Figure 6A). In contrast, the MDH1 mutant converted a significant portion of [^{13}C]pyruvate into all the carbon atoms of galactose (Figure 6B). The identification of these resonances as galactose and not glucose is based upon the differences in the chemical shift values of the ^{13}C resonances of these sugars as reported by Bock and Pedersen (1983). This was also true for the ICDH and αKGDC mutants (data not shown). Multiplets were not observed in the galactose resonances, so it is likely that the ^{13}C labeling was randomized. The scrambling at fumarase can produce [2-^{13}C]- and [3-^{13}C]-pyruvate from [3-^{13}C]pyruvate, and the scrambling at glycerol can produce [1-^{13}C]- and [3-^{13}C]glycerol from [3-^{13}C]glycerol without producing multiply labeled intermediates.

Acetate Oxidation. Even though the MDH1, NAD-ICDH1, and αKGDC1 defective yeast strains were unable to grow on acetate, one would expect that they could utilize [2-^{13}C]acetate in the presence of unlabeled aspartate or malate. Wild-type cells oxidize acetate readily, but acetate oxidation was negligible in the mutant strains (Figure 7). The greatest

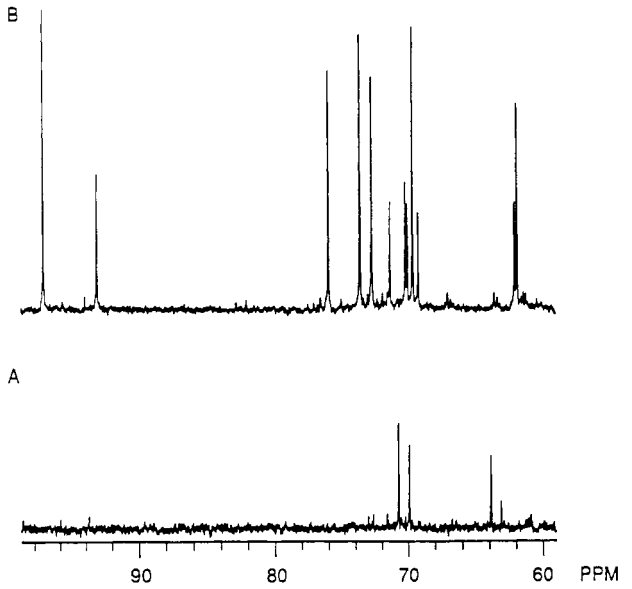

FIGURE 6: ^{13}C NMR spectra showing the effects of mitochondrial mutation on gluconeogenesis in *Saccharomyces cerevisiae*. (A) Parental strain; (B) mitochondrial malate dehydrogenase mutant. The resonances in the top spectrum correspond to ^{13}C-enriched galactose.

oxidation was observed in the *IDH2* mutant yeast strain, which produced a detectable quantity of [4-^{13}C]citrate and some [4-^{13}C]glutamate (Figure 7C). The ^{13}C NMR spectrum of the *IDH2* mutant cell extracts indicated that the intensity of glutamate C3 was lower than that of glutamate C4 (compared to a C4/C3 ratio of 1.2 in wild type), indicating that the *IDH2*$^-$ mutant cells were metabolizing acetate in a condition far from steady state. In the case of the αKGDC and MDH1 mutant cells, even less ^{13}C was incorporated into glutamate.

DISCUSSION

Oxidation of [3-^{13}C]pyruvate by wild-type yeast is very efficient. The major intermediates detected by ^{13}C NMR were glutamate, alanine, and acetate. Mutations that deleted TCA cycle enzymes changed this metabolic pattern; the main synthetic product was galactose instead of glutamate, while acetate and alanine were produced in quantities similar to those produced in wild-type cells. Although there were some differences in acetate and alanine formation in the different mutant cells, the most significant and general change was the decrease in glutamate synthesis from [3-^{13}C]pyruvate. The pool size of unlabeled glutamate was not decreased in the mutant strains (Table II), indicating that this metabolite could still be formed directly or indirectly from other pathways. A genetic defect in αKGDC significantly increased the acetate pool size, both labeled and unlabeled, indicating that a significant part of [3-^{13}C]pyruvate and endogenous metabolites were oxidized to acetate. The MDH1 mutant had a significantly larger alanine pool size, but the ^{13}C fractional enrichment was lower than that found in wild-type cells. This suggests that the MDH1 mutant preferentially converted endogenous intermediates to alanine.

