Reversible Transdominant Inhibition of a Metabolic Pathway

IN VIVO EVIDENCE OF INTERACTION BETWEEN TWO SEQUENTIAL TRICARBOXYLIC ACID CYCLE ENZYMES IN YEAST∗

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The enzymes of the Krebs tricarboxylic acid cycle in mitochondria are proposed to form a supramolecular complex, in which there is channeling of intermediates between enzyme active sites. While interactions have been demonstrated in vitro between most of the sequential tricarboxylic acid cycle enzymes, no direct evidence has been obtained in vivo for such interactions. We have isolated, in the Saccharomyces cerevisiae gene encoding the tricarboxylic acid cycle enzyme citrate synthase Cit1p, an “assembly mutation,” i.e. a mutation that causes a tricarboxylic acid cycle deficiency without affecting the citrate synthase activity. We have shown that a 15-amino acid peptide from wild type Cit1p encompassing the mutation point inhibits the tricarboxylic acid cycle in a dominant manner, and that the inhibitory phenotype is overcome by a co-overexpression of Mdh1p, the mitochondrial malate dehydrogenase. These data provide the first direct in vivo evidence of interaction between two sequential tricarboxylic acid cycle enzymes, Cit1p and Mdh1p, and indicate that the characterization of assembly mutations by the reversible transdominant inhibition method may be a powerful way to study multienzyme complexes in their physiological context.

An important and unresolved general problem in cellular biochemistry concerns the metabolic organization in cells. Considering a metabolic pathway, a basic question is, “Are the enzymes and the metabolites randomly dispersed inside the cell, or is there a cellular organization such that the sequential enzymes of this pathway remain in a close proximity allowing especially the intermediary metabolites to be passed directly from one enzyme of the sequence to the next one?” Whereas no direct evidence has ever supported the view of the cell as a bag of uniformly dispersed enzymes, the general concept of an intracellular organization of enzymes and channeling of intermediates has in many respects received support from a large body of experimental data during the last 20 years (reviewed in Refs. 1–3). This type of organization is often referred to as “microcompartmentation” (4), and the term “metabolon” has been introduced (5) to describe supramolecular complexes of sequential metabolic enzymes and structural components.

Such “integrated” pathways may present important advantages for metabolic integration and regulation, such as protection of labile intermediates, prevention of intermediates from escaping into the bulk solution where they may be sequestered by other enzymes for use in different metabolic pathways, conservation of cellular solvent capacity, reduction of transition times, and enhancement of metabolic fluxes.

Experimental data support the existence of metabolons for many metabolic pathways and especially for the Krebs tricarboxylic acid cycle. For instance, specific interactions have been demonstrated in vitro between six of the eight sequential tricarboxylic acid cycle enzymes including citrate synthase (CS)† and malate dehydrogenase (MDH) (reviewed in Ref. 3). In the last few years, evidence was presented concerning the channeling of oxaloacetate in vitro between yeast mitochondrial CS (Cit1p) and MDH (Mdh1p) (6) as well as between the corresponding pig enzymes (7, 8).

However, no direct evidence has been obtained in vivo for the interactions between sequential Krebs tricarboxylic acid cycle enzymes, mainly because these interactions are weak and many of the resulting complexes dissociate during isolation due to dilution effects. A possible strategy to demonstrate the in situ existence and the physiological relevance of such complexes is to screen for and characterize mutations that affect specifically these interactions, i.e. mutations that affect the metabolic pathway without affecting the enzymatic activities. Such mutations, which we refer to as “assembly mutations,” have already been isolated in Saccharomyces cerevisiae. For instance, McCammon (9), who screened for mutants unable to grow on acetate, isolated three mutants with defects in the ACO1 gene (encoding mitochondrial aconitase) that display a wild type level of aconitase activity.

Here, we describe the serendipitous isolation of an assembly mutation in the CIT1 gene encoding Cit1p and its characterization using an adaptation and an extension of the transdominant genetic analysis initially described by Caponigro et al. (10).

Transdominant genetic experiments involve introduction and overexpression in wild type cells of small molecules and selection for those that inhibit a particular biological process. This is a new and promising approach to identify and characterize protein interactions in vivo (10–12). These authors used expression libraries designed to encode inhibitory proteinaceous molecules (peptides and protein fragments) and performed a selection for those that inhibit the pheromone response pathway in yeast. Peptide and protein fragments that

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† Deceased.

