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PURIFICATION OF THE MITOCHONDRIAL CITRATE TRANSPORTER IN YEAST

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Summary - We present a method of partial purification of mitochondrial citrate transporter of the yeast Saccharomyces cerevisiae. Based on functional evidence of interaction between citrate transport and citrate synthase, we have used an affinity column containing pig heart citrate synthase (PHCS) for the purification. The purified preparation shows two protein components whose M_r is ~50K and 60K. The specific activity of our purest fractions is 2.6 μmoles/min which compares favorably to that of purified beef liver enzyme and purified rat liver enzyme.

We have shown previously that mitochondrial citrate synthase (CS1) binds to a protein of the inner membrane (IM) of mitochondria (1,2). The yeast peroxisomal isozyme of citrate synthase (CS2) does not bind to inner mitochondrial membranes (3). Mutant yeast cells lacking CS1 were incapable of carrying out citrate transport even though citrate transporter (also referred to as citrate carrier and the tricarboxylic acid carrier) could be demonstrated to be present at normal levels in $CS1^-$ mitochondria (4). We hypothesized that the binding of CS1 to the IM was to the citrate transporter.

The citrate transporter of yeast mitochondria has not been characterized, although there is one report on the citrate transporter in oleaginous yeast (5). On the other hand, the mitochondrial citrate transporter of rat liver (6) and beef liver (7) have been purified to apparent homogeneity.

In order to continue our studies on the possible interaction of citrate synthase and citrate transporter it was necessary to obtain the S. cerevisiae citrate transporter, and in this paper we

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report a purification procedure for the partial purification of this protein.

Materials and Methods

All manipulations were carried out at 4°C unless otherwise indicated.

Materials. Pig heart citrate synthase (PHCS) was from Boehringer (Mannheim, Germany), Triton X-114, asolectin, and Amberlite XAD-2 were from Fluka (Buchs, Switzerland). Hydroxylapatite (Bio-Gel HTP) was from BioRad (Richmond, CA). CNBr-activated Sepharose 4B and all other biochemicals were from Sigma (St. Louis, MO).

Asolectin was purified as described previously (8). The resulting asolectin solution was stored at below 0°C. Amberlite XAD-2 was washed with isopropanol and then extensively rinsed with distilled water.

Preparation of Immobilized Pig Heart Citrate Synthase. CNBr-activated Sepharose 4B (0.30 g) was swollen and washed for 15 min with 1 mM HCl on a sintered glass filter. It was then suspended in 1 ml 0.1 M $NaHCO_3$ (pH 9) and mixed with 1 ml (10 mg) PHCS in 3.2 M $(NH_4)SO_4$ end-over-end for 2 h at room temperature. The resulting enzyme-gel preparation was washed on a sintered glass filter with 0.1 M $NaHCO_3$ and then incubated as above in 5 ml 0.1 M Tris-Cl buffer (pH 8) in order to block remaining active groups. Finally, non-covalently adsorbed protein was removed by washing three times with 10 ml portions of 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8) and 0.1 M Na-acetate, 0.5 M NaCl (pH 4), respectively.

Solubilization of Yeast Mitochondria. Yeast mitochondria were isolated by the method of Daum et al. (9) from PSY142 (10) cells grown in YPD media 500 ml cultures to stationary phase. The mitochondria were suspended (2 g/ml) in 20 mM MOPS, 100 mM NaCl, 20 mM trisodium citrate (pH 7.2) and stored at -70°C. Thawed mitochondrial suspension (1 or 2 ml) was mixed with an equal volume of 20 mM MOPS, 20 mM trisodium citrate, 6% (w/v) Triton X-114, and 4 mg/ml asolectin (fresh), and the resulting solution was incubated with end-over-end mixing for 20 min.

Hydroxylapatite Treatment. The Triton X-114 solubilized mitochondria were applied directly to a column (1.5 cm diameter) containing Bio-Gel HTP (1 g dry weight/ml solution applied) which had been equilibrated in 5 mM MOPS, 5 mM trisodium citrate, 2% (w/v) Triton X-114 (pH 7.2) (buffer A), and various concentrations of NaCl. The column was eluted with the same buffer, and 1 ml fractions containing protein were pooled. Alternatively, a batch procedure was applied involving mixing the solubilized mitochondria with the equilibrated Bio-Gel HTP end-over-end for 15 min followed by centrifugation at 5,000 ×g for 5 min. The resulting supernatant was saved and the Bio-Gel HTP resuspended in the same buffer, and the centrifugation was repeated. The two supernatants were pooled.

Affinity Chromatography. The solution resulting from the hydroxylapatite step was loaded on a column (diameter 0.9 cm) containing 0.7 ml packed PHCS-Sepharose 4B equilibrated in buffer A containing the same concentration of NaCl. The column was eluted as described in the legend of Figure 1.

