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Identification of the yeast ACR1 gene product as a succinate-fumarate transporter essential for growth on ethanol or acetate

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Abstract The protein encoded by the *ACR1* gene in *Saccharomyces cerevisiae* belongs to a family of 35 related membrane proteins that are encoded in the fungal genome. Some of them are known to transport various substrates and products across the inner membranes of mitochondria, but the functions of 28 members of the family are unknown. The yeast *ACR1* gene was introduced into *Escherichia coli* on an expression plasmid. The protein was over-produced as inclusion bodies, which were purified and solubilised in the presence of sarkosyl. The solubilised protein was reconstituted into liposomes and shown to transport fumarate and succinate. Its physiological role in *S. cerevisiae* is probably to transport cytoplasmic succinate, derived from isocitrate by the action of isocitrate lyase in the cytosol, into the mitochondrial matrix in exchange for fumarate. This exchange activity and the subsequent conversion of fumarate to oxaloacetate in the cytosol would be essential for the growth of *S. cerevisiae* on ethanol or acetate as the sole carbon source.

Key words: Yeast mitochondrion; Transport; *ACR1* gene; Succinate-fumarate exchange

1. Introduction

The glyoxylate pathway is one of the main anaplerotic routes for replenishing the intermediates of the tricarboxylic acid cycle that have been withdrawn for biosynthetic purposes. In the yeast *Saccharomyces cerevisiae*, this pathway is essential for aerobic growth on either ethanol or acetate as the sole carbon source. Ethanol is converted to acetate via acetaldehyde, and then the acetate is activated to acetyl-CoA, and fed into the glyoxylate and tricarboxylic acid cycles. Two inducible enzymes in the yeast glyoxylate pathway, isocitrate lyase and malate synthase, are in the cytosol [1,2], whereas in plants they are confined to the glyoxysome [3]. The other enzymes implicated in the pathway (malate dehydrogenase, citrate synthase and aconitase) are present in both the cytosol and the mitochondrion, where they participate in the tricarboxylic acid cycle. Thus, succinate, one of the principal products of this pathway, is produced in the cytosol from isocitrate. How does it replenish the succinate pool available to the tricarboxylic acid cycle in the matrix of the mitochondrion? In order to investigate this and related questions, mutant strains of *S. cerevisiae* have been selected for their inability to grow on ethanol as the sole carbon source. One such mutation is in the *ACR1* gene [4]. It encodes a member of a family of 35 membrane proteins [5], whose biochemically characterised members are involved in the transport of substrates and products across the inner membranes of mitochondria [6–8]. Identified members in *S. cerevisiae* include three isoforms of the ADP/ATP translocase [9–11], and the phosphate [12], dicarboxylate [5,13], citrate [14] and ornithine [15] carriers.

In the present work, we have identified ACR1 as a transporter for succinate and fumarate. Therefore, it is probably responsible for carrying cytosolic succinate produced by the glyoxylate pathway into the mitochondrion in exchange for fumarate, which is transported from the mitochondrial matrix to the cytosol.

2. Materials and methods

2.1. Materials

NEM Life Science Products (Milan, Italy) supplied the sodium salt of α-[1-^{14}C]ketoglutaric acid. Egg-yolk phospholipids (egg lecithin) were obtained from Fluka (Milan, Italy), and cardiolipin and sarkosyl (*N*-lauroylsarcosine) from Sigma Chemical Company (Milan, Italy). The molecular weight marker kit 12.3–78.0 kDa was purchased from BDH (Poole, UK).

2.2. Amplification of the ACR1 gene from yeast genomic DNA

Oligonucleotide primers were synthesised with the sequences 5′-TAGGGATCCCATATGTCTCAAAAAAAGAAGGCTTCC-3′ and 5′- CGAAAGCTTTTACTTTAATGGCTTTGGCTTTGG-3′. They correspond to the extremities of the coding sequence for *ACR1* (nucleotides 609464–610429 on the positive strand of chromosome X; GenBank accession numbers Z25485, Z49595), followed by the stop codon TAA, and with *Nde*I and *Hin*dIII sites at the 5′ and 3′ ends, respectively. The sequence of interest was amplified from *S. cerevisiae* genomic DNA, cloned into the expression vector pMW172, transformed into *Escherichia coli* DH5α cells, and transformants were selected and screened by direct colony PCR, as described before [5,15]. The sequence of the insert coding for ACR1 was verified by the modified dideoxy chain termination method [16].