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† C. V. dedicates this paper to the memory of Paul A. Srere.

‡ The abbreviations used are: CS, citrate synthase; Aco1p, yeast mitochondrial aconitase; Cit1p, yeast mitochondrial CS; Cit2p, yeast peroxisomal CS; GFP, green fluorescent protein; MDH, malate dehydrogenase; Mdh1p, yeast mitochondrial MDH; WT, wild type; ORF, open reading frame.
permit cell division in the presence of pheromone were recovered and characterized. Several were derived from proteins that were already known to be involved in the pathway, and other inhibitors represented novel peptide sequences allowing identification of proteins that may negatively influence the pheromone response. To designate collectively the transdominant proteinaceous inhibitors, Caponigro et al. (10) introduced the term “perturbagens” by analogy with “mutagens”; instead of producing mutations in genes, perturbagens act at the level of the proteins, disrupting specific biochemical interactions in cells to generate a mutant phenocopy. In fact, a perturbagen acts as the product of a gene carrying a dominant negative mutation (13), i.e. a gene encoding an altered protein that when overexpressed disrupts the function of the wild type protein. The power of the transdominant inhibition analysis lies mainly in three features: (a) the interactions are detected in their physiological context; (b) the physiological relevance of the interaction is directly reflected by the mutant phenocopy resulting from its inhibition; and (c) the identification and the characterization of the perturbagens allows a direct access to the structure of the interaction (i.e. binding site, residues involved, etc.).

In this study, we did not use randomly generated perturbagens but rather a specific peptide based on the location of the assembly mutation previously identified in order to confirm that this mutation does affect an interacting site of the enzyme. To identify targets for the inhibitors, Caponigro et al. (10), Norman et al. (11), and Geyer et al. (12) used the two-hybrid technique of Fields and Song (14). Here, the interacting partner was identified by searching for proteins that are able, when co-overexpressed with the perturbagen, to reverse the transdominant inhibition (i.e. to restore a wild type phenotype) as a consequence of a “back-titration” of the perturbagen.

The results reported here provide the first direct in vivo evidence of interaction between two sequential Krebs tricarboxylic acid cycle enzymes (Cit1p and Mdh1p) and suggest that the reversible transdominant inhibition method may be a powerful tool to identify weak protein-protein interactions in vivo.

**EXPERIMENTAL PROCEDURES**

*Strains, Growth Media, and Transformation Procedures—* The *S. cerevisiae* null mutant *cit1* (*cit1::LEU2*) and *cit1cit2* (*cit1::LEU2 cit2::URA3*) strains used in this study originated from the haploid parental strain FYF5 (*MATa ura3-52 leu2-1 his3Δ200 trp1Δ63*), isogenic to the reference strain S288c (ATCC 26108). FYF5 was used in all the transdominant inhibition experiments. The yeast media were synthetic complete (SC) media prepared as described by Sherman et al. (15) with either 2% glucose (SCD) or 2% sodium acetate (SCAc) as the carbon source. SCD-U-W and SCAc-U-W were like SCD and SCAc, respectively, but lacked uracil and tryptophan. Rich media consisted of 1% yeast extract and 2% peptone (YP) with either 2% glucose (YPD), 2% (w/v) lactic acid (YPL), or 2% sodium acetate (YPAc) as the carbon source. YPL medium contained also 1% KH₂PO₄ and was adjusted to pH 5.5 with NaOH. Two percent agar (Difco) was added for plates. Yeast transformations were conducted using the lithium acetate protocol of Gietz et al. (16).

The host *Escherichia coli* strain used for cloning was XL1-Blue (recA1, end A1, gyrA96, thi-1, hsdR17, proAB, lacY1, lacZΔM15, Tn10(tetR)). Preparations and transformation of competent cells were performed according to Morris (17). Bacterial transformant cells were grown on LB plates supplemented with 100 μg/ml ampicillin.