Reconstitution of the Tricarboxylate Carrier. The tricarboxylate carrier was reconstituted into asolectin liposomes by removing Triton X-114 with Amberlite XAD-2. Liposomes were prepared by

sonicating 45 mg washed asolectin per ml 20 mM MOPS, 20 mM NaCl (pH 7.2) for 20-30 min in a sonicating bath (Laboratory Supplies, Co.-Hicksville, NY). Amberlite beads were pre-equilibrated with 9 mg/ml asolectin by end-over-end mixing for 30 min. Since the assay for citrate exchange required the removal of the trisodium citrate used as a stabilizer, each sample was passed through a Sephadex G-25M column equilibrated with 10 mM MOPS, 40 mM NaCl, 10 mM trisodium DL-isocitrate, 2% (w/v) Triton X-114 (pH 7.2). The void volume was collected and immediately mixed with liposomes in a 4:1 (v/v) ratio. DL-isocitrate was added to a final concentration of 50 mM, and the resulting solution was mixed end-over-end for 15 min after which pre-equilibrated Amberlite (500 mg/ml) was added, and the mixing was continued for 2 h and 30 min.

Assay of Citrate Exchange Activity. The citrate/isocitrate exchange was measured spectrophotometrically at 25°C by following the production of NADPH at 340 nm as described by Lüthy and Azzi (11) except the NADP concentration was 0.15 mM instead of 0.075 mM. The initial velocity was used to calculate the maximal activity when appropriate, however, many samples showed a lag of 2-3 min before a maximal, linear rate was achieved which was then used as activity measured.

Production of Polyclonal Antibodies. Protein (600 μg) from the hydroxylapatite treatment was reconstituted into asolectin liposomes, mixed with an equal volume of complete Freund's Adjuvant (Gibco-Grand Island, NY), and injected intracutaneously into the foot pad of 6-8 week old female BALBC mice (50 μg/100 μl/mouse). On day 54 after immunization the mouse sera was tested by a simple enzyme immunosorbent assay (12) and by Western blot using the solution from the hydroxylapatite treatment as antigen. Mice were boosted 3× (50 μg injections intraperitoneally) without adjuvation at 30-day intervals.

Other Methods. Protein concentration in the fractions from the coupling of pig heart citrate synthase on Sepharose 4B was determined by the Bradford method (13) whereas all other fractions were analyzed by a modified Lowry procedure in the presence of 1% SDS (14). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of samples precipitated with 30% (v/v) acetone was performed on Phast System (Pharmacia-Uppsala, Sweden) using PhastGel gradient 8-25 as described in the manual.

Results and Discussion

Based upon our observation of the functional coupling of citrate transport to citrate synthase in yeast mitochondria, we postulated that a physical interaction occurred between the two proteins (4). We therefore incorporated a step using a CS affinity column in the purification (Table I and Fig. 1). We have used primarily the assay described by Lüthy and Azzi (11), and the specific activity of our best fractions is 8.2 μmol/min/mg which compares favorably with those reported for the rat liver and bovine liver transporters. The transport activity is inhibited 1, 2, 3 benzyl tricarboxylate (Table II).

Table I

Purification of Citrate Carrier from Yeast

Fraction	Specific Activity (μmol/min mg)	Total Activity (μmol/min)
Best Preparation		
Solubilized mitochondria	0.062	0.316
Hydroxylapatite pass-through	0	-
NaCl eluate from PHCS-Sepharose	8.25	0.041
Summary of Eight Different Experiments		
Solubilized mitochondria	0.025-0.153 (0.070)	0.003-0.316
Hydroxylapatite pass-through	0	-
Desorbed eluate from PHCS-Sepharose	0.414-8.25 (2.64)	0.015-0.041

Data represent the range of specific activity obtained. The average value is presented within parentheses (n=18-19).

SDS-PAGE analyses were carried out on varying fractions (Fig. 2). The polypeptide pattern of the non-binding material in peak 1 resembles the pattern of the hydroxylapatite fraction which shows that most of the proteins loaded on the PHCS column did not bind. The material desorbed at 10 mM NaCl (peak 2) also exhibit a large number of protein bands which indicate nonspecific binding. In contrast, the protein fractions desorbed at 50 and 100 mM NaCl

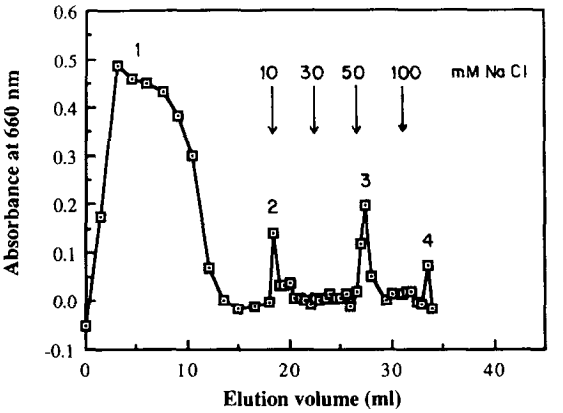

Figure 1. Protein profile from the affinity chromatography of the NaCl-free hydroxylapatite fraction on PHCS-Sepharose. After loading and washing with NaCl-free buffer (5 mM MOPS, 5 mM trisodium citrate, 2% (w/v) Triton X-114, pH 7.2), the column was eluted with the same buffer containing 2.5% (w/v) ethyleneglycol, 1 mg/ml asolectin, and the indicated NaCl concentrations.

