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Bacterial Overexpression of Putative Yeast Mitochondrial Transport Proteins

June A. Mayor,[1] David Kakhniashvili,[2] David A. Gremse,[2] Clayton Campbell,[2] Reinhard Krämer,[3] Andreas Schroers,[3] and Ronald S. Kaplan[1]

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Thirty-two genes have been identified within the genome of the yeast *Saccharomyces cerevisiae* which putatively encode mitochondrial transport proteins. We have attempted to overexpress a subset of these genes, namely those which encode mitochondrial transporters of unknown function, and have succeeded in overexpressing 19 of these genes. The overexpressed proteins were then isolated and tested for five well-characterized reconstituted transport activities (i.e., the transport of citrate, dicarboxylates, pyruvate, carnitine, and aspartate). Utilizing this approach, we have clearly identified the yeast mitochondrial dicarboxylate transport protein, as well as two additional lower-magnitude transport functions (i.e., tricarboxylate and dicarboxylate transport activities). The implications of these results and the considerations relevant to this approach are discussed.

KEY WORDS: Mitochondria; transporter; overexpression; yeast; membrane proteins; crystallization.

INTRODUCTION

A major challenge in the field of mitochondrial transport proteins has been the correlation of structure with function. Thus, of the approximately two dozen anion and cation transport functions that have been identified within the mitochondrial inner membrane, the primary sequence is known for less than half of the corresponding transport proteins (Kaplan *et al.*, 1995; Palmieri *et al.*, 1996; Tzagoloff *et al.*, 1996; Kakhniashvili *et al.*, 1997; Palmieri *et al.*, 1997; for a review see Kaplan, 1996a). This paucity of structural information has been due primarily to the difficulty in purifying a sufficient quantity of functional protein from native sources. Recently, two major advances have dramatically altered the landscape, namely, the complete sequencing of the genome of the yeast *Saccharomyces cerevisiae* and the development of procedures for overexpressing mitochondrial transport proteins in *E. coli* (Fiermonte *et al.*, 1993; Wohlrab and Briggs, 1994; Kaplan *et al.*, 1995).

With this background in mind, here we report results from our investigations which sought to: (i) identify those yeast genes encoding proteins which display the structural characteristics that typify all mitochondrial carriers of known sequence; and (ii) overexpress the protein products encoded by these genes and then assay these products for five reconstituted transport functions.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Bacterial Overexpression, and Purification of the Resulting Protein Products

Each of the 26 identified genes that potentially encode mitochondrial transport proteins of unknown function was amplified by PCR[4] using total *Saccha-*

[1] Department of Biological Chemistry, Finch University of Health Sciences, The Chicago Medical School, 3333 Green Bay Road, North Chicago, Illinois 60064.
[2] Department of Pharmacology, University of South Alabama College of Medicine, Mobile, Alabama 36688.
[3] Institute of Biochemistry, University of Cologne, 50674 Cologne, Germany.

[4] The abbreviations used are: BTC, 1,2,3-benzenetricarboxylate; $C_{12}E_8$, dodecyl octaoxyethylene ether; CTP, citrate transport protein; DTP, dicarboxylate transport protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NEM, *N*-ethylmaleimide; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; pCMB, *p*-chloromercuribenzoic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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romyces cerevisiae genomic DNA (Novagen; strain S288C) as the template. A forward primer was designed corresponding to the beginning of the ORF of a given gene and a reverse primer was constructed corresponding to the reverse complement of sequence that is 3′ to the termination codon at the end of a given ORF. The engineered primers typically contained *Nde*I and *Bam*HI restriction sites, respectively. However, with those ORFs that contain internal *Nde*I and/or *Bam*HI sites, alternative restriction sites were engineered. Amplifications and subsequent subcloning through NovaBlue- and BL21(DE3)-competent cells were essentially carried out as described previously (Kakhniashvili *et al.*, 1997).