*Plasmid Constructions—* All of the vectors used in this study are summarized in Table I. All of the constructs were cloned in either pRS314, pRS424, or pRS426. The vectors encoding the hybrid Ctp proteins (pRS-C-hCit2/1 and pRS-C-hcit2/1*) have been performed from pRS341/MDH1, respectively from pRS314. The vector pGAL-CL6GFP was used to amplify the coding region of green fluorescent protein (GFP), alone or fused to the 3'-flanking region of *CIT1*. Plasmids used for PCR amplifications of *ACO1*, *CIT1*, *CIT2*, and *MDH1* sequences were pRS-C-Aco1, pRS-C-Cit1, pRS-C-CIT2/SKL, and pRS314/MDH1, respectively.

The oligonucleotides were obtained from Life Technologies, Inc., or IDT. All enzymatic reactions were performed as recommended by the manufacturers (Roche Molecular Biochemicals, Life Technologies, Inc., Qiagen, and Stratagene), and all recombinant manipulations were done according to Maniatis et al. (21). Plasmids were prepared using commercially available kits (Qiagen, Promega). Cloned Pfu polymerase (Stratagene) was used in all polymerase chain reaction amplifications, except in those of the construction pRS-C-hcit2/1* that were performed with Taq DNA polymerase (Roche Molecular Biochemicals). All of the constructs were sequenced on an ABI 377 automated sequencer.

*Yeast Cellular Fractionation—* Yeast precultures were grown under selective pressure in synthetic glucose medium (SCD-W or SCD-U-W). Aliquots of the precultures were used to inoculate either 250-ml cultures of a rich medium (YPL or YPAc) or 750-ml cultures of SCD-U-W medium. Cultures were grown to early log phase. Cellular fractionation was performed as described previously (20).

*Immunoblot Analysis of GFP Constructs—* Mitochondrial proteins were separated on a 12% SDS-polyacrylamide gel and transferred to

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**TABLE I  Vectors used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>pRS314</td>
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<td>Ref. 18</td>
</tr>
<tr>
<td>pRS424</td>
<td>2-μm, TRP1-based yeast-<em>E. coli</em> shuttle vector</td>
<td>Ref. 18</td>
</tr>
<tr>
<td>pRS426</td>
<td>2-μm, URAs3-based yeast-<em>E. coli</em> shuttle vector</td>
<td>Ref. 18</td>
</tr>
<tr>
<td>pGAL-CL6GFP</td>
<td>ARS-CEN, URAs3-based vector containing GFP ORF fused to the 3'-flanking region of <em>CIT1</em></td>
<td>Ref. 19</td>
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<td>pRS-C-ACO1</td>
<td>pRS314 containing ACO1</td>
<td>G. J. Lauquin</td>
</tr>
<tr>
<td>pRS-C-CIT1</td>
<td>pRS314 containing CIT1</td>
<td>Ref. 20</td>
</tr>
<tr>
<td>pRS-C-LSCIT2/SKL</td>
<td>pRS314 containing CIT2 ORF (without its three last codons and fused to <em>CIT1</em> leader sequence) under control of CIT1 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pRS314/MDH1</td>
<td>pRS314 containing MDH1</td>
<td>L. McAlister-Henn</td>
</tr>
<tr>
<td>pRS-C-hCit2/1</td>
<td>pRS314 containing a “wild type” hybrid construct between <em>CIT2</em> and <em>CIT1</em> (see “Results”) under control of <em>CIT1</em> promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pRS-C-hcit2/1*</td>
<td>Mutated version of pRS-C-hCit2/1 (see “Results”)</td>
<td>This study</td>
</tr>
<tr>
<td>pRS-C-CIT1/PLS</td>
<td>pRS314 containing CIT1 promoter and <em>CIT1</em> leader sequence</td>
<td>Ref. 20</td>
</tr>
<tr>
<td>pRS-E-GFP</td>
<td>pRS424 containing GFP ORF fused to <em>ACO1</em> leader sequence, under control of <em>ACO1</em> promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept1</td>
<td>As pRS-E-GFP except that GFP ORF is fused in 3’ to the sequence encoding Pept1</td>
<td>This study</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept0</td>
<td>As pRS-E-GFP except that GFP ORF is fused in 3’ to a multicloning site encoding a 19-amino acid unrelated peptide</td>
<td>This study</td>
</tr>
<tr>
<td>pRS-E-ACO1</td>
<td>pRS426 containing ACO1</td>
<td>This study</td>
</tr>
<tr>
<td>pRS-E-CIT1</td>
<td>pRS426 containing <em>CIT1</em> ORF under control of <em>ACO1</em> promoter</td>
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<tr>
<td>pRS-E-CIT2</td>
<td>pRS426 containing CIT2 ORF (without its three last codons and fused to <em>CIT1</em> leader sequence) under control of <em>ACO1</em> promoter</td>
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</tr>
<tr>
<td>pRS-E-MDH1</td>
<td>pRS426 containing MDH1 ORF under control of <em>ACO1</em> promoter</td>
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The results of this work showed that the following plasmid construction, which led to the serendipitous isolation of an assembly mutation in the CIT1 gene, was performed.

