Cooperation between Enzyme and Transporter in the Inner Mitochondrial Membrane of Yeast

REQUIREMENT FOR MITOCHONDRIAL CITRATE SYNTHASE FOR CITRATE AND MALATE TRANSPORT IN SACCHAROMYCES CEREVISIAE*

Attila Sandor‡§, John H. Johnson‡§, and Paul A. Sreere‡||

From the ‡Research Service, Department of Veterans Affairs Medical Center and the §Department of Biochemistry and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75216 and the ||University Medical School, Institute of Biochemistry, Szüret ut 12, H-7624, Hungary

We have characterized 1,2,3-benzenetricarboxylic acid-sensitive, mersalyl-insensitive citrate uptake by mitochondria from two strains of Saccharomyces cerevisiae by describing the time course, $K_\text{m}$ and $V_\text{max}$ values, pH dependence, and response to inhibitors. In unloaded mitochondria from PSY142 CS1- cells, a mutant that lacks mitochondrial citrate synthase, both citrate uptake and efflux were reduced 7- and 8-fold, respectively, compared with the parental strain. No malate uptake was detectable in mitochondria from CS1- cells, while in the parental strain, uptake was 5.4 nmol/min/mg of protein. In contrast, mutations in peroxisomal citrate synthase (CS2-) or in other tricarboxylic acid cycle enzymes did not result in changes in mitochondrial citrate transport, suggesting a specific functional role for mitochondrial citrate synthase in citrate transport. More important, liposomes containing protein extracts from CS1-mitochondria showed the same citrate and malate transport rates as liposomes made from protein extracts of parental strain mitochondria. Thus, an apparently normal amount of both the citrate transporter and the dicarboxylate carrier is present in CS1-mitochondria, but both function abnormally in undisrupted mitochondria. We suggest that cooperation between the citrate transporter and mitochondrial citrate synthase is necessary for normal function of the transporter.

Physical ordering of multi-enzyme complexes within metabolic pathways that catalyze sequential reactions has recently been recognized as a form of compartmentation of "soluble" enzymes that provides the pathway with the organization necessary for the most efficient use of substrates (for a review, see Ref. 1). Demonstration of discreet substrate binding patterns (2) predict that the mitochondrial tricarboxylic acid cycle enzymes are organized into a complex. The term metabolon has been used to describe this form of compartmentation. Evidence has been presented that several of the tricarboxylic acid cycle enzymes are bound to the inner mitochondrial membrane (3). Grigorenko et al. (4) provided preliminary evidence that the loss of mitochondrial citrate synthase results in a reduction of citrate transport activity without a loss of citrate transporter protein. Subsequent searches for mitochondrial membrane-binding proteins using affinity chromatography have identified the citrate transporter (5) and the dicarboxylate carrier (6) as being capable of being bound to immobilized citrate synthase and malate dehydrogenase, respectively. These results suggest that the tricarboxylic acid cycle has a vectorial organization that includes transport of substrates across the mitochondrial membrane. Similar observations have been made for the transport and metabolism of glucose (7), carnitine (8), and ornithine (9).

We have begun a series of structure/function studies to probe further into the interactions of the tricarboxylic acid cycle metabolon and the citrate transporter. To accomplish this, we have measured the effects of mutations in tricarboxylic acid enzymes of Saccharomyces cerevisiae mitochondria on the uptake of citrate and malate into both intact mitochondria and mitochondrial protein extracts reconstituted into liposomes.

EXPERIMENTAL PROCEDURES

Materials—1,2,3-Benzenetricarboxylic acid (BTC),\* α-cyano-3-hydroxycinnamic acid, mersalyl, rotenone, antimycin, aprotinin, phenylmethylsulfonyl fluoride, benzamidine, lysate, cardiolipin (diphosphatidyglycerol), and Dowex 1-X8 (100 mesh) were obtained from Sigma. Phenyl succinate and n-butylmalonic acid were from Aldrich. Phosphatidylcholine was from Avanti Polar Lipids, Inc. (Birmingham, AL). [1,5-3C]citric acid, [U-14C]malic acid, and [U-14C]glucose were from Amersham Corp. All other chemicals were of the highest purity commercially available.