Bacterial growth and the subsequent induction of overexpression with IPTG (1 mM) were conducted essentially as previously reported (Kakhniashvili *et al.*, 1997). Expressed protein was then solubilized from isolated inclusion bodies (Kaplan *et al.*, 1995) with 1.2% (w/v) sarkosyl, followed by centrifugation at 314,000 × *g* for 30 min. The resulting supernatants contained overexpressed protein.

Incorporation of Overexpressed Protein Into Liposomes and Measurement of Reconstituted Transport

Citrate Transport

Phospholipid vesicles were prepared by bath sonication of dried asolectin (233.2 mg) in 2.1 ml of Buffer A (120 mM Hepes, 50 mM NaCl, 1 mM EDTA, pH 7.4). Sarkosyl-solubilized inclusion body protein (25–200 µl) was then incorporated into the preformed vesicles via the freeze–thaw–sonication technique generally as previously detailed (Kaplan *et al.*, 1995). BTC-sensitive [1,5-^{14}C]citrate/citrate exchange was measured at 1–4 min essentially as previously reported (Kaplan *et al.*, 1990a, 1995).

Pyruvate Transport

30–100 µl of solubilized protein was added to a mix of 525 µl of preformed phospholipid vesicles, 113 µl of Buffer A, 150–220 µl of Buffer B (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.0; 1.0 mM dithioerythritol; added to maintain a constant final volume), and 75 µl of 10% (v/v) Triton X-114. Following the freeze–thaw–sonication procedure, the α-cyano-4-hydroxycinnamate-sensitive [1-^{14}C]pyruvate uptake was measured at 2 and 10 min essentially as previously described (Kaplan *et al.*, 1990b).

Dicarboxylate Transport

30–100 µl of sarkosyl-solubilized protein was added to a mix consisting of 525 µl of asolectin vesicles that had been prepared in Buffer C (120 mM Hepes, 50 mM KCl, 1 mM EDTA, pH 7.4), 0–70 µl of Buffer C (to maintain a constant final volume), 97 µl of Buffer D (10 mM potassium phosphate, 20 mM KCl, 1 mM EDTA, pH 7.2), 190 µl of 100 mM malate, and 43 µl of 10% (v/v) Triton X-114. *n*-Butylmalonate-sensitive and pCMB-sensitive [2-^{14}C]malonate/malate exchange was then measured over a time interval of 1–6 min essentially as previously described (Kaplan *et al.*, 1990b).

Carnitine Transport

75 µl of sarkosyl-solubilized inclusion body protein was incorporated into asolectin vesicles utilizing a procedure similar to that described above with the dicarboxylate carrier except Buffer D was omitted and 15–20 mM carnitine (final concentration) was included in place of malate. Following the freeze–thaw–sonication step, extraliposomal carnitine was removed via chromatography on Sephadex G-50. Eluted proteoliposomes were assayed for transport at 30°C as follows. Proteoliposomes (90 µl) were preincubated with 10 µl of either water (experimental tube) or pCMB (0.3 mM final concentration; control tube) for 45 sec. Transport was subsequently triggered via the addition of 10 µl of 13 mM L-[methyl-^{3}H]carnitine (Amersham; specific radioactivity approximately 6 × 10^4 dpm/nmol; final concentration 1.2 mM). 2–15 min later 90 µl of the reaction mix was removed and diluted with 480 µl of Buffer C plus pCMB (0.3 mM final concentration). Extraliposomal radiolabel was then separated from the intraliposomal label by centrifugation through Sephadex essentially as previously described (Kaplan *et al.*, 1990b). It should be noted that in certain experiments NEM (final concentration = 1 mM) was used in place of pCMB at all steps. Both reagents have been shown to effectively inhibit the mitochondrial carnitine transporter (Indiveri *et al.*, 1990). The inhibitor-sensitive carnitine transport rate was calculated by subtracting the control value from the experimental value.