This plasmid was constructed from pRS314 (18), a centromere-based vector carrying the yeast TRP1 gene as a selectable marker, and designed to encode a chimeric Citp protein including the first 40 amino acids of Cit1p, followed by the C-terminal two-thirds of Cit2p. The polymerase chain reaction amplifications of the fragment containing codons 1–177 of the CIT2 gene and of that including the last 283 codons of CIT1 and its 3′-flanking region were first performed with Taq DNA polymerase (Roche Molecular Biochemicals), and the subsequent sequencing of the construct revealed the presence of mutations in the chimeric coding region. The resulting plasmid, where the CIT1 promoter drives the expression of a gene encoding a mutated hybrid Citp protein (hCitp*), was termed pRS-C-hcit2/1*. The same construct was performed again but using cloned Pfu polymerase (Stratagene), which is known to exhibit the lowest error rate of any thermostable polymerase (28). The sequencing of the resulting construct, termed pRS-C-hCit2/1, did not reveal the presence of any mutation.

These two plasmids as well as pRS-C-CIT1 encoding native Cit1p were used to transform the yeast null mutants Δcit1 and Δcit1 Δcit2, which do not grow on acetate as the sole carbon source (29). The phenotype of these yeast strains harboring the plasmids pRS-C-CIT1, pRS-C-hcit2/1, pRS-C-hcit2/1*, and pRS-C-hcit2/1* was examined to identify any potential growth advantage. As expected, the Δcit1 Δcit2 double mutant did not grow on acetate, while the Δcit1 double mutant harboring the plasmid pRS-C-hcit2/1 grew much more poorly with a significantly increased lag time and exhibited a decreased cell density in stationary phase when compared with the transformants expressing either native Cit1p or "wild type" hCitp.

Mitochondrial and cytosolic CS activities were determined in these three Δcit1 Δcit2 transformants as well as in parental wild type and mutant strains (as controls). As shown in Table II, the transformant expressing the plasmid-encoded native Cit1p protein displayed more mitochondrial CS activity than the wild type strain. This is a common observation, probably due to the fact that a centromere-based plasmid is generally present in slightly more than one copy per cell. Similar cellular levels of CS activity were found in the transformant expressing "wild type" hCitp. Indeed, the transformant expressing the mutated hCitp* protein displayed less mitochondrial CS activity than the other transformants. But, interestingly, this difference cannot explain the difference observed for acetate utilization between these transformants, since the mutated hybrid protein nevertheless displayed a level of mitochondrial CS activity comparable with that of the original parental strain, which clearly grows on acetate. Moreover, a collection of Citp hybrid mutants were tested for their ability to grow on acetate medium (SCAc). The same complementation results were obtained with both mutant strains, and only those obtained with the double mutant strain are shown (Fig. 1).

The results of this work indicate that the mutation affecting the chimeric CIT2/1 gene we have constructed here shows no correspondence between the level of mitochondrial CS activity and the ability of the corresponding cells to grow fully on acetate. In other words, we have isolated an altered form of Cit1p that, although fully catalytically active, does affect the metabolic process in which it is involved (i.e. the Krebs tricarboxylic acid cycle). Consequently, this mutation is designated an assembly mutation.

FIG. 1. Ability of the plasmid-encoded Citp proteins to restore the growth on acetate of the double Δcit1 Δcit2 mutant. Strains were grown overnight in liquid synthetic glucose medium, washed twice in sterile water, and resuspended to 2 × 10⁴ cells/ml (1 A₅₆₀ unit corresponds to 2.4 × 10⁴ cells/ml for the strains used here). This resuspen- sion was then serially diluted in 10-fold steps, down to 2 × 10³ cells/ml. For each strain, 5-μl drops (10⁵ to 10 cells from the left to the right) were spotted onto acetate plates (SCAc medium). The plates were photographed after incubation at 30 °C for 4 days.

