Identification by bacterial expression and functional reconstitution of the yeast genomic sequence encoding the mitochondrial dicarboxylate carrier protein

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Abstract The inner membranes of mitochondria contain a family of transport proteins of related sequence and structure. The DNA sequence of the genome of *Saccharomyces cerevisiae* encodes at least 35 members of this family. Three of them can be recognised as known isoforms of the ADP-ATP translocase and two others as the phosphate and citrate carriers. The transport functions of the remainder cannot be identified with certainty. One of them, encoded on yeast chromosome xii, shows a fairly close sequence relationship to the known sequence of the bovine mitochondrial oxoglutarate-malate carrier. The yeast protein has been obtained by over-expression in *Escherichia coli*, reconstituted into phospholipid vesicles and shown to have transport properties characteristic of the mitochondrial carrier for dicarboxylate ions, such as malate, and also phosphate, previously biochemically characterised, but not sequenced, from both mammalian and yeast mitochondria. This is the first example of the biochemical identification of an unknown membrane protein encoded in the yeast genome since the completion of the genomic sequence.

Key words: Dicarboxylate carrier; Transport; Mitochondria

1. Introduction

The inner membranes of mitochondria contain a family of related proteins that transport various substrates and products through the membrane. Their sequences are characterised by three tandem related sequences of about 100 amino acids, each of them probably being folded into two anti-parallel transmembrane α-helices linked by an extensive hydrophilic sequence. The three repeats are joined together by shorter hydrophilic sequences. This arrangement was identified first [1] in the published sequence of the ADP/ATP translocase [2], and subsequently in the uncoupling protein from brown fat mitochondria [3], and in the mitochondrial phosphate [4], oxoglutarate-malate [5], citrate [6] and carnitine carriers [7]. The various repeats are all related to each other throughout the family, and several characteristic sequence features are conserved [8–11]. The sequences of proteins of unknown function from various species also belong to the family, whereas the biochemical properties of other carrier proteins from mitochondrial membranes, exemplified by the dicarboxylate carrier, have been determined but their sequences are not known [8–11].

As described below, we have examined the proteins encoded in the sequence of the genome of *Saccharomyces cerevisiae* for members of the mitochondrial carrier family, and we have identified 35 members. They include three isoforms of the ADP/ATP translocase [12–14] and the phosphate and citrate carriers [15,16]. Otherwise the transport functions of the other 30 members of the family cannot be identified with certainty. One of them, named xii-C1 (the only mitochondrial carrier encoded on chromosome xii), is quite closely related in sequence to the bovine oxoglutarate-malate carrier protein. Its coding sequence has been cloned into a bacterial expression vector. The over-expressed yeast protein has been reconstituted into phospholipid vesicles and identified from its transport characteristics as the carrier for dicarboxylate ions. From earlier work, this dicarboxylate carrier is known to catalyse an electroneutral exchange of dicarboxylates, such as malate, and also inorganic phosphate [17,18] by a simultaneous antiport reaction mechanism [19,9]. It also transports sulphate and thiosulphate [20,21]. It can be inhibited by the impermeant dicarboxylate analogue butylmalonate [22], by bathophenanthroline and by sulphydryl reagents (excluding *N*-ethylmaleimide) [23,24]. The protein has been isolated previously, but not sequenced from the mitochondria of rat liver [25] and yeast [26], and has an apparent molecular mass of 28 kDa. In liver, it plays an important role in gluconeogenesis, urea synthesis and sulphur metabolism, and much less activity is present in cardiac muscle [27].

2. Materials and methods

2.1. Materials

Amersham Radiochemicals (Amersham, UK) supplied L-[1,4(2,3)-^{14}C]malic acid and [^{33}P]phosphate. Egg-yolk phospholipids (egg lecithin) were obtained from Fluka, Dorset, UK, and sarkosyl (*N*-lauroylsarcosine) from Sigma Chemical Company, Poole, UK.

2.2. Computer search for family members

The proteins encoded in the yeast genome were compared with the sequences of the mitochondrial carriers whose function is also known with the aid of the program BLAST [28].