Yeast Strains—In the PSY142 parent strain (MATa, ura3-52, leu2-2, leu2-112, lys2-801), the CIT1 gene was disrupted by LEU2 (CIT1::LEU2) to produce the CS1- mutant. The CIT2 gene was disrupted by URA3 (CIT2::URA3) to make the CS2- mutant. The CS1-mutant of the BWG1-7a parental strain (Mato, ade1-100, his3-11,15, leu2-2, leu2-112, ural-3-52) was produced in the same way (10). The strains above were obtained from Dr. L. P. Guarente. The following mutants with the common feature of inability to grow on acetate (acetate\* collection) were from M. T. McCammon and J. M. Goodman (University of Texas Southwestern): mitochondrial malate dehydrogenase mutant (MDH1\*) and isocitrate dehydrogenase mutant for subunit 1 (ICDH1\*) and subunit 2 (ICDH2\*) from YM01 (MATa, ade1-100, his3-11,15, leu2-3,112, trpl-1, ural-3-1, can1-100). The mitochondrial fumarase mutant (FUM1\*) was derived from the W303–1A parental strain (MATa, ade1-1, his3-1,15, leu2-3,112, trpl-1, ural-3-1, can1-100).

Media and Growing Conditions—Cells were grown overnight in 20 ml of YP (1% yeast extract, 2% Bacto-peptone) or selective medium containing 2% glucose with shaking at 30 °C. These cultures were transferred into 500 ml of YP with 2% galactose, and growth was allowed to continue for 24 h. All media were supplied with 20 μm potassium phosphate, pH 7.0, in order to buffer accumulating acetate when acetate\* strains were grown.

Preparation of Mitochondria—The procedure for isolating mitochondria was essentially that of Daum et al. (11). Cells were collected and

\* This work was supported by grants from the Department of Veterans Affairs, NIDDK Grant DK13335, National Science Foundation Grant MCB-9117385, and OTKA Grant 0909. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Veterans Affairs Medical Center, 4500 S. Lancaster Rd., Dallas, TX 75216. Tel.: 214-376-5451 (ext. 5605); Fax: 214-372-9534.

‡ The abbreviation used is: BTC, 1,2,3-benzenetricarboxylic acid.
washed with distilled water by centrifugation at 2800 x g for 5 min (4000 rpm; Beckman J-10 rotor). Cells were incubated in 0.1 M Tris, pH 9.4 (0.15-0.2 g (wet weight)/ml in this and subsequent steps), in the presence of 10 mM diethiothreitol for 15 min at 30 °C. Cells were then washed twice in 1.2 M sorbitol in 20 mM potassium phosphate, pH 7.4 (buffer A), and incubated at 30 °C in buffer B with 3 mg of lyticase/ml for 1 h. After 1 h, an additional 1 mg of lyticase/kg of cells was added, and the digestion was continued for 1 additional h. Spheroplasts were collected by centrifugation; washed twice with 1.2 M sorbitol; and suspended in 0.6 M mannitol, 0.1% bovine serum albumin, 20 mM potassium phosphate, pH 7.2, supplemented with protease inhibitors (1 mg benzamidine, 1 mg phenylmethylsulfonyl fluoride, 2 units of aprotinin/100 ml) at 4 °C. Spheroplasts were homogenized twice with three strokes each in a tight fitting motor-driven Potter homogenizer (after the first three strokes, the solution was chilled on ice for 2 min). The homogenate was centrifuged at 7000 x g for 5 min (4000 rpm; Beckman J-20 rotor). The supernatant solution was decanted and centrifuged in the same rotor at 17,500 x g for 5 min (12,000 g) to sediment yeast mitochondria.

Transport Studies on Intact Mitochondria—The mitochondrial pellet was resuspended in 0.55 M mannitol, 20 mM potassium phosphate, pH 6.8, 0.1% bovine serum albumin, 5 mM rotenone, and 5 μM antimycin (buffer B). This 5-20 mg suspension of mitochondria (10-20 mg of protein/ml) was placed on ice, and transport was assayed immediately.