Table II

Inhibition of Citrate/Isocitrate Exchange Activity in Yeast Citrate Carrier Reconstituted in Asolectin Liposomes

	Fraction	
	PHCS-Sepharose	Solubilized mitochondria
Control 1	2.6 (0.53-7.8)	
4 mM BTA (10)	1.5 (0-4.3)	
8 mM BTA (4)	0	
Control 2	4.1	
Non-immunized + sera (2)	6.4	
+ anti-sera (2)	3.1	
Control 3	-	0.12 (0.9-0.15)
+ hydroxyapatite	-	0

Data show the range with the average given in parenthesis. The BTA inhibition was measured in seven different preparations while the others were measured from one preparation. n=total number of measurements; where n=2 the average value is shown.

(peaks 3 and 4) are dominated by two bands of an apparent molecular weight of 50 kD and 60 kD, respectively. None of these bands represent a major band in the loaded hydroxylapatite fraction which, together with the relatively high ionic strength that was necessary to elute them, indicate a strong and specific interaction between these proteins and the immobilized PHCS.

The fractions in Figure 1 were also reconstituted into asolectin liposomes and assayed for citrate exchange activity. Such activity was detected in all of the samples except the hydroxylapatite fraction and peak 1 (non-binding material).

Table I summarizes the citrate exchange activities from eight different experiments in which hydroxylapatite fractions containing 30 or 50 mM NaCl were loaded on a PHCS column (pre-equilibrated in the same salt concentration) which was then washed with the same salt concentration and finally eluted with buffer containing 100 mM NaCl or 0.05 mM CoA and 0.05 mM OAA. Both elution methods yielded the same polypeptide pattern with two dominant protein bands. The latter desorption agents, CoA and OAA, are known to cause a conformational change in PHCS (15) which further strengthens the notion of a specific interaction between these proteins and PHCS.

Reconstituted material from the hydroxylapatite fraction revealed no citrate exchange activity. Addition of this material to active samples caused a total inhibition of their activity which

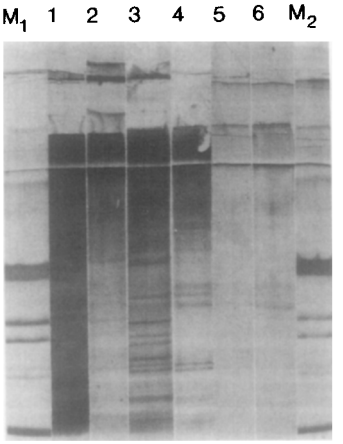

Figure 2. SDS polyacrylamide gel electrophoresis of fractions obtained during the purification of citrate transporter from yeast mitochondria. (1) Triton X-114 solubilized mitochondria; (2) Hydroxylapatite pass-through; (3) pig heart citrate synthase-Sepharose (PHCS-Sepharose) pass-through fraction; (4) material desorbed from the PHCS-Sepharose by 10 mM NaCl; (5) material desorbed from the PHCS-Sepharose by 50 mM NaCl; (6) material desorbed from the PHCS-Sepharose by 100 mM NaCl. (M_1 and M_2) Molecular weight markers: bovine serum albumin (66,000), ovalbumin (45,000), pepsin (34,700), trypsinogen (24,000), β-lactoglobulin (18,000), and lysozyme (14,300). Lane M_1 and lanes 1-4 represent one gel while lanes 5-6 and M_2 are from another gel.

further support the presence of an inhibitor in the hydroxylapatite fraction (Table II).

Polyclonal antibodies raised against the hydroxylapatite pass-through fraction were mixed with reconstituted purified citrate carrier, and they caused a 24% reduction in the citrate exchange activity. Incubation with a non-immune mouse sera stimulated the activity (Table II). The antibodies react with several bands in the molecular weight interval between 50,000 and 60,000 (data not shown).

Since mitochondrial citrate levels should reflect both acetyl CoA formation and utilization, it is reasonable to propose that intra- and extramitochondrial citrate levels control rates of exchange. The most efficient means of vectorial control of intramitochondrial levels of citrate would be a coupling of the exchanger with citrate synthase.

In cells such as yeast, which contain a glyoxylate cycle, there also exists a nonmitochondrial citrate synthase. Such cells may have little need for a mitochondrial citrate carrier, but one exists nevertheless. A literature search has found only one study on yeast citrate carrier, and this was in an oleaginous yeast that

produces lipids (5). In these yeasts (which contain cytosolic ATP citrate lyase) a large quantity of cytosolic citrate is needed for fatty acid biosynthesis. It was shown that palmityl CoA was an inhibitor for the yeast citrate carrier.

Our recent work with the physical coupling of tricarboxylic acid cycle enzymes places us in a position to test directly for formation of a complex between catabolic enzymes and a transport protein and elucidate the role of such a complex as a vectorial control point of metabolism.

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