2.3. Amplification of the coding sequence from yeast genomic DNA

Oligonucleotide primers were synthesised with the sequences 5′-TAGGGATCCCATATGTCAACCAACGCAAAAGAGTCT-3′ and 5′-CGAAAGCTTTTACTTGTCTTCCTTTGGCATGCC-3′. They consist of the sequences at the extremities of the coding sequence for xii-C1 (nucleotides 827 869–826 973 on the negative strand of chromosome xii; Genbank accession number U19028), with additional *Nde*I and *Hin*dIII sites, respectively. The sequence of interest was amplified from *S. cerevisiae* genomic DNA (10 ng; kindly donated by Dr. R. Arkowitz) by 30 cycles of PCR. The amplified DNA contained a 0.9 kb product visible by ethidium bromide staining after electrophoresis in a 1% agarose gel. The product was cloned into the expression vector pMW172, which was transformed into *E. coli* DH5α cells. Transformants selected on 2×TY plates containing am-

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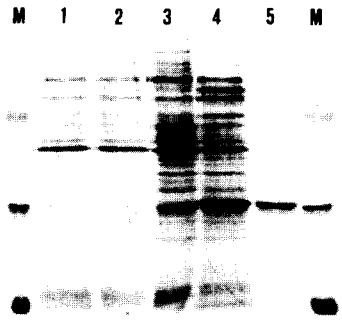

Fig. 1. Expression in *E. coli* and purification of the yeast xii-C1 protein. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye. Lanes M, markers (bovine serum albumin, carbonic anhydrase and cytochrome *c*); lanes 1–4, *E. coli* C41(DE3) containing the expression vector, without (lanes 1 and 3), and with the coding sequence for xii-C1 (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 analyzed in each sample. Lane 5, purified yeast xii-C1 protein (3.5 μg) originating from bacteria shown in lane 4.

picillin (100 μg/ml) were screened by direct colony PCR, and by restriction digestion of the purified plasmid DNA. The sequence of the insert coding for xii-C1 was verified by the modified dideoxy chain termination method [29].

2.4. Bacterial expression of the yeast xii-C1 protein

The protein was over-expressed at 37°C in *E. coli* C41(DE3) [30], and inclusion bodies were obtained as described previously for the oxoglutarate-malate carrier [31]. Control cultures containing the empty pMW172 vector were processed in parallel. The crude inclusion body pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA; 2 ml) and fractionated by centrifugation at 131 000×*g* for 4.5 h at 4°C, on a step gradient made of 10 ml of 40%, 15 ml of 53% and 4 ml of 70% (w/v) solutions of sucrose dissolved in the buffer. The xii-C1 protein collected in a grey-gold band at the interface between the 53% and 70% sucrose layers [31]. The inclusion bodies were washed at 4°C first with TE buffer, then twice with a buffer containing 10 mM PIPES, pH 7.0, Triton X-114 (3%, w/v) and 1 mM EDTA, and then once again with TE buffer. The xii-C1 protein was solubilised from the inclusion bodies in buffer containing 10 mM Tris-HCl, pH 7.0, sarkosyl (2.5%, w/v) and 0.1 mM EDTA. Insoluble material was removed by centrifugation (258 000×*g* for 1 h at 4°C).

Samples taken from cultures at various points of growth and purified inclusion bodies were examined by SDS-PAGE in 17.5% gels [31]. The proteins were either stained with Coomassie blue dye or transferred to poly(vinylidene difluoride) membranes, stained with Coomassie blue dye, and their N-terminal sequences determined with a pulsed liquid protein sequencer (Applied Biosystems 477A). The yield of purified yeast protein 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. Reconstitution of the yeast xii-C1 protein into liposomes

The solubilised protein was diluted 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 for the bovine mitochondrial oxoglutarate-malate [31]. Similar protocols have been used subsequently for the citrate and phosphate carriers [15,16].

2.6. Activity assays

External substrate was removed from proteoliposomes on a Sephadex G-75 column. Transport at 25°C was started by adding [^{14}C]malate or [^{33}P]phosphate to the proteoliposomes, and was terminated after 60 s (unless indicated otherwise) by addition of either 30 mM butylmalonate or a mixture containing 30 mM pyridoxal 5′-phosphate and 10 mM bathophenanthroline [32]. In control samples, the inhibitor was added at the beginning together with the labelled substrate. Extraliposomal labelled substrate was removed [32], and the radioactivity in the liposomes was measured [32]. Transport activity was calculated by subtracting the respective control from the experimental values. Transport of other substrates by the reconstituted yeast protein was assayed in a similar way. Effects of inhibitors and externally added substrates on the [^{14}C]malate/phosphate exchange were studied in liposomes containing xii-C1, and loaded with 20 mM phosphate. The exchange reaction was started by adding 1 mM [^{14}C]malate, and was stopped after 1 min. Thiol reagents, pyridoxal 5′-phosphate, carboxyatractyloside and α-cyanocinnamate were added 3 min before the radioactive substrate; other inhibitors and external substrates were added together with [^{14}C]malate. All inhibitors and substrates were used at a concentration of 20 mM, except for *N*-ethylmaleimide which was used at 2 mM, and organic mercurials, carboxyatractyloside and α-cyanocinnamate at 0.15 mM. The control transport values of uninhibited malate/phosphate exchange in three independent experiments ranged between 3244 and 3628 μmol/min/g protein.