2.3. Bacterial expression and functional reconstitution of ACR1

The ACR1 protein was over-expressed at 37°C in *E. coli* CO214 (DE3) [15]. Control cultures containing the empty pMW172 vector were processed in parallel. Inclusion bodies were obtained and purified as described previously [5,15,17]. ACR1 was solubilised from the purified inclusion bodies in the presence of sarkosyl (2%, w/v), diluted 1.5-fold with a buffer containing 2 mM PIPES, pH 7.0, 0.6% (w/v) Triton X-114 and 0.2 mM EDTA, and then reconstituted into liposomes in the presence of appropriate substrates, as described before [5,15,17], except that cardiolipin (1 mg/ml) was included in the reconstitution mixture.

2.4. Protein chemical characterisation of ACR1

Samples taken from cultures at various points of growth and purified inclusion bodies were examined by SDS-PAGE in 17.5% gels. Either the proteins were stained with Coomassie blue dye or they were transferred to poly(vinylidene difluoride) membranes, stained

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with Coomassie blue dye, and their N-terminal sequences determined with a pulsed liquid protein sequencer (Applied Biosystems Model 477A). The yield of purified ACR1 per litre of bacterial culture was estimated from a Coomassie blue-stained SDS-PAGE gel with an LKB 2202 Ultroscan laser densitometer, with carbonic anhydrase as protein standard.

2.5. Activity assays

External substrate was removed from proteoliposomes on a Sephadex G-75 column. The transport activity at 25°C was determined by measuring the flux of ^{14}C-substrate from either outside to inside (forward exchange) or inside to outside (backward exchange) [18]. In backward exchanges, the internal substrate pool of the proteoliposomes was made radioactive by carrier-mediated exchange equilibration with 5 μM [^{14}C]oxoglutarate added at high specific radioactivity. After 30 min, residual external radioactivity was removed by passing the proteoliposomes again through a column of Sephadex G-75. In forward exchange reactions, transport was started by adding radioactive substrate to the proteoliposomes, and in backward exchanges, by adding non-radioactive substrate. In both cases, exchange was stopped by addition of 30 mM pyridoxal 5-phosphate and 10 mM bathophenanthroline. In control samples, the inhibitors were added at the beginning together with the external substrate. Finally, the external substrate was removed [18] and the radioactivity in the liposomes was measured [18]. In forward exchange kinetic experiments, the transport rate was calculated in μmol/min per mg protein from the radioactivity taken up by the proteoliposomes within 1 min. In backward exchange experiments, the decrease in radioactivity inside the liposomes before equilibrium was fitted to a single exponential decay equation plus offset. The initial rate was derived from this curve. The backward exchange rates were expressed as apparent velocities (v'), which are proportional to the actual transport rates (v), the product of v' and the volume of liposomes containing the active ACR1. The active internal volume can be determined only with low accuracy, but it is constant for each experiment. Therefore, the relative backward exchange velocities v' are suitable for the kinetic investigations described here. A similar procedure has been used before in kinetic analysis of the aspartate/glutamate, oxoglutarate, dicarboxylate and carnitine carriers [19–22].

Table 1
The dependence on internal substrate of the transport properties of proteoliposomes containing bacterially expressed yeast ACR1

Internal substrate	Oxoglutarate transport (nmol/min/mg protein)	% of exchange rate
None (Cl^- present)	16	1.1
Succinate	1425	97.6
Fumarate	1460	100
Methylfumarate	1422	97.4
Oxoglutarate	908	62.2
Oxaloacetate	898	61.5
L-Malate	393	26.9
Maleate	80	5.5
Malonate	79	5.4
Citrate	191	13.1
cis-Aconitate	337	23.1
Threo-D_S-isocitrate	393	26.9
Phosphoenolpyruvate	152	10.4
Glutamate	39	2.7
Aspartate	58	4.0
Phosphate	61	4.2
Sulphate	53	3.6
Pyruvate	92	6.3
Carnitine	35	2.4
Ornithine	57	3.9
ADP	19	1.3
ATP	26	1.8

Proteoliposomes containing ACR1 were pre-loaded internally with various substrates (concentration 20 mM). Transport was started by the external addition of 1.5 mM [^{14}C]oxoglutarate, and stopped after 1 min. Similar results were obtained in three independent experiments.