Aspartate Transport

Overexpressed protein was reconstituted into phospholipid vesicles via hydrophobic chromatography employing Triton X-100 and $C_{12}E_8$ plus widely varying protein/lipid and detergent/lipid ratios essentially as previously described (Krämer and Heberger, 1986; Dierks and Krämer, 1988). Following reconstitution, pyridoxal-5′-phosphate-sensitive [^{14}C]aspartate/aspartate exchange was measured as previously detailed (Dierks and Krämer, 1988).

RESULTS AND DISCUSSION

The strategy on which the present investigation was based consisted of overexpression of protein products that were identified as putative mitochondrial transport proteins followed by attempted reconstitution of function in liposomal systems. This approach arose from our earlier work (Kaplan *et al.*, 1995) with the mitochondrial citrate transport protein (i.e., CTP). In that study, we used a similar strategy to prove that a yeast gene encoding a protein sequence that displayed a reasonably high identity to the rat liver mitochondrial citrate transport protein sequence (Kaplan *et al.*, 1993) did in fact encode the yeast mitochondrial CTP.

With this background in mind, we proceeded to identify yeast genes that potentially encode mitochondrial transport proteins as follows. First, BLAST and TFastA comparisons of known yeast mitochondrial transporter sequences were performed against the GenBank and the Saccharomyces Genome Databases. Yeast protein sequences resulting from these searches were then examined to determine whether: (i) they constitute basic proteins that are approximately 280–370 amino acid residues in length; (ii) they contain significant elements of the mitochondrial signature sequence Pro-X-(Asp/Glu)-X-(Val/Ile/Ala/Leu/Thr/Met)-(Lys/Arg) -X-(Lys/Arg)-(Leu/Ile/Val/Met/Phe/Tyr) which typically repeats 2–3 times within a given transporter sequence; (iii) dot matrix analysis reveals the presence of three sequence domains approximately 100 amino acids in length which are related to each other, as well as to the domains present within mitochondrial transport proteins of previously identified function; and (iv) hydropathy analysis predicts the presence of six membrane-spanning domains within each sequence. Utilizing this approach, we identified 32 genes which encode protein sequences (Table I) that substantially meet the above criteria and thus we thought were likely to represent mitochondrial transport proteins. As indicated in Table I, at the start of this work, six of these sequences had already been functionally identified [i.e., the protein products of AAC1 (Adrian *et al.*, 1986), AAC2 (Lawson and Douglas, 1988), AAC3 (Kolarov *et al.*, 1990), MIR 1 (Wohlrab and Briggs, 1994), CTP1 (Kaplan *et al.*, 1995), and FLX1 (Tzagoloff *et al.*, 1996)]. The remaining twenty-six putative carrier sequences of unknown function were then prioritized for overexpression based upon how closely they fulfilled the above criteria.

Several comments regarding the use of this strategy are in order. First, it is important to note that this approach intrinsically selects for mitochondrial transport proteins which display structural properties closely resembling those of mitochondrial anion carriers of known sequence. Thus, if any mitochondrial carriers exist which display markedly different structural properties, they are unlikely to be identified by this approach. Accordingly, the estimate that 32 yeast genes encode mitochondrial transport proteins should be considered as a minimum estimate. Second, with genes YIL006w, RIM2, YP9367.01c, and ORF N2312, only a portion of the ORF was subcloned and expressed (i.e., nucleotides 14,444–15,403, 989–2,011, 333–1, 304, and 15,845–16,882, respectively). With genes YIL006w and RIM2, this decision was based on the fact that inspection of the amino terminal sequence, as well as alignment of the deduced protein sequences with other mitochondrial carrier sequences (D. R. Nelson, personnel communication), suggested the existence of targeting pre-sequences that are likely cleaved during formation of the mature protein. Hence, these portions of the sequences were not included in the final protein products. Similarly, since gene YP9367.01c appears to encode a mitochondrial transporter sequence embedded within a considerably longer sequence (D. R. Nelson, personnel communication), we elected to express only the putative transporter sequence. Finally, if one assumes a sequencing error in gene ORF N2312 such that 1 G within a string of 9 G's (at positions 16,444–16,452) is omitted (D. R. Nelson, personal communication), the consequent frame shift yields an embedded sequence within this ORF (i.e., nucleotides 15,845–16,882) whose deduced amino acid sequence displays characteristics typifying mitochondrial transport proteins. It is this embedded sequence which we overexpressed.