**Table II.**

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Plasmid (centromeric)</th>
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<tr>
<td>WT</td>
<td></td>
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<tr>
<td>Δcit1 Δcit2</td>
<td></td>
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</tr>
<tr>
<td>Δcit1 Δcit2</td>
<td>pRS-C-CIT1</td>
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<tr>
<td>Δcit1 Δcit2</td>
<td>pRS-C-hCit2/1</td>
<td></td>
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<tr>
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In Vivo Interaction between Cit1p and Mdh1p

Identification of the cit1 Assembly Mutation—The sequencing of the chimeric CIT2/1 gene from vector pRS-C-hcit2 revealed the deletion of three bases within the CIT1 coding region (Fig. 3): G\(^{1069}\) (codon 357), G\(^{1075}\) (codon 359), and A\(^{1089}\) (codon 363). Since these three bases are not consecutive, these deletions result not only in the loss of one residue in the encoded protein but also in a change of sequence of five consecutive amino acid residues, as illustrated in Fig. 3. We termed this mutation Δcit1.

The whole peptide shown in Fig. 3 (from Pro\(^{354}\) to Pro\(^{366}\)) is fully conserved in Cit2p and in pig heart CS, where it forms a solvent exposed loop (from Pro\(^{316}\) to Pro\(^{328}\)) between two fully conserved in Cit2p and in pig heart CS, where it forms a peptide encompassing the mutation site of Δcit1 to behave as a transdominant inhibitor (a perturbagen) of the Krebs tricarboxylic acid cycle, i.e. to evaluate the ability of this peptide, when overexpressed in cells, to inhibit the growth on acetate of a wild type strain.

The peptide we have chosen to test is a 15-amino acid peptide (termed Pept1) corresponding to the wild type sequence shown in Fig. 3 plus one residue on the amino terminus (Val\(^{355}\)) and one residue on the carboxyl terminus (Arg\(^{377}\)). Peptides can be stably expressed inside the cell as fusions with an inert carrier protein as, for instance, the adsorption protein (pII) of fd phage (30), thioredoxin from Escherichia coli (31) GFP (10, 32), or a catalytically inactive version of staphylococcal nuclease (11). In this study, we expressed Pept1 as a C-terminal fusion with GFP. In fact, the total sequence fused to the GFP coding region encoded a 19-amino acid peptide because of four construction residues encoded by two HpaI restriction sites that were introduced just upstream and downstream of the sequence encoding Pept1. To express and target the GFP fusion in yeast mitochondria, we used the promoter and the leader sequence of the ACO1 gene. The ACO1 promoter, which is subject to catabolite repression (33) and is fully derepressed on AT that is a characteristic of high efficiency promoters in S. cerevisiae (34). The combined use of a multicopy vector and the ACO1 promoter was designed to improve overexpression of the gene encoding the GFP-Pept1 fusion under the desired experimental conditions and, thus, to observe a transdominant inhibition effect. The mitochondrial targeting sequence of ACO1 was chosen for construction ease and has been shown, in the context of other work, to be efficient for mitochondrial targeting of other proteins (such as cytosolic MDH, Mdh2p, and peroxisomal MDH, Mdh3p). Finally, to assure the stability of the GFP mRNA in yeast, the coding region of GFP (or GFP-Pept1 fusion) was fused to the 3'-flanking region of CIT1.