There was a significant decrease in the ^{13}C enrichment of alanine in all TCA cycle mutants presented with [3-^{13}C]-pyruvate. Thus, the decreased ^{13}C labeling of glutamate in the mutant cells can be partially explained by a decreased ^{13}C enrichment in the acetyl-CoA pool, assuming that most of the acetyl-CoA is derived directly from pyruvate via the PDC. The [2-^{13}C]acetate oxidation data suggest there may be other reasons why less ^{13}C is found in glutamate in the mutant

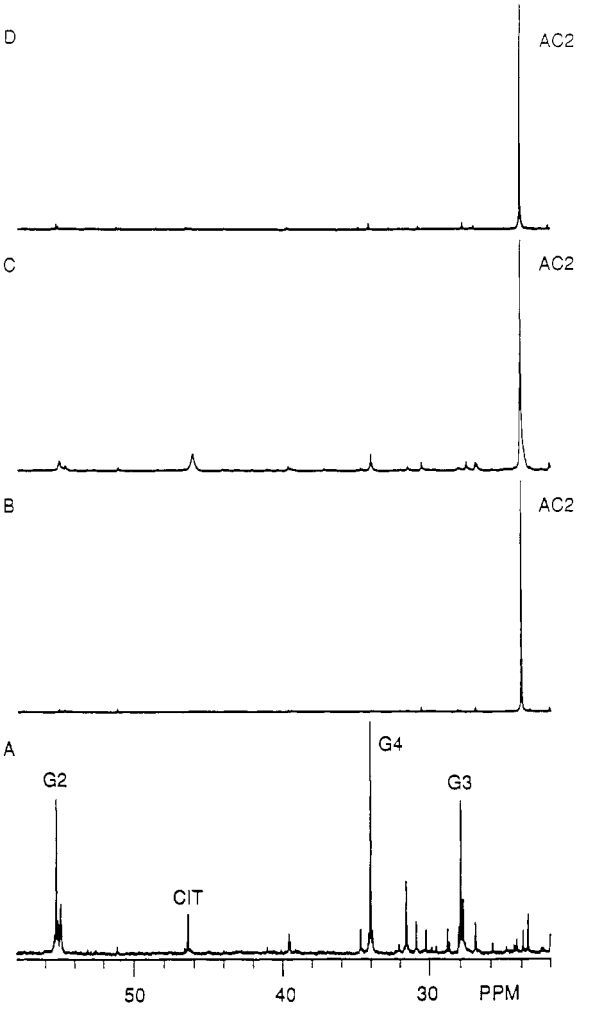

FIGURE 7: ^{13}C spectra showing the effects of mutation on oxidation of [2-^{13}C]acetate. (A) Parental strain; (B) mitochondrial MDH1 mutant; (C) NAD-ICDH1 mutant (*idh2-1*); (D) αKGDC mutant. Resonances labeled G2, G3, and G4 correspond to the glutamate C2, C3, and C4 carbons. CIT corresponds to the citrate C2 and C4 carbons, and AC2 corresponds to the acetate C2 carbon.

strain. At the end of the incubation period with [2-^{13}C]acetate, the wild-type cells had used up virtually all of the acetate. In the mutant cells a significant amount of [2-^{13}C]acetate remained, and ^{13}C labeling in glutamate was negligible. In the case of the αKGDC and MDH1 mutants, which are not directly blocked in glutamate synthesis, this may result from the disruption of TCA cycle enzyme organization. Alternatively, acetyl-CoA formation from either pyruvate or acetate may be limiting in the mutants.