As depicted in Table II, four significant findings have emerged from this work. First, of the 26 gene products that we attempted to overexpress, we succeeded

Table I. Identification of Thirty-Two Yeast Genes Which Potentially Encode Mitochondrial Transport Proteins[a]

Transport function	Gene name	Accession number	Amino acid length	Molecular mass (kDa)	Isoelectric point
ADP/ATP	AAC1	M12514	309	34.1	10.5
ADP/ATP	AAC2	J04021	318	34.4	10.6
ADP/ATP	AAC3	Z35954	307	33.3	10.6
Phosphate	MIR1	Z49577	311	32.8	10.1
Citrate	CTP1	U17503	299	32.2	10.5
FAD	FLX1	L41168	311	34.4	10.7
?	ACR1	Z25485	321	35.4	10.7
?	YOR50-12	X92441	307	34.0	10.5
?	YEL006W	U18530	335	37.0	10.6
?	ARG11	Z75038	292	31.6	9.9
?	YKL522 (PMT)	S44213	324	35.2	10.4
?	MRS3	X87371	314	34.5	8.3
?	YLR348C	U19028	298	33.0	10.5
?	PET8	U02536	284	31.0	10.5
?	YMC2	Z35973	329	36.6	10.0
?	YMC1	X67122	307	33.4	10.3
?	YM9408.03	Z48756	314	34.2	10.7
?	YM8520.15c	Z49700	368	41.0	10.0
?	SHM1 (YHM1)	U08352	300	33.2	10.8
?	ORF YGR096w	Z72881	314	35.1	10.8
?	ORF YDL119c	Z74167	307	34.2	10.8
?	ORF YGR257c	Z73042	366	40.8	10.1
?	YER053c	U18796	300	33.5	10.2
?	YOR3193c	X94335	327	34.8	10.6
?	YIL006w	Z38113	319	35.9	10.6
?	RIM2	Z21487	340	37.9	10.7
?	YP9367.01c	Z49274	324	36.4	10.4
?	YPL134C	U43703	310	34.2	10.7
?	MRS4	X56444	304	33.3	8.0
?	YP9531.04c	Z49919	326	35.9	10.3
?	YHR002w	U10555	357	40.8	10.4
?	ORF N2312	X89016	345	38.0	10.3

[a] Each gene encoding an unidentified transport function at the start of this work (denoted by ?) was subcloned and overexpression was subsequently attempted as described under Experimental Procedures. Biochemical parameters were calculated from the deduced amino acid sequence using the Genetics Computer Group PeptideSort program. Protein products corresponding to genes MRS4, YP9531.04c, and YHR002w contained an additional amino terminal Met-Ala-Ser sequence, whereas the protein product resulting from ORF N2312 contained an additional amino terminal Met-Ala, and the RIM2 product contained an additional Met. These additions arose from the restriction site engineering required to accomplish the necessary subcloning and are not included in the calculated parameters depicted in this table. Additionally, the products denoted for genes YIL006w, RIM2, YP9367.01c, and ORF N2312 correspond to only the portion of the open reading frame that was amplified (see text for further details).