Three GFP plasmids have been constructed, PRS-E-GFP, pRS-E-GFP-Pept1, and pRS-E-GFP-Pept0, respectively, designed to encode GFP alone, GFP fused to Pept1, and GFP fused to a 19-amino acid random unrelated peptide (encoded by a multicloning site, partially derived from vector pPROEX-1). All of these plasmids were constructed from pRS424 (18), a multicloning site, partially derived from vector pPROEX-1). Three GFP plasmids have been constructed, PRS-E-GFP, pRS-E-GFP-Pept1, and pRS-E-GFP-Pept0, respectively, designed to encode GFP alone, GFP fused to Pept1, and GFP fused to a 19-amino acid random unrelated peptide (encoded by a multicloning site, partially derived from vector pPROEX-1). All of these plasmids were constructed from pRS424 (18), a 2-μm multicopy vector containing the yeast gene TRP1 as a selectable marker. The wild type yeast strain FYF5 was co-transformed with either pRS424 or each of the three GFP constructs along with pRS426 (18), a 2-μm multicopy vector with the yeast URA3 gene for selection. Vector pRS426 was the parental vector of another set of constructions designed to encode specific proteins that were subsequently tested for their ability to back-titrate Pept1 (see below). The correct expression and transport into mitochondria of each GFP construct were ascertained by Western blot analysis (with monoclonal anti-GFP antibodies) of mitochondrial extracts from the resulting Trp\(^+\) Ura\(^+\) transformants (Fig. 4). The efficiency of mitochondrial...
drial extract preparation was confirmed by measuring, in mitochondrial and cytosolic fractions, the activities of NAD-dependent isocitrate dehydrogenase, a mitochondrial marker enzyme. The results indicated that at least 94% of NAD-dependent isocitrate dehydrogenase activity was found in the mitochondria (data not shown). The transformants were tested for their ability to grow on acetate (Fig. 5, rows 1–4). As expected, no difference in growth was observed between the transformant strain harboring the parental vectors (row 1) and the strain expressing GFP alone (without any foreign peptide) (row 2). However, a very significant decrease in growth on acetate was observed in the transformant expressing the GFP-Pept1 fusion (row 3), suggesting that this peptide does behave as a transdominant inhibitor of the Krebs tricarboxylic acid cycle and that original mutation cit1Δ3 does affect a protein-interacting site that is relevant for the Krebs tricarboxylic acid cycle. The strain harboring the control vector pRS-E-GFP-Pept0, while growing significantly better than the pRS-E-GFP-Pept1 transformant (compare rows 4 and 3), showed however somewhat reduced growth relative to the positive control (compare rows 4 and 1). This may be due to the fact that the peptide Pept0 is not totally “innocent” with regard to the Krebs tricarboxylic acid cycle. In other words, this peptide may present some similarities with protein-protein interacting sites involved in this pathway. In order to confirm that the Krebs tricarboxylic acid cycle enzyme activities are not affected by the overexpression of GFP-Pept1 fusion (i.e. that the inability of the corresponding transformant to utilize acetate is not due to an inactivation of these enzymes), CS, MDH, aconitase, and NAD-dependent isocitrate dehydrogenase activities were determined in mitochondrial extracts from the four transformants (Table III). NAD-dependent isocitrate dehydrogenase activity in the cytosolic extracts was also measured to demonstrate the efficiency of fractionation. As shown in Table III, CS activity was increased only 2-fold in mitochondrial extracts from the transformant harboring the plasmid pRS-E-MDH1 was able to grow on acetate (row 8), indicating that Mdh1p was able to back-titrated Pept1. Mitochondrial localization and overexpression of Aco1p, Cit1p, Cit2p, or Mdh1p was confirmed by measuring aconitase, CS, and MDH activities in the mitochondrial extracts of these transformants (Table III). NAD-dependent isocitrate dehydrogenase activities were also determined in both mitochondrial and cytosolic extracts to demonstrate the efficiency of fractionation. As shown in Table III, CS activity was increased only 2-fold in mitochondrial extracts from the transformant harboring the plasmid pRS-E-CIT2. However, the mitochondrial form of Cit2p lacking the carboxyl-terminal tripeptide SKL is known to be very unstable in protein extracts (20).

DISCUSSION

Dissecting the molecular mechanism of a biological process requires, among other approaches, identifying the proteins and protein-protein interactions that mediate the process. To that end, a number of genetic approaches have been developed, especially in the yeast S. cerevisiae, to directly assay interactions between known proteins and to isolate novel interacting partners for a protein of interest (reviewed in Ref. 37). A new approach that offers an alternative strategy to classical genetics involves the isolation, from peptide display libraries, of transdominant inhibitors (perturbagens) of a particular process or pathway (10–12).

In this study, we have used an adaptation and an extension of this approach to characterize an assembly mutation in the S. cerevisiae CIT1 gene encoding mitochondrial CS, Cit1p. The results of this characterization provided evidence that this enzyme interacts in vivo with mitochondrial MDH, Mdh1p (one of its sequential enzymes in the Krebs tricarboxylic acid cycle) and that this interaction involves the Pro354–Pro366 region of Cit1p; the corresponding peptide (termed Pept1) proved to be a transdominant inhibitor of the Krebs tricarboxylic acid cycle, and the inhibitory phenotype was overcome by an overexpression of Mdh1p.