For uptake studies, 200 μl of buffer B supplemented with 1.5 mM [1,5-14C]citrate (2000 cpdm/mmol) were equilibrated at 30 °C in 1.5-ml Eppendorf tubes for 2 min. To this solution were added 100 μl of an ice-cold mitochondrial suspension, resulting in a final citrate concentration of 10 μM. At the indicated times, or in the zero-time tubes before the addition of mitochondria, 100 μl from the 160 mM BTC stock solution (40 mM final concentration) were added. Suspensions were diluted with 900 μl of ice cold 40 mM BTC, and the tubes were gently vortexed and centrifuged in an Eppendorf centrifuge (Model 5415) for 1 min at 14,000 rpm (8000 x g). The supernatant solution was decanted, the wall of the tube was wiped to remove residual radioactivity, and the pellet was dissolved in 100 μl of concentrated formic acid and transferred into vials for determination of radioactivity. Uptake was found to be linear for at least 30 s and was relatively insensitive to pH between pH 6 and 8. For malate uptake studies, the same procedure was employed with the exception that [U-14C]malate was used as substrate and butyl malonate was the specific inhibitor.

In studies of citrate efflux, mitochondria were preloaded with 5 mM [1,5-14C]citrate (specific activity of 2000 cpdm/mmol) at 5 °C. To monitor loading, 100-μl aliquots were taken, added to 900 μl of 40 mM ice-cold BTC, and centrifuged, and the radioactivity in the pellets was measured as described above. Mitochondria from the PSY142 parental strain were usually loaded for 10 min, while those from the CS1 mutant were loaded for 15 min. To measure citrate efflux, 100 μl of loaded mitochondria were added to 200 μl of buffer B pre-equilibrated at 30 °C for 2 min with or without 1 mM unlabeled citrate as specified. After 30 s, or in the zero-time tubes before the addition of mitochondria, 100 μl from the 160 mM BTC stock solution were added (40 mM final concentration). The tubes were centrifuged as described above, and 350 μl of the supernatant were used to determine radioactivity. Extramitochondrial space was determined with [1,5-14C]citrate (10,000 cpdm/mmol) to start transport. The reaction was quenched with 10 mM BTC (final concentration) after 5 min in the two sample tubes or by adding the BTC before the [1,5-14C]citrate to the two zero-time tubes. A 200-μl aliquot of each reaction mixture was then added to a Dowex 1-X8 column as described above and eluted with 1.3 ml of buffer C into scintillation vials.

Protein Determination—Mitochondrial extracts were collected by centrifugation in an Eppendorf centrifuge (14,000 rpm for 1 min), precipitated with 5 volumes of cold acetone (−20 °C), and recentrifuged. The pellets were suspended in 0.1 N NaOH, and protein was determined with the bicinchoninic acid assay (Fierce) using bovine serum albumin as a standard.

RESULTS

Citrato Uptake by Intact Mitochondria from the PSY142 Strain—Fig. 1 shows the citrate concentration dependence of citrate uptake by mitochondria isolated from the PSY142 strain of S. cerevisiae. From the data in Fig. 1A, it is apparent that there are two kinetic components to this uptake. One shows a linear concentration dependence throughout the concentrations studied, while the other displays saturation kinetics. The nonsaturable uptake is similar to that seen with pyruvate uptake into yeast mitochondria (13) and is thought to be either a second, low affinity citrate transporter or nonspecific uptake of citrate. The saturable component of the curve has an apparent K<sub>m</sub> for citrate of 240 μM and a V<sub>max</sub> of 21.8 nmol/min/mg of protein. This K<sub>m</sub> exceeds the rat liver value by a factor of 2 (14).

Table I compares the effects of different inhibitors on citrate uptake into these mitochondria, and differences in the inhibitor sensitivity of yeast and rat liver mitochondrial citrate transport are noted. The concentrations of α-cyano-3-hydroxyquinamic acid required to inhibit the yeast transporter are higher than those required for the rat liver transporter. This is con-
Mutants-To measurement made in Table I is butyl malonate-inhibitable synthase deletions affect dicarboxylate carrier activity. Mitochondrial isocitrate dehydrogenase has no effect. Since the inhibition is by association with citrate synthase, detergent extraction of these extracts into liposomes by freeze-thaw sonication shows that the transport activity of proteoliposomes from CS1- cells is indistinguishable from the rates obtained with the parental line or CS2- cells (Table IV). Malate uptake was also found to be independent of the cell type from which the extract was prepared (Table IV). These data suggest that although transport is compromised in the intact mitochondria, similar amounts of citrate transport protein with similar transport activity are extracted from the membranes of mitochondria with the CS1- mutation compared with the wild type and CS2- cells. These data also suggest that disruption of the metabolic complex results in an alteration in transporter function without an alteration in protein levels.