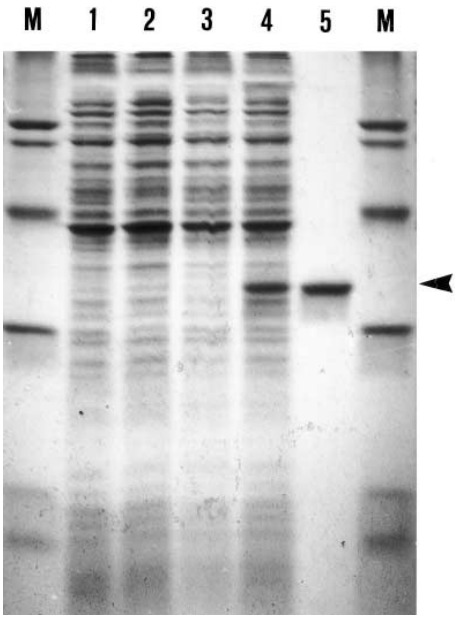

Fig. 1. Expression of yeast ACR1 in *E. coli*. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye. The position of ACR-1 is indicated by an arrow on the right. Lanes M, markers (hen egg ovotransferrin, bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c*); lanes 1–4, *E. coli* C0214(DE3) containing the expression vector, without (lanes 1 and 3), and with the coding sequence of ACR1 (lanes 2 and 4). Samples were taken at the time of induction (lanes 1 and 2) and 5 h later (lanes 3 and 4). The same number of bacteria was analysed in each sample. Lane 5, purified ACR1 (12 μg) originating from bacteria shown in lane 4.

3. Results and discussion

3.1. Bacterial expression of ACR1

ACR1 over-expressed in *E. coli* C0214(DE3) (see Fig. 1, lane 4) has an apparent molecular mass of about 35.5 kDa (its calculated molecular mass is 35,341, including the initiator methionine). The protein, which appears as a single band in SDS-PAGE after purification from the inclusion bodies (Fig. 1, lane 5), was not detected in bacteria harvested immediately before induction of expression (lane 2), nor in cells lacking the ACR1 coding sequence in the expression vector, harvested after induction (lane 3). The N-terminal sequence of the over-expressed protein was SQKKKASHPA, identical to the predicted sequence of residues 2–11 of ACR1. About 75 mg of purified protein (see Fig. 1, lane 5) was obtained per litre of bacterial culture.

3.2. Substrate specificity and inhibitor sensitivity

The reconstituted ACR1 catalysed a [^{14}C]oxoglutarate/oxoglutarate exchange, but not the homo-exchanges of phosphate, pyruvate, malonate, glutamate, aspartate, glutamine, carnitine, ornithine, ADP and ATP (external concentrations 1 mM, internal concentrations 20 mM, transport for 10 min). No oxoglutarate/oxoglutarate exchange activity was associated with sarkosyl-solubilised material from bacterial cells either lacking the expression vector for ACR1, or harvested immediately before induction of expression of ACR1.

The substrate specificity of ACR1 was investigated in greater detail by measuring the uptake of [^{14}C]oxoglutarate into proteoliposomes that had been pre-loaded with a variety of

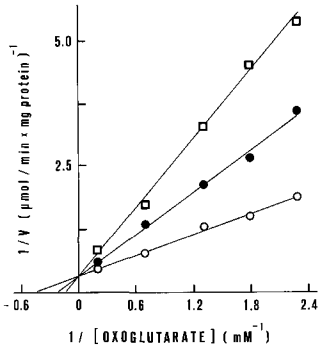

Fig. 2. Dependence of the rate of [^{14}C]oxoglutarate/fumarate exchange in proteoliposomes containing reconstituted ACR1 on the external oxoglutarate concentration, and competitive inhibition by succinate and fumarate. [^{14}C]Oxoglutarate was added at the concentrations indicated to proteoliposomes containing 20 mM fumarate. Except in the controls (○), 0.8 mM succinate (●) or 0.6 mM fumarate (□) was added simultaneously with the [^{14}C]oxoglutarate.

substrates (see Table 1). In the absence of internal substrate, uptake of labelled oxoglutarate was not observed. The highest activities were detected in the presence of internal succinate, fumarate and methylfumarate. There was also significant activity with internal oxoglutarate and oxaloacetate. To a much lesser extent, L-malate, *cis*-aconitate and isocitrate were exchanged for external [^{14}C]oxoglutarate, and to a very low extent so were citrate and phosphoenolpyruvate. Virtually no exchange was observed with internal maleate, malonate, glutamate, aspartate, phosphate, sulphate, pyruvate, carnitine, ornithine, ADP, ATP and (not shown) glutamine, alanine and leucine. These results indicate clearly that radioactive oxoglutarate is transported into proteoliposomes containing ACR1, only in exchange for an internal counter-substrate, the best of which are succinate and fumarate.