3. Results and discussion

3.1. Expression of the yeast xii-C1 protein in bacteria

The yeast xii-C1 protein was highly expressed in *E. coli* C41(DE3) (see Fig. 1, lane 4). Its apparent molecular mass was about 31 kDa (the calculated molecular mass for xii-C1 is 32 991, including the initiator methionine residue), and the N-terminal sequence determined for residues 1–10 (STNAKESAGK) corresponds exactly to the anticipated sequence. The over-expressed protein accumulated in the bacterial cytosol as inclusion bodies, and the protein was readily purified by centrifugation and washing steps (see Fig. 1, lane 5). A 1 litre culture of *E. coli* C41(DE3) yielded about 25 mg of the purified yeast protein.

3.2. Substrate specificity and inhibitor sensitivity

In the search for the function of xii-C1, the substrates for known mitochondrial transporters were tested, but the yeast xii-C1 protein did not catalyse the homo-exchange of oxoglutarate, adenosine diphosphate, pyruvate, citrate, glutamate, aspartate, carnitine, ornithine and glutamine (external concen-

Table 1
Transport properties of proteoliposomes containing bacterially expressed yeast xii-C1 protein

Internal substrate	Substrate transported (μmol/10 min/g protein)	
	[^{14}C]Malate	[^{33}P]Phosphate
None (Cl^- present)	74	43
Malate	2364	937
Phosphate	2110	963
Malonate	1790	950
Succinate	1365	934
Sulphate	939	481
Thiosulphate	922	549
Oxoglutarate	325	156
Fumarate	56	24
Aspartate	108	19
Glutamate	80	27
Citrate	83	10
ADP	99	58
Pyruvate	71	19
Carnitine	65	63
Ornithine	76	48

Proteoliposomes containing xii-C1 were loaded internally with the substrate (concentration 20 mM). Transport was started by the external addition of 0.1 mM [^{14}C]malate or 0.1 mM [^{33}P]phosphate, and stopped after 10 min. Similar results were obtained in four experiments; a representative experiment is shown.

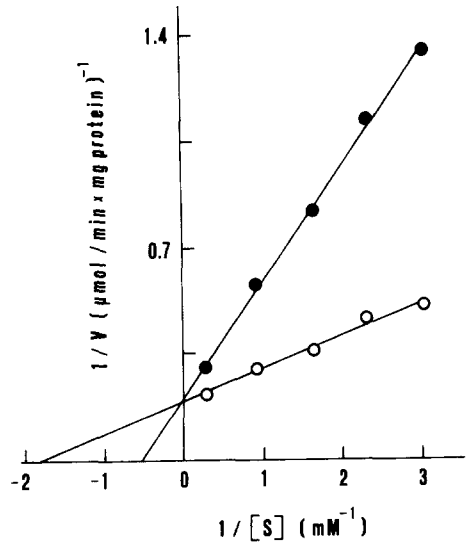

Fig. 2. Lineweaver-Burk plots of the [^{14}C]malate/malate and [^{33}P]phosphate/malate exchanges in liposomes reconstituted with the bacterially expressed yeast xii-C1 protein. Radioactive substrates were added at the concentrations indicated to proteoliposomes containing 20 mM malate; ●, phosphate: ○, malate.

tration 0.5 mM for 10 min), nor did it catalyse a phosphate/phosphate exchange inhibitable by *N*-ethylmaleimide (characteristic of the phosphate carrier). However, the reconstituted yeast protein did catalyse a [^{14}C]malate/phosphate exchange reaction that could be inhibited by butylmalonate, characteristic reactions of the dicarboxylate carrier protein established previously in rat and yeast mitochondria [17,18,20,25,26]. No such exchange activity was detected either in material from bacterial cells lacking the expression vector for xii-C1, or in cells harvested immediately before induction of expression of xii-C1.

The substrate specificity of the yeast xii-C1 protein was examined in greater detail by measuring the uptake of [^{14}C]malate and of [^{33}P]phosphate into proteoliposomes which had been pre-loaded with various substrates (see Table 1). The highest activities were observed in the presence of internal L-malate, malonate, phosphate and succinate. To a lesser extent, sulphate and thiosulphate were exchanged for both external malate and phosphate. A very low activity was also found in the presence of internal oxoglutarate (about 10–15% of the sample with internal malate). No significant exchange was observed with internal fumarate, aspartate, glutamate, citrate, ADP, pyruvate, carnitine or ornithine. The residual activity in the presence of these substrates was approximately the same as the activity observed in the presence of sodium chloride.