Proof of this regulatory mechanism would be to observe modulation of citrate transport activity after the readdition of mitochondrial citrate synthase to the proteoliposome preparation. Unfortunately, the addition of mitochondrial citrate synthase had no effect on citrate uptake by proteoliposome preparations from either CS1- or parental cell mitochondria (data not shown). Since the extract from parental cell mitochondria contains as much as 92% of the citrate synthase activity found in the intact organelle, it is of interest to determine whether the lack of transport activity of extracts from CS1- cells compared with the wild type and CS2- cells is due to the lack of the mitochondrial enzyme or due to its inability to serve as a substrate for the transporter. The results of these experiments are described in the next section.

The Transporter as a Similar Level of Inhibition in Parental and CS1- Mitochondria

Mitochondria were prepared and citrate and malate uptake was tested as specified under "Experimental Procedures." Values are averages of triplicate measurements.

Table I: Citrate Uptake by Reconstituted Proteoliposomes Containing CS1- and CS2- Mitochondria

Table II: Uptake of [1,5-14C]and [U-14C]Malate into Yeast Mitochondria from Different Parental and Mutant Cells

Table III: BTC-sensitive efflux of [1,5-14C]citrate from preloaded yeast mitochondria of PSY142 cells and its CS1- mutant

* ND, not detectable.

A new method for preparing proteoliposomes suggested that the transport activity of reconstituted systems might be improved by readdition of additional amounts of mitochondrial citrate synthase. These results suggest that the lack of transport activity in extracts from CS1- cells is not due to the lack of the enzyme but rather to its inability to serve as a substrate for the transporter. The results of these experiments are described in the next section.

Mitochondria from different parental and mutant cells were prepared and citrate and malate uptake was tested as specified under "Experimental Procedures." Values are averages of triplicate measurements.

Table I: Citrate Uptake by Reconstituted Proteoliposomes Containing Mitochondria from Cells with Mutations in Tricarboxylic Acid Cycle Enzymes

Table II: Uptake of [1,5-14C]citrate and [U-14C]malate into yeast mitochondria from different parental and mutant cells

Table III: BTC-sensitive efflux of [1,5-14C]citrate from preloaded yeast mitochondria of PSY142 cells and its CS1- mutant

* ND, not detectable.

A new method for preparing proteoliposomes suggested that the transport activity of reconstituted systems might be improved by readdition of additional amounts of mitochondrial citrate synthase. These results suggest that the lack of transport activity in extracts from CS1- cells is not due to the lack of the enzyme but rather to its inability to serve as a substrate for the transporter. The results of these experiments are described in the next section.

Mitochondria from different parental and mutant cells were prepared and citrate and malate uptake was tested as specified under "Experimental Procedures." Values are averages of triplicate measurements.

Table I: Citrate Uptake by Reconstituted Proteoliposomes Containing Mitochondria from Cells with Mutations in Tricarboxylic Acid Cycle Enzymes

Table II: Uptake of [1,5-14C]citrate and [U-14C]malate into yeast mitochondria from different parental and mutant cells

Table III: BTC-sensitive efflux of [1,5-14C]citrate from preloaded yeast mitochondria of PSY142 cells and its CS1- mutant

* ND, not detectable.

A new method for preparing proteoliposomes suggested that the transport activity of reconstituted systems might be improved by readdition of additional amounts of mitochondrial citrate synthase. These results suggest that the lack of transport activity in extracts from CS1- cells is not due to the lack of the enzyme but rather to its inability to serve as a substrate for the transporter. The results of these experiments are described in the next section.

Mitochondria from different parental and mutant cells were prepared and citrate and malate uptake was tested as specified under "Experimental Procedures." Values are averages of triplicate measurements.