As mentioned under “Results,” Pept1 is fully conserved in pig CS. We have previously published a model of a docking orientation between mitochondrial MDH, CS, and aconitase generated by computer modeling studies using the porcine high definition structures of these three enzymes (38). This quinary structure model, based on the structural and experimental constraints from our results obtained with the yeast fusion proteins (6), showed substantial interacting surface areas with spatial and electrostatic complementarities that made the

Fig. 4. Evidence of mitochondrial expression of the GFP constructs. Protein extracts (80 μg) from mitochondrial fractions prepared from the yeast strain FYF5 transformed with pRS426 and either pRS424 (lane 1), pRS-E-GFP (lane 2), pRS-E-GFP-Pept1 (lane 3), or pRS-E-GFP-Pept0 (lane 4) were used for immunoblot analysis with monoclonal anti-GFP antibody as described under “Experimental Procedures.” Pure GFP (10 ng, lane 5) was used as positive control. Transformant strains were precultured under selective pressure on synthetic glucose medium (SCD-U-W) before transfer into rich lactate (YPL) medium for optimal expression of the GFP constructs (which are under control of the ACO1 promoter).
complex thermodynamically stable. By examining the electrostatic potential of each docking surface, we identified the charged amino acids from the putative docking sites and determined which could be involved in electrostatic interactions with complementarily charged amino acids of the other molecule. Interestingly, Arg$^{362}$ of Cit1p, which is changed to a residue of the opposite charge (Glu) in the cit1Δ3 mutation (Fig. 3), corresponds in pig CS to Arg 324, which was found to be possibly involved in an interacting amino acid pair with Glu$^{246}$ or Glu$^{276}$ of pig mitochondrial MDH (38). Moreover, the whole wild type peptide shown in Fig. 3 is also conserved in Cit2p, which has been recently shown to be able to replace Cit1p both in vivo and in vitro for interaction with Mdh1p but not with Aco1p. This is also consistent with our docking model, where the CS surface proposed to interact with aconitate is distinct from that corresponding to Pept1 (38).

Results reported here correlate with previous in vivo experiments supporting the involvement of Cit1p in a mitochondrial complex. Disruption of the CIT1 gene results in cells that are unable to grow on acetate (29) despite the fact that the inner mitochondrial membrane is provided with a citrate transporter (39), which could allow the CS extramitochondrial isoform (Cit2p) to act as a shunt for the missing Cit1p. In addition, introduction in null cit1 mutant cells of a catalytically inactive but structurally unchanged Cit1p protein resulted in restoration of tricarboxylic acid cycle function and growth on acetate (40), suggesting that the protein plays a key structural role in cycle function that is independent of its catalytic function. This was also supported by recent results showing that a cytosolically localized form of Cit1p was incompetent for restoration of growth of a Δcit1 strain on acetate (20), suggesting that mitochondrial localization of Cit1p is essential for its function in the tricarboxylic acid cycle. Moreover, using a 5-fluorotryptophan-labeled Cit1p, Haggie and Brindle (41) have shown by 19F NMR

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Enzyme activities$^a$</th>
<th>Growth on acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP1</td>
<td>URA3</td>
<td></td>
</tr>
<tr>
<td>pRS424</td>
<td>pRS426</td>
<td>1</td>
</tr>
<tr>
<td>pRS-E-GFP</td>
<td>pRS426</td>
<td>2</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept1</td>
<td>pRS426</td>
<td>3</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept0</td>
<td>pRS426</td>
<td>4</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept1</td>
<td>pRS-E-CIT1</td>
<td>5</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept1</td>
<td>pRS-E-CIT2</td>
<td>6</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept1</td>
<td>pRS-E-ACO1</td>
<td>7</td>
</tr>
<tr>
<td>pRS-E-GFP-Pept0</td>
<td>pRS-E-MDH1</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$ Specific activities are expressed in nmol of citrate (for CS), NADH (for MDH and NAD-dependent isocitrate dehydrogenase), or cis-aconitate (for aconitate) formed per min per mg of mitochondrial or cytosolic protein ± S.E. of three independent experiments.