with 19. Overexpression was judged successful by: (i) the appearance of a substantial quantity of protein in the sarkosyl-solubilized inclusion body fraction (i.e., greater than 5 mg of protein per Liter of starting *E. coli* culture); and (ii) confirmation via SDS-PAGE that the predominant overexpressed protein product was of approximately the expected molecular mass (data not shown). With the 19 overexpressed proteins, the level of expression ranged from 7–75 mg of protein with a median value of 44 mg. Based on SDS-PAGE, the overexpressed proteins were typically of high purity. It should be noted that these studies consisted of a single attempt at overexpression of each gene employing one pET vector [i.e., pET-21a(+)]. Since we have previously observed that expression of a given mitochondrial protein varies depending on the pET vector employed (Kaplan, 1996b), it is certainly possible that expression of the remaining 7 genes (which we were unable to overexpress) can be achieved with different pET vectors.

Table II. Overexpression and Functional Reconstitution Data Obtained with Twenty-Six Yeast Genes Encoding Putative Mitochondrial Transport Proteins of Unidentified Function[a]

Gene name	Expressed protein	Citrate transport	Pyruvate transport	Dicarboxylate transport	Carnitine transport	Aspartate transport
ACR1	29	—	—	—	—	—
YOR50-12	56	—	—	—	—	—
YEL006W	61	—	—	—	—	—
ARG11	3	—	—	ND	ND	ND
YKL522 (PMT)	53	—	—	+	—	—
MRS3	44	—	—	—	—	—
YLR348C	29	—	—	+++	ND	—
PET8	71	—	—	—	—	—
YMC2	12	—	—	—	—	—
YMC1	10	—	—	—	—	—
YM9408.03	59	+	—	—	—	—
YM8520.15c	2	—	—	ND	ND	ND
SHM1 (YHM1)	27	—	—	—	—	—
ORF YGR096w	7	—	—	—	—	—
ORF YDL119c	2	—	—	ND	ND	ND
ORF YGR257c	1	—	—	ND	ND	ND
YER053c	55	—	—	—	—	—
YOR3193c	2	—	—	ND	ND	ND
YIL006w	29	—	—	—	—	—
RIM2	48	—	—	—	—	—
YP9367.01c	39	—	—	—	—	—
YPL134C	40	—	—	—	—	—
MRS4	65	—	—	—	—	—
YP9531.04c	2	ND	ND	ND	ND	ND
YHR002w	0	ND	ND	ND	ND	ND
ORF N2312	75	—	—	—	—	—

[a] A dash denotes no significant reconstituted transport activity; + denotes a low-magnitude reconstituted transport activity; + + + denotes a high-magnitude reconstituted transport function; ND denotes not determined. Expressed protein refers to mg of protein present in the sarkosyl-solubilized inclusion body fraction normalized to 1 liter of starting *E. coli* culture. Other conditions were as described under Experimental Procedures.

A second important result to emerge from this work has been the identification of the gene encoding the mitochondrial dicarboxylate transport protein (DTP) via this overexpression/functional reconstitution approach. Since the DTP overexpression has recently been described in detail (Kakhniashvili *et al.*, 1997), only the highlights will be mentioned. Utilizing our overexpression protocol, we obtain approximately 28 mg of DTP (per liter of *E. coli* culture) at a purity of 75% in the sarkosyl-solubilized inclusion body fraction. Upon reconstitution, this protein displays a high specific activity (V_{max} = 3.0 μmol/min/mg protein; pCMB-sensitive [^{14}C]malonate/malate exchange was measured) and a substrate specificity and inhibitor sensitivity nearly identical to the native transporter. Thus, the overexpressed DTP represents ideal material for the initiation of both structural and functional studies. Also, this work provided the first primary structure for the mitochondrial DTP from any organism. Palmieri *et al.* (1996) have recently reported similar results.

