Identification of the yeast ARG-11 gene as a mitochondrial ornithine carrier involved in arginine biosynthesis

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Abstract The ARG-11 gene in *Saccharomyces cerevisiae* encodes a protein with the characteristic features of 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 29 members of the family are unknown. The yeast ARG-11 protein has been over-produced as inclusion bodies in *Escherichia coli*. It has been solubilized in the presence of sarkosyl, re-constituted into liposomes and shown to transport ornithine in exchange for protons. Its main physiological role is probably to take ornithine synthesized from glutamate in the mitochondrial matrix to the cytosol where it is converted to arginine.

Key words: ARG-11 gene; Arginine biosynthesis; Ornithine carrier; Transport; Yeast mitochondrion

1. Introduction

The biosynthesis of arginine in *Saccharomyces cerevisiae* involves five enzymes in the matrix of mitochondria that produce ornithine from imported glutamate, and three enzymes in the cytosol that convert ornithine exported from the mitochondrion into arginine [1]. How are the mitochondrial and cytosolic segments of this pathway linked together? Yeast cells with mutations in the ARG-11 gene grow poorly in the absence of arginine [2], and in one mutant, MG409 (Arg11-1), the glutamate pool was increased and the arginine and ornithine pools were decreased [3]. Therefore, it has been proposed that the protein encoded by ARG-11 provides one link between the intra-mitochondrial and cytosolic parts of the arginine biosynthetic pathway, either by importing glutamate into the organelle, or by exporting ornithine from the mitochondrion into the cytosol [2]. From the genomic sequence of *Saccharomyces cerevisiae*, it is now known that ARG-11 is on chromosome XV, and that the gene encodes a protein with a sequence containing the characteristic features of a family of intrinsic membrane proteins that are involved in the transport of substrates and products across the inner membranes of mitochondria [4–6]. Thirty-five members of this family have been identified in the genomic sequence of *Saccharomyces cerevisiae* [7]. They include three isoforms of the ADP/ATP translocase [8–10], and the transport proteins for phosphate [11], citrate [12] and dicarboxylates [7,13], but the functions of the other members of the family are unknown.

As described below, the protein encoded by ARG-11 has been over-produced and reconstituted by a procedure originally described for the bacterial over-production and functional reconstitution of the oxoglutarate-malate carrier protein from bovine mitochondria [14], and employed subsequently for the over-production and reconstitution of the phosphate [11], citrate [12] and dicarboxylate [7,13] carriers from yeast mitochondria. It has been shown by study of its transport characteristics to be an ornithine carrierer. It transports arginine and lysine less efficiently than ornithine, but citrulline is not transported.

2. Materials and methods

2.1. Amplification of the ARG-11 gene from yeast genomic DNA

Oligonucleotide primers were synthesised with the sequences at the extremities of the coding sequence of ARG-11 (nucleotides 570803–569925 on the negative strand of chromosome XV; Genebank accession numbers X90518, X94335), with additional *Nde*I and *Eco*RI sites, respectively. The sequence of interest was amplified from *S. cerevisiae* genomic DNA, cloned into the expression vector pMW172 and transformed into *E. coli* DH5α cells as described before [7]. Transformants selected on 2XTY plates containing ampicillin (100 μg/ml) were screened by direct colony PCR and by restriction digestion of purified plasmid DNA. The sequence of ARG-11 was verified by the modified dideoxy chain termination method [15].

2.2. Bacterial expression and functional reconstitution of the ARG-11 protein

The over-production of the protein as inclusion bodies in the bacterial cytosol was accomplished as described before for the bovine oxoglutarate-malate [14] and yeast dicarboxylate [7] carriers, except that the host cells were *E. coli* C0214(DE3), a mutant of *E. coli* C41(DE3) selected for its ability to over-express the phosphate carrier protein without toxic effects (G. Fiermonte and J.E.W., unpublished work) as described for the selection of the host strains *E. coli* C41(DE3) and C43(DE3) [16]. Inclusion bodies were purified, and the protein solubilised and reconstituted into liposomes in the presence of substrates, as described previously [7,14].