$^b$ Mitochondrial (mitoch.) and cytosolic extracts were prepared from cells grown under selective pressure in synthetic glucose medium (SCD-U-W) and harvested in early log phase.

$^c$ BDL, below detection limit for activity.
that this enzyme is motionally restricted in yeast mitochondria, consistent with its participation in a multienzyme complex.

Currently, the most widely used approach to identify and analyze protein-protein interactions is the two-hybrid system (14). Despite the proven success of the method, it does not directly provide information about the physiological relevance of the interactions, since nuclear localization of the interacting partners is required to obtain reporter gene expression. Moreover, many proteins (e.g., proteins with competing organellar sequences) may not be amenable to this localization, or the nuclear context may not be appropriate to detect some interactions that physiologically occur in another organelle. Indeed, alternative systems have been developed for analysis of interactions in cellular compartments other than the nucleus (42, 43), but again, most of the interactions are detected out of their physiological context.

The strength of the transdominant inhibition method lies precisely in the fact that it allows the study of protein-protein interactions in their natural cellular context, the physiological relevance of the interaction being directly reflected by the inhibitory phenotype resulting from overexpression of the perturbagen (i.e., resulting from disruption of the interaction). Another interesting advantage of this approach is that it does not require the use of specifically engineered reporter yeast strains; any wild type strain can be used as long as it contains the auxotrophic markers required for selection of plasmids.

The originality of the method used in this study lies mainly in two aspects. First, the perturbagen was not selected from a combinatorial library but was specifically designed based on a previously characterized assembly mutation affecting one of our proteins of interest. Consequently, a generalization of this approach would initially require generation of such mutations specifically in genes related to the biological process of interest. This could be performed by polymerase chain reaction mutagenesis, either based on the intrinsic error frequency of Taq DNA polymerase (44) (for instance, mutation cit1ΔΔ3 was isolated by using Taq DNA polymerase under standard conditions) or using conditions that increase the infidelity of the polymerase (45–47). The difficulty that is probably the main drawback of this approach concerns the distinction of assembly mutations from the others. Such a screen requires that proteins of interest possess another role (other than the interactions of interest) that can be easily assayed (such as an enzymatic activity) and that is also required for the biological process in question. For this reason, the approach used in this study may be applicable only to a restricted number of processes. The second original aspect of our method concerns the identification of the interacting partner(s). Caponigro et al. (10), Norman et al. (11), and Geyer et al. (12) used the two-hybrid technique with the perturbagen as the bait. In addition to the weaknesses of this method discussed above, the major drawback of two-hybrid analysis is the frequency of false positives as mentioned by Norman et al. (11). By screening for proteins that are able, when co-overexpressed with the perturbagen, to overcome the inhibitory phenotype, the chances for isolating a false positive are considerably decreased, since the interaction between the perturbagen and its partner is directly detected through the recovery of the biological process in which this interaction is physiologically relevant. The main limitation of this method, however, concerns the targeting of proteins when the metabolic pathway studied takes place in a specific organelle such as mitochondria or peroxisomes. In these cases, this approach may be limited to tests of a restricted number of proteins suspected to be good candidates for the interacting partner (as in the case of the study described in this report).

Despite the growing body of evidence for the organization of the enzymes of the tricarboxylic acid cycle in a multienzyme complex in which there is channeling of cycle intermediates, the concept of a tricarboxylic acid cycle metabolon has remained controversial, since it has been difficult to isolate an intact complex from the cell. The relatively weak interactions between the enzymes that are favored in vivo by the very high protein concentrations in the mitochondrial matrix (48, 49) are disrupted by the dilution that occurs during cell extraction. The generalization of the reversible transdominant inhibition analysis, as described in this study, to the whole tricarboxylic acid cycle seems to be a powerful tool to access a detailed understanding of how tricarboxylic acid cycle enzyme organization at the ultrastructural level underlies function of the cycle and especially the channeling of intermediates.

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In Vivo Interaction between Cit1p and Mdh1p

Reversible Transdominant Inhibition of a Metabolic Pathway: IN VIVO EVIDENCE OF INTERACTION BETWEEN TWO SEQUENTIAL TRICARBOXYLIC ACID CYCLE ENZYMES IN YEAST

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