A third significant finding from the present investigation was obtained with the protein product encoded by yeast gene YM9408.03. Upon incorporation into liposomes, this overexpressed protein catalyzes a low but significant citrate/citrate exchange (i.e., approximately 90 nmol/min/mg protein). Interestingly, this exchange is not inhibited by BTC, the classical inhibitor of the citrate carrier (Palmieri *et al.*, 1972), but is inhibited by the sulfhydryl reagent mersalyl. The citrate uptake reaction demonstrates a requirement for intraliposomal substrate. An examination of the external substrate specificity of this transporter indicates that nonradioactive citrate, α-ketoglutarate, and succinate effectively inhibit the [^{14}C]citrate uptake, whereas isocitrate, malate, malonate, phosphate, and phosphoenolpyruvate do not. Thus, we conclude that gene

YM9408.03 does in fact encode a functional mitochondrial transport protein with a substrate specificity that is distinct from previously characterized mitochondrial carriers. Whether citrate, α-ketoglutarate, and succinate are in fact physiological substrates for this carrier, or whether as yet untested anions constitute the *bona fide* substrates, remains an unresolved issue at this time. Finally, our findings that: (i) the protein is expressed in high abundance; (ii) SDS-PAGE analysis indicates that upon extraction from inclusion bodies it is rather pure (i.e., greater than 70%); and (iii) it displays some function upon reconstitution in liposomes, suggest that this protein might be an excellent candidate for crystallization trials.

A fourth finding worthy of comment concerns the protein product encoded by yeast gene YKL522. Upon incorporation into liposomes, the overexpressed protein catalyzes a low, but significant [^{14}C]malonate uptake (i.e., 1–2 nmol/min/mg protein; malate was employed as the intraliposomal counteranion) which is inhibited by either *n*-butylmalonate or pCMB. An important, and as yet unanswered, question is whether this protein in fact represents a second dicarboxylate carrier that is characterized by a low intrinsic activity, or whether: (i) the extraction/reconstitution conditions need to be further optimized; and/or (ii) other anions are the preferred substrates. Clearly, this represents an important area for future investigation.

Finally, several additional points concerning this approach merit discussion. First, the power of this strategy is demonstrated by four clear-cut examples, obtained from multiple laboratories, in which an overexpressed yeast mitochondrial protein product has been unambiguously identified with function, namely the citrate (Kaplan *et al.*, 1995), dicarboxylate (Kakhniashvili *et al.*, 1997; Palmieri *et al.*, 1996), phosphate (Wohlrab and Briggs, 1994), and most recently ornithine (Palmieri *et al.*, 1997) transport proteins. With both the gene and its overexpressed protein product in hand, one can immediately launch both structural and functional studies. In relation to this, we believe that a reasonable argument can be made that crystallization trials should be initiated with all of the overexpressed proteins listed in Table II (even those of unknown function) in parallel with the search for reconstituted function, the idea being that by increasing the pool of candidate proteins one is increasing the likelihood of obtaining high quality crystals with at least one of these transporters. Second, it is important to note that a negative result in a reconstitution assay does not in fact prove that a given protein does not constitute a given transporter, but rather that under the conditions utilized for overexpression, solubilization, and reconstitution, transport was not obtained. Clearly, one can envision difficulties encountered with protein renaturation/denaturation during the solubilization step and/or that the reconstitution conditions employed (i.e., the substrates placed inside and outside the liposome; the inhibitors tested; the lipid composition, etc.) might not be suitable to support a given protein's function. Thus, negative results are not conclusive and must be interpreted cautiously. Moreover, an observed low-level transport activity (e.g., the reconstitution data obtained with the protein encoded by gene YKL522) may represent either an inherent property of a given transport protein or may be due to less than optimal experimental conditions which must then be intensively probed. Finally, the data presented in Table II also demonstrate a difficulty inherent in this approach, namely, in many cases the search for a given protein's transport function will require intensive investigation involving considerable variation in the extraction and reconstitution parameters. Along these lines, we plan to continue to probe the reconstitution conditions necessary to identify the functions of the gene products listed in Table II. Included in these efforts will be an expansion of the array of transport functions tested.

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