The [^{14}C]oxoglutarate/fumarate exchange reaction catalysed by reconstituted ACR1 was inhibited by sulphydryl reagents (mersalyl, *p*-chloromercuribenzene sulphonate and mercuric chloride), by pyridoxal 5-phosphate and by bathophenanthroline (see Table 2). The impermeable dicarboxylate and tricarboxylate analogues phenylsuccinate, benzylmalonate and 1,2,3-benzenetricarboxylate inhibited the reconstituted transport activity strongly. In contrast, inhibitors of the pyruvate, ADP/ATP and phosphate carriers, α-cyanocinnamate, carboxyatractyloside and *N*-ethylmaleimide, respectively [23–25], had little or no effect on the activity of ACR1.

The ability of non-radioactive potential substrates to inhibit the oxoglutarate/fumarate exchange was examined also (Table 2). In general, the exchange was prevented by external addition of each of the substrates that are transported by ACR1 (Tables 1 and 2), and it was unaffected by substrates of other mitochondrial carriers such as phosphate, glutamate and ornithine. However, poor substrates, such as isocitrate and phosphoenolpyruvate, as well as the dicarboxylates maleate and malonate, which are not transported at all by the ACR1 protein, inhibited the exchange activity significantly, suggesting that they bind to the substrate binding site of ACR1.

3.3. Kinetic characteristics of reconstituted ACR1

In order to obtain kinetic information about the [^{14}C]oxoglutarate/fumarate exchange, the dependence of the exchange rate on substrate concentration was investigated by changing the concentration of externally added [^{14}C]oxoglutarate at a constant internal concentration of 20 mM fumarate. The K_m and V_{max} values for oxoglutarate exchange at 25°C from a typical experiment (see Fig. 2) were 2.1 mM and 3.0 μmol/min per mg protein, respectively. The average values of K_m and V_{max} from 24 experiments were 2.0 ± 0.11 mM and 2.8 ± 0.7 μmol/min per mg protein, respectively. The rather high standard error of the V_{max} values probably arises from variations in the amount of active carrier molecules present in the different preparations of the purified carrier. Succinate and fumarate inhibited the [^{14}C]oxoglutarate/fumarate exchange in a competitive manner (see Fig. 2). The inhibition constants, K_i, 0.77 mM for succinate and 0.26 mM for fumarate, show that the carrier has a higher affinity for these two dicarboxylates than for oxoglutarate.

Since ACR1 catalyses a counter-exchange of internal and external molecules across the membrane, the half-saturation

Table 2
Effect of inhibitors and externally added substrates on the [^{14}C]oxoglutarate/fumarate exchange by proteoliposomes containing ACR1

Reagent	Inhibition (%)
Experiment 1	
None	–
Mersalyl	94
p-Chloromercuribenzenesulphonate	96
Mercuric chloride	75
N-Ethylmaleimide	9
Pyridoxal 5′-phosphate	94
Bathophenanthroline	97
α-Cyanocinnamate	6
Carboxyatractyloside	7
Phenylsuccinate	79
Butylmalonate	40
Benzylmalonate	80
1,2,3-Benzenetricarboxylate	82
Experiment 2	
None	–
Succinate	87
Fumarate	99
Methylfumarate	95
Oxoglutarate	90
Oxaloacetate	81
L-Malate	54
Maleate	30
Malonate	23
Citrate	33
cis-Aconitate	63
Threo-D_s-isocitrate	95
Phosphoenolpyruvate	57
Phosphate	8
Sulphate	9
Glutamate	−2
Ornithine	2
Pyruvate	10
Carnitine	−1

Proteoliposomes were loaded with 20 mM fumarate and transport was started by adding 1.5 mM [^{14}C]oxoglutarate. The incubation time was 1 min. Thiol reagents were added 2 min before the labelled substrate; the other inhibitors and external substrates were added together with [^{14}C]oxoglutarate. All inhibitors and substrates were used at a concentration of 15 mM, except for mercurials and α-cyanocinnamate (0.1 mM), *N*-ethylmaleimide (2 mM), carboxyatractyloside (20 μM), pyridoxal 5′-phosphate and bathophenanthroline (1 mM). In experiments 1 and 2, respectively, the control values of uninhibited oxoglutarate/fumarate exchange were 1336 and 1207 nmol/min per mg protein. Similar results were obtained in three independent experiments.