Mutation of the NAD-ICDH1 also resulted in less ^{13}C incorporation into glutamate (Figure 4B) even though the NADP-ICDH is present in the mitochondrial compartment (Keys & McAlister-Henn, 1988). The fact that a detectable quantity of citrate was formed in the NAD-ICDH1 mutant (Figure 4A) which was not detected in the wild-type cells indicates that citrate synthase was active and that glutamate synthesis was inhibited at the NAD-ICDH1 step. MDH1, CS, aconitase, and mitochondrial NADP–isocitrate dehydrogenase (NADP-ICDH) were all present in the NAD-ICDH1 mutant in quantities similar to those observed in wild-type cells (Keys & McAlister-Henn, 1988), and thus the lack of glutamate synthesis cannot be attributed to a lack of enzyme activity. The most logical explanation of these data is that NAD-ICDH1 is an integral part of TCA cycle and that the NADP-dependent isozyme cannot perform its function because this enzyme is not involved in the organization of TCA cycle

enzymes (Keys & McAlister-Henn, 1988; Repetto & Tzagoloff, 1989).

Previous studies on TCA cycle yeast mutants (CS1$^-$, MDH1$^-$, and NAD-ICDH1$^-$) have shown that they are unable to grow on acetate as a sole carbon source. This occurs even though isozymes of CS and MDH exist outside the mitochondria and a NADP-ICDH exists inside the mitochondria. When an inactive CS1 (CS1*) was expressed in a CS1 null background, some growth on acetate was restored (Kispal et al., 1989). These data were interpreted using the metabolon model for the TCA cycle within the mitochondrion. This model was based on several lines of evidence (Srere, 1992) which showed (1) specific interactions between sequential Krebs cycle enzymes, (2) binding of Krebs TCA cycle enzymes to proteins of the inner surface of the inner mitochondrial membrane, and (3) kinetic enhancement of Krebs TCA cycle reactions in preparations of opened mitochondria with bound TCA cycle enzymes compared to completely disrupted mitochondria.

According to the metabolon model, a missing enzyme causes a loss of structure of the TCA cycle enzyme complex, and, in spite of possible shunting enzyme activity outside the mitochondria (MDH2 and CS2) or shunting enzyme activity inside the mitochondria (NADP-ICDH), the disrupted TCA cycle metabolon does not function as well as the intact complex. Thus, even an inactive CS1 protein (CS1*), which could restore a folded structure without catalytic function, was able to enhance overall TCA cycle activity and allow some growth on acetate (Kispal et al., 1989). There is other recent evidence from [^{13}C]propionate metabolism in yeast that can be interpreted as tight channeling through a TCA cycle metabolon (Sumegi et al., 1991a).

The results of [3-^{13}C]pyruvate metabolism presented in this paper extend these growth observations. Yeast cells deficient in CS1 are able to grow on pyruvate (but not acetate) with the energy presumably coming from the oxidation of pyruvate to acetate. In mutants lacking MDH1, the formation of TCA cycle intermediates can theoretically still occur since both acetyl-CoA from the pyruvate dehydrogenase complex and OAA from pyruvate carboxylase can be formed from pyruvate (see Figure 1). It should be possible to observe the conversion of [3-^{13}C]pyruvate to Krebs cycle intermediates and glutamate. However, the results presented here indicate that this does not occur in vivo. Similar arguments could be used for the other TCA cycle mutants used here. The similarity in metabolic effects of disruption in various steps of the TCA cycle suggests that these effects are due either to disruption of the TCA cycle metabolon or to accumulation of some unknown common inhibitor of cycle function.

One could also invoke changes occurring in other enzymes outside the TCA cycle as a consequence of the mutation that results in the metabolic alterations observed. In the case of CS1$^-$ mutants, changes in other TCA cycle enzymes (PDC and αKGDC) have been observed, but the changes were only 2-fold (Kispal et al., 1989). On the other hand, no significant TCA cycle enzyme alterations were measured in the NAD-ICDH1$^-$ mutants. It is also possible that a critical enzyme which we have not yet measured has been altered. Alternatively, one can propose that changes in unseen metabolite levels have caused an inhibition of the Krebs TCA cycle activity and a shift in pyruvate utilization to galactose production. However, for several candidates for such effects such as succinate, fumarate, and citrate, whose resonances are visible, no changes in concentrations were observed. It therefore seems reasonable to maintain the metabolon concept until data can be obtained that substantiate alternate theories.