The effects of inhibitors on the [^{14}C]malate/phosphate exchange reaction catalysed by the reconstituted yeast xii-C1 protein were also examined (see Section 2). The exchange reaction was inhibited by the substrate analogues butylmalonate (86% inhibition), benzylmalonate (82%), phenylsuccinate (60%) and phthalate (78%), as well as by the sulphydryl reagents mersalyl (97%) and *p*-chloromercuriphenylsulphonate (95%), and by the inhibitors bathophenanthroline (99%) and pyridoxal 5′-phosphate (96%). Butylmalonate was a more effective inhibitor of the malate/phosphate exchange than phenylsuccinate, as is the dicarboxylate carrier in mitochondria [20]. The oxoglutarate carrier is more sensitive to the latter inhibitor than to the former [33]. Inhibitors of other characterised mitochondrial carriers, such as *N*-ethylmaleimide, carboxyatractyloside, 1,2,3-benzenetricarboxylate and α-cyanocinnamate, had no effect on the activity of xii-C1. In addition, the reconstituted malate/phosphate exchange was inhibited by the external addition of the well-known substrates of the dicarboxylate carrier, namely malate (93% inhibition), phosphate (85%), malonate (93%), succinate (76%), sulphate (54%) and thiosulphate (61%). It was also inhibited to a much lesser extent by oxoglutarate (25%), and it was virtually unaffected by fumarate, citrate, ADP, aspartate, glutamate, pyruvate, carnitine, glutamine and ornithine. The same results were obtained in three independent experiments.

The transport characteristics of the yeast xii-C1 protein and the effects of inhibitors on transport are the same as those determined previously for the dicarboxylate carrier in mitochondria [17,18,20–24], and after its purification from mitochondria and reconstitution into liposomes [25,26].

3.3. Kinetic characteristics of the reconstituted yeast xii-C1 protein

The dependence of the exchange rate on substrate concentration was studied by changing the concentration of external [^{14}C]malate at a constant internal concentration of 20 mM malate or 20 mM phosphate. The data from a typical experiment are shown in Fig. 2. With both external substrates, linear functions were obtained that intersected the ordinate close to a common point. Therefore, the V_{max} value is independent of the type of substrate, as observed previously with the dicarboxylate carrier in mitochondria [18] and after purification [34]. However, the different slopes indicate that the K_m for phosphate is more than three times higher than for malate (the mean values from four experiments are 1.65 ± 0.19 and 0.56 ± 0.09 mM for phosphate and malate, respectively). Thus, the yeast xii-C1 protein is also similar in these respects to the rat liver dicarboxylate carrier, where its K_m values for phosphate and malate are 1.5 mM and 0.26 mM, respectively, in mitochondria [18], and 1.41 mM and 0.49 mM, respectively, after purification [34]. From the inhibition constants (K_i values) of different substrates for the [^{14}C]malate/phosphate exchange, the order of affinity was: malate ≈ malonate > phosphate ≈ succinate > thiosulphate > sulphate ≫ oxoglutarate, in close agreement with affinities measured with the rat liver dicarboxylate carrier [18,34–36]. The V_{max} value for reconstituted xii-C1 (5.6 ± 0.7 µmol/min/mg protein at 25°C) is higher than the value reported for the rat liver dicarboxylate carrier in mitochondria (70 nmol/min/mg protein at 9°C) [18], and it is virtually the same as the value reported for the purified and reconstituted rat liver carrier [34].

3.4. Conclusions

From its transport properties and kinetic characteristics, there is no doubt that the yeast xii-C1 protein is a dicarboxylate carrier protein. As yet, there is no definitive proof that this protein is the same as the dicarboxylate carrier detected in the inner membranes of yeast mitochondria, which has not been analysed by protein sequencing. However, it is likely to be so. With the exception of the phosphate and citrate carriers in mammals [4,6], but not in yeast [15,16], the mitochondrial carrier proteins do not have N-terminal extensions that are removed during entry to the organelle, and none is associated with xii-C1. However, with the possible exceptions of the

maize brittle1 protein [37]and the PMP-47 protein from *Candida boidinii* [38], the characterised members of this family of carriers have been found exclusively in mitochondrial membranes. Therefore, this is the most likely sub-cellular localisation of xii-C1.

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