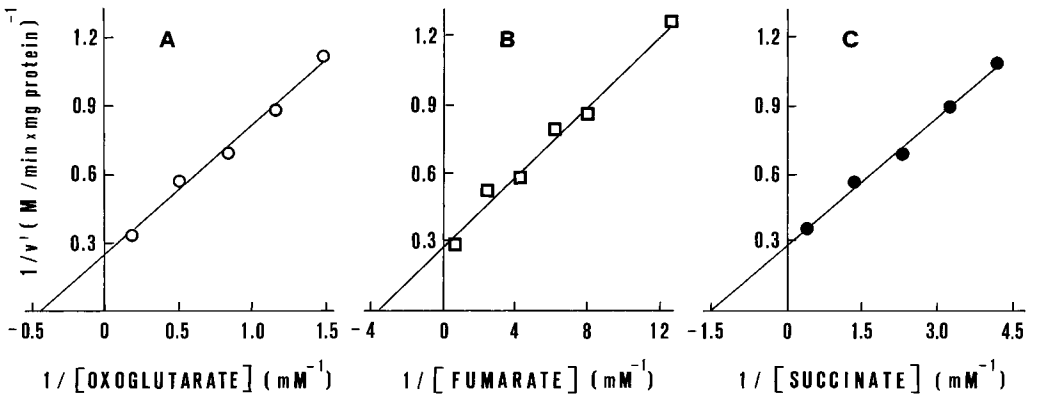

Fig. 3. K_m values of reconstituted ACR1 for external oxoglutarate, succinate and fumarate. Lineweaver-Burk plots were obtained from backward exchange measurements of initial [^{14}C]oxoglutarate efflux rates. Non-radioactive oxoglutarate (A), fumarate (B) or succinate (C) was added at the concentrations indicated to proteoliposomes containing 20 mM [^{14}C]oxoglutarate. See Section 2 for explanation of v'.

constants, K_m, for transport of non-radioactive succinate and fumarate can be determined by applying the backward exchange method [18]. Therefore, the internal substrate pool of proteoliposomes containing 20 mM oxoglutarate was labelled radioactively by carrier-mediated equilibration with external [^{14}C]oxoglutarate. During this step, the internal substrate concentration remained unchanged (oxoglutarate/oxoglutarate homo-exchange). After removal of residual external radioactivity, export of [^{14}C]oxoglutarate was initiated by addition of different concentrations of non-radioactive succinate or fumarate (in the absence of externally added substrate, no efflux of radioactive oxoglutarate was observed, even after 60 min incubation).

In order to verify that the backward exchange technique is applicable also to reconstituted ACR1, the K_m value for external oxoglutarate was measured by the backward exchange procedure in the presence of 20 mM internal [^{14}C]oxoglutarate and external oxoglutarate ranging from 0.66 to 5 mM (see Fig. 3A). The mean values obtained from this and three similar experiments (1.86 ± 0.25 mM) agreed well with those from the forward exchange determinations (average values 2.0 ± 0.11 mM from 24 experiments). It should be noted that the different intercepts on the ordinates of Figs. 2 and 3A, respectively, do not represent different V_{max} values for forward and backward exchange measurements, as the backward exchange activities are given as apparent velocities (see Section 2).

Having established the validity of the backward exchange method, the external K_m values for fumarate and succinate were determined by this procedure. A linear reciprocal plot was obtained over ranges of 0.08–2.5 mM and 0.24–2.5 mM for external fumarate and succinate, respectively, with a constant internal oxoglutarate concentration of 20 mM (Fig. 3B,C). From a series of experiments of this type, a transport affinity (K_m) of 0.36 ± 0.08 mM for fumarate and 0.69 ± 0.12 mM for succinate were calculated (see Table 3). These K_m values are similar to the inhibition constants (K_i) of succinate and fumarate, determined by measuring the reconstituted [^{14}C]oxoglutarate/fumarate exchange in the presence of different oxoglutarate concentrations and with a constant concentration of either succinate or fumarate (Fig. 2). The mean values and standard errors of transport affinities and inhibition constants for several substrates of the reconstituted ACR1 protein are summarised in Table 3. Fumarate and succinate are the best substrates for reconstituted ACR1. Both the transport affinities and inhibition constants of fumarate are 2-fold lower than those of succinate, but they are about 6-fold lower than those of oxoglutarate and oxaloacetate, and 15-fold lower than those of malate. The transport affinities of citrate, isocitrate and phosphoenolpyruvate (which are the physiological substrates of the tricarboxylate carrier from both rat liver and *S. cerevisiae* [26,14], are very high (> 6 mM) and therefore they are not important physiologically. The dicarboxylates and tricarboxylates listed in Table 3, as well as phosphoenolpyruvate, are all competitive inhibitors, since they were found to increase the apparent K_m without