Table I: Citrate Uptake by Reconstituted Proteoliposomes Containing Mitochondria from Cells with Mutations in Tricarboxylic Acid Cycle Enzymes

Table II: Uptake of [1,5-14C]citrate and [U-14C]malate into yeast mitochondria from different parental and mutant cells

Table III: BTC-sensitive efflux of [1,5-14C]citrate from preloaded yeast mitochondria of PSY142 cells and its CS1- mutant

* ND, not detectable.
**Table IV**

Transport of [1,5-3H]citrate and [U-14C]malate by mitochondria and proteoliposomes from the PSY142 parental strain and its citrate synthase mutants

| Yeast strain | Intact mitochondria | Extract built into liposomes |
|--------------|---------------------|------------------------------|
|              | Citrate             | Malate                       |
|              | nmol × mg protein⁻¹× min⁻¹ | nmol × mg protein⁻¹× 5 min⁻¹ |
| Parental     | 27.9                | 10.70                        |
| CS1⁻         | 4.0                 | ND                           |
| CS2⁻         | 31.8                | 9.1                          |

* ND, not detectable.

**Discussion**

The mitochondrial citrate transporter catalyzes the exchange of citrate for citrate, malate, isocitrate, succinate, and phosphoenolpyruvate (17—19). The mammalian mitochondrial citrate transporter has been well studied (20, 21), and the rat liver transporter has been recently cloned (22). Here, we have characterized further the mitochondrial citrate transporter from *S. cerevisiae*, and subtle kinetic differences and inhibitor sensitivity differences suggest that the yeast protein may not be identical to the mammalian protein. Using this characterization of a system amenable to genetic manipulation, we have asked fundamental questions about the organization of mitochondrial membrane transport proteins and mitochondrial matrix enzymes.

We (22, 27—30) and others (24—26) have reported physical interactions between mitochondrial matrix enzymes in both mammalian and yeast cells. We (3) and others (26) have shown that these enzymes are associated with mitochondrial membrane-binding proteins and that these proteins could be the respective transport proteins for the enzyme substrates or products. Demonstration of these complexes would make metabolic pathways like the tricarboxylic acid cycle a closed loop system for substrates from the mitochondrial membrane surface through the pathway.

In this study, we have probed for functional associations of the mitochondrial citrate and malate transporters with tricarboxylic acid cycle enzymes by measuring citrate and malate transport in mitochondria with deletions in the tricarboxylic acid cycle enzymes. We have found that deficiencies in mitochondrial, but not cytosolic, citrate synthase result in severely impaired citrate uptake and efflux as well as malate uptake. Other mutations in tricarboxylic acid cycle enzymes had little or no effect on citrate uptake. Mutation of malate dehydrogenase did impair malate uptake, as would be expected from the work of Lançar-Benba et al. (6), who clearly demonstrated interaction of malate dehydrogenase with the dicarboxylate carrier of mitochondria when they were able to purify the dicarboxylate carrier with a malate dehydrogenase affinity column. Interestingly, disruption of nearest neighbor tricarboxylic acid cycle enzymes to malate dehydrogenase had a large impact on malate uptake, while no effect was observed with disruptions in more distal enzymes in the cycle.

When extracts of mitochondria from mutant strains were prepared and reconstituted into liposomes, citrate and malate uptake rates were indistinguishable from the rates observed with parental mitochondrial extracts. This finding indicates that there was no difference in the levels of transport protein in these respective mitochondria, but that there was regulation of the activity of the transporters. More important, it should be noted that while malate uptake was undetectable in the mitochondria from mutants lacking mitochondrial citrate synthase, butyl malonate-sensitive malate uptake was the same in extracts from the mutant and parental strains in the proteoliposome.

These data support the previous demonstration that citrate synthase and fumarase associate with the malate dehydrogenase enzyme (25) and suggest that this complex associates with the citrate transporter and the dicarboxylate carrier in the mitochondrial membrane. Disruption of any member of the complex results in dissociation of the complex from the citrate transporter. We propose that the vectorial organization of the tricarboxylic acid cycle metabolism includes the citrate transporter and the dicarboxylate carrier of the mitochondrial membrane.

Acknowledgments—We thank Penny Kerby for secretarial assistance and Ginny Poffenberger for technical assistance. We are indebted to Dr. M. T. McCammon for supplying the mutant yeast cells.