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REFERENCES

Bock, K., & Pedersen, C. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 45.

Cupp, V. R., & McAlister-Henn, L. (1991) *J. Biol. Chem. 266*, 22199–22205.

Gancedo, C., & Serrano, R. (1989) in *The Yeasts* (Rose, A. H., & Harrison, J. S., Eds.) 2nd ed., Vol. 3, pp 205–259, Academic Press, New York.

Keys, D. A., & McAlister-Henn, L. (1988) *J. Bacteriol. 172*, 4280–4287.

Kispal, G., Rosenkrantz, M., Guarente, L., & Srere, P. A. (1988) *J. Biol. Chem. 263*, 11145–11149.

Kispal, G., Evans, C. T., Malloy, C., & Srere, P. A. (1989) *J. Biol. Chem. 264*, 11204–11210.

Malloy, C. R., Sherry, A. D., & Jeffrey, F. M. H. (1988) *J. Biol. Chem. 263*, 6964–6971.

Malloy, C. R., Sherry, A. D., & Jeffrey, F. M. H. (1990) *Am. J. Physiol. 259*, H987–H995.

McCammon, M. T., Veenhuis, M., Trapp, S. B., & Goodman, J. M. (1990) *J. Bacteriol. 172*, 5816–5827.

Minard, K. I., & McAlister-Henn, L. (1991) *Mol. Cell. Biol. 11*, 370–380.

Repetto, B., & Tzagoloff, A. (1989) *Mol. Cell. Biol. 9*, 2695–2705.

Robinson, J. B., Jr., Inman, L., Sumegi, B., & Srere, P. A. (1987) *J. Biol. Chem. 262*, 1786–1790.

Srere, P. A. (1987) *Annu. Rev. Biochem. 56*, 89–124.

Srere, P. A. (1990) *Trends. Biochem. Sci. 15*, 411–412.

Srere, P. A. (1992) The Molecular Physiology of Citrate, in *Current Topics in Cellular Regulation* (Welch, G. R., Ed.) Vol. 33, pp 261–275, Academic Press, Inc., New York.

Steffan, J. S., & McAlister-Henn, L. (1991) *Arch. Biochem. Biophys. 287*, 276–282.

Sumegi, B., & Porpaczy, Z. (1990) in *Structural and Organizational Aspects of Metabolic Regulation* (Srere, P. A., Jones, M. E., & Mathews, C., Eds.) UCLA Symposium on Molecular and Cellular Biology, New Series, Vol. 133, pp 229–244, Alan R. Liss, Inc., New York.

Sumegi, B., Sherry, A. D., & Malloy, C. R. (1990) *Biochemistry 29*, 9106–9110.

Sumegi, B., Sherry, A. D., Malloy, C. R., Evans, C., & Srere, P. A. (1991a) *Biochem. Soc. Trans. 19*, 1002–1005.

Sumegi, B., Porpaczy, Z., & Alkonyi, I. (1991b) *Biochim. Biophys. Acta 1081*, 121–128.

Sumegi, B., Porpaczy, Z., Srere, P. A., & Sherry, A. D. (1991c) in *Biochemistry and Physiology of Thiamine Diphosphate Enzymes* (Bisswanger, H., & Ullrich, J., Eds.) pp 232–241, VCH Publisher, Weinheim, Germany, Cambridge, UK, and New York.

Sumegi, B., Porpaczy, Z., McCammon, M. T., Sherry, A. D., Malloy, C. R., & Srere, P. A. (1992) in *Current Topics in Cellular Regulation* (Welch, G. R., Ed.) Vol. 33, pp 249–260, Academic Press, Inc., New York.