Table 3
Kinetic parameters of reconstituted ACR1

Substrate	K_m (mM)	K_i (mM)
Fumarate	0.36 ± 0.08 (6)	0.32 ± 0.06 (5)
Succinate	0.69 ± 0.12 (6)	0.72 ± 0.07 (6)
Oxoglutarate (backward exchange)	1.86 ± 0.25 (4)	n.d.
Oxoglutarate (forward exchange)	2.00 ± 0.11 (24)	n.d.
Oxaloacetate	1.93 ± 0.28 (3)	2.15 ± 0.23 (4)
L-Malate	5.26 ± 0.95 (3)	5.32 ± 0.51 (3)
Citrate	n.d.	9.86 ± 0.88 (4)
Threo-D$_s$-isocitrate	6.17 ± 0.97 (3)	0.37 ± 0.04 (4)
Phosphoenolpyruvate	> 9.0 (2)	4.40 ± 0.53 (3)

The K_m values were determined by the backward exchange method using the experimental conditions described in Fig. 3B,C for fumarate and succinate. The K_i values were calculated from double reciprocal plots of the rate of [^{14}C]oxoglutarate/fumarate exchange versus substrate concentration, performed under the experimental conditions described in Fig. 2. The competing anions were added together with [^{14}C]oxoglutarate at the appropriate concentrations. The values are averages ± S.E.M. (n); n.d., not determined.

changing the V_{max} of the [^{14}C]oxoglutarate/fumarate exchange (data not shown). The inhibition constants of these compounds are virtually coincident with their transport affinities, except for isocitrate and phosphoenolpyruvate where the K_i values are significantly lower than the respective K_m values. The inhibition constant of isocitrate (0.37 mM) is particularly low, demonstrating that although it is poorly transported by ACR1, nonetheless it has a high affinity for the protein. In this context, it is worth noting that ACR1 and the yeast citrate carrier are 30% identical in sequence and ACR1 is closer to the citrate carrier than any other member of the family.

3.4. Conclusions

The experiments described in this paper strongly suggest that ACR1 is a transport protein in the inner membranes of yeast mitochondria that connects the anaplerotic production of succinate by the glyoxylate cycle in the cytosol with the tricarboxylic acid cycle and electron transfer by complex II (succinate dehydrogenase:ubiquinone reductase) in the mitochondrion. The cytoplasmic fumarate can be converted first to malate, and then to oxaloacetate, which is indispensable for gluconeogenesis and for the oxidation of acetyl-CoA that has not been consumed by the glyoxalate cycle. Therefore, a mutation in *ACR1* would prevent the anaplerotic replenishment of oxaloacetate and block the tricarboxylic acid cycle. The consequent accumulation of acetyl-CoA could explain the low levels of acetyl-CoA synthase activity found in the ACR1 defective strain [4]. The regulatory function of the succinate-fumarate transporter on acetyl-CoA synthase and the proposed mitochondrial location of ACR1 will need to be verified by further experimentation. Neither the sequence of ACR1 nor that of isocitrate lyase has any of the characteristic features that are associated with targetting to the glyoxysome [1].

The procedure employed in this work for the bacterial overproduction of ACR1 as inclusion bodies, and its functional reconstitution was first described for the bovine oxoglutarate/malate carrier [17]. With minor modifications, it has proved to be a way of also producing active phosphate [12], citrate [14], dicarboxylate [5,13] and ornithine [15] carriers, and, as in the present instance, it has allowed the hitherto unknown yeast genes for the dicarboxylate and ornithine carriers to be identified. The prospects for identifying the biochemical functions of further members of the family of mitochondrial carrier proteins by similar means are excellent, and more distantly, the procedure could provide sufficient amounts of active transport proteins for crystallisation, thereby overcoming one of the major obstacles in their structural analysis [27].

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