**References**

1. Srere, P. A. (1987) *Annu. Rev. Biochem.* 56, 89–124
2. Sumegi, B., Sherry, A. D., and Malloy, C. R. (1980) *Biochemistry* 29, 9106–9110
3. D’Ouza, S. F., and Srere, P. A. (1983) *J. Biol. Chem.* 258, 4706–4709
4. Grigorenko, E. V., Small, W. C., Penson, L., and Srere, P. A. (1990) *J. Mol. Biol.* 213, 215–219
5. Persson, L. O., and Srere, P. A. (1992) *Biochem. Biophys. Res. Commun.* 6, 76–76
6. Lançar-Benba, J., Foucher, B., and Saint-Macary, P. (1994) *Biochem. Biophys. Acta* 1196, 213–216
7. Bissau, L. F., and Freidel, D. G. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 1730–1734
8. Pande, S. V., and Murthy, M. S. R. (1988) in *Microcompartmentation* (Jones, D. P., ed.) pp. 89–114, CRC Press, Inc., Boca Raton, FL
9. Cohen, N. S., Chung, C.-W., and Rajam, L. (1987) *J. Biol. Chem.* 262, 203–208
10. Kim, K., Rosenkrantz, M. S., and Guarente, L. P. (1986) *Mol. Cell. Biol.* 6, 1936–1942
11. Daum, G., Böhm, P. C., and Schatz, G. (1982) *J. Biol. Chem.* 258, 13028–13033
12. Kaplan, R. S., Mayor, J. A., and Johnston, N., and Oliver, J. L. (1990) *J. Biol. Chem.* 265, 13379–13385
13. Bricuet, M. (1977) *Biochim. Biophys. Acta* 459, 280–289
14. Kaplan, R. S., Parlo, R. A., and Coleman, P. S. (1986) *Methods Enzymol. 125*, 671–696
15. McGowan, J. D., and Klingenberg, M. (1971) *Eur. J. Biochem.* 20, 302–309
16. Bissacca, F., DelPalm, A., and Palmieri, F. (1986) *Biochim. Biophys. Acta* 977, 171–176
17. Chappell, B., and Harroff, K. N. (1967) in *Biochemistry of Mitochondria* (Slater, E. C., Kanuga, Z., and Wojtaczak, L., eds.) pp. 75–91, Academic Press, Ltd., London
18. Connovers, T. E. (1987) *Trends Biochem. Sci.* 12, 88–89
19. Palmieri, F., and Quagliariello, E. (1978) in *Bioenergetics at Mitochondria and Cellular Levels* (Wojtaczak, L., and Shen, F. E., and Shorohov, J., eds.) pp. 5–38, Nencki Institute of Experimental Biology, Warsaw, Poland
20. Chajes, D., and Aziz, A. (1989) *J. Biol. Chem.* 264, 14677–14680
21. Gris, M. D., Chajes, D., Mertens, W., and Aziz, A. (1996) *Eur. J. Biochem.* 240, 681–684
22. Kaplan, R. S., Mayor, J. A., and Wood, D. O. (1993) *J. Biol. Chem.* 268, 13892–13899
23. Halper, L. A., and Srere, P. A. (1977) *Arch. Biochem. Biophys.* 184, 529–534
24. Sumegi, B., Gyoci, L., and Alkonyi, I. (1980) *Biochim. Biophys. Acta* 616, 155–166
25. Beckmann, S., and Kanarick, L. (1981) *Eur. J. Biochem.* 117, 527–535
26. Fahien, L. A., Kmiotek, E. H., Woldegiorgis, G., Everson, M., Shrago, E., and Marshall, M. (1985) *J. Biol. Chem.* 260, 6089–6097
27. Tyiska, L., Williams, J. S., Brest, L. G., Hudson, P. C., Clark, B. J., Robinson, J. B., Jr., and Srere, P. A. (1986) *NATO ASI Ser. Ser. B. Life Sci.* 128, 177–189
28. Sumegi, B., and Srere, P. A. (1984) *J. Biol. Chem.* 259, 15400–15405
29. Brest, L. G., and Srere, P. A. (1987) *J. Biol. Chem.* 262, 313–326
30. Kaplan, G., Evans, C. T., Malloy, C., and Srere, P. A. (1990) *J. Biol. Chem.* 264, 11204–11210