2.3. Protein chemical characterization of the over-expressed protein

Purified inclusion bodies were analyzed by SDS-PAGE in 17.5% gels [14]. The protein was either stained with Coomassie blue dye or transferred to poly(vinylidene difluoride) membranes, stained with Coomassie blue dye, and its N-terminal sequence determined. The yield of purified yeast protein per litre of bacterial culture was estimated as before [7].

2.4. Activity assays

External substrate was removed from proteoliposomes on a Sephadex G-75 column in the presence of a reversible inhibitor of efflux, 5 μM *p*-chloro-mercuri-benzene sulphonate. Transport was started by adding 5 mM dithioerythritol (DTE) and [^{3}H]ornithine or [^{14}C]lysine (from NEM Life Science Products, Milan, Italy) and was terminated

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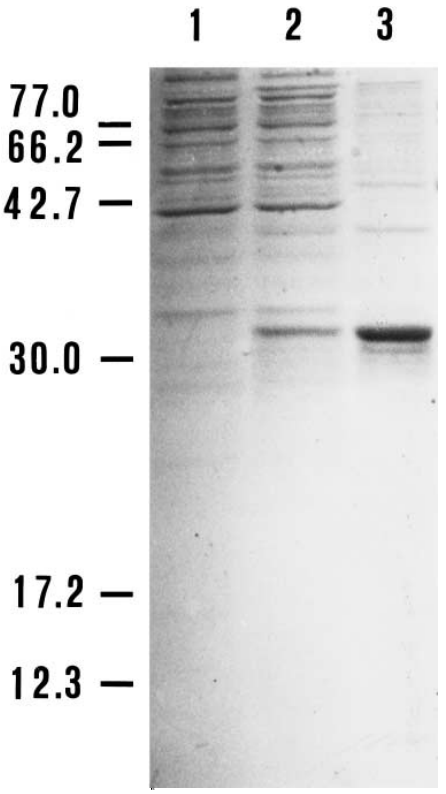

Fig. 1. Expression of the yeast ARG-11 gene product in *E. coli*. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye. The positions of molecular weight markers are shown on the left in kDa. Lanes 1 and 2, *E. coli* C0214(DE3) containing the expression vector without (lane 1) and with (lane 2) the coding sequence. Samples were taken 5 h after induction. The same number of bacteria was analyzed in each sample. Lane 3, purified ARG-11 protein (4 μg) from bacteria in lane 2.

after 40 s (unless indicated otherwise) by addition of 20 mM pyridoxal 5′-phosphate and 10 mM bathophenanthroline [17]. In control samples, the inhibitor and the radiolabelled substrate were added at the beginning. For efflux measurements, the proteoliposomes containing 20 mM ornithine were prelabelled by carrier-mediated exchange equilibration [17] and efflux started by adding 5 mM DTE and stopped by the mixture of pyridoxal 5′-phosphate and bathophenanthroline. External substrate was removed and the internal radioactivity was measured [17]. In uptake experiments, the experimental values were corrected by subtracting the control value. In efflux experiments, the rate in $-\Delta$dpm/min was obtained from the decrease of internal radioactivity from a single-exponential decay equation. Transport of other substrates by the reconstituted yeast ARG-11 protein was assayed similarly.

Table 1
Transport of ornithine and lysine by the ARG-11 protein: dependence on internal substrate

Internal substrate	Substrate transported (μmol/5 min per g protein)	
	[^{3}H]Ornithine	[^{14}C]Lysine
None (sucrose present)	59	43
L-Ornithine	864	720
L-Lysine	630	605
L-Arginine	760	670
D-Ornithine	553	418
D-Lysine	475	302
α-Methyl-L-ornithine	346	266
L-Histidine	52	36
L-Citrulline	50	29
L-Glutamate	26	22
L-Threonine	52	57
L-Proline	61	43
L-Glutamine	35	50
2-Oxoglutarate	69	47
Phosphate	43	36
ADP	55	68

Proteoliposomes containing the ARG-11 protein were pre-loaded internally with various substrates (concentration 30 mM). Transport was started by addition of 5 mM DTE and 0.05 mM [^{3}H]ornithine or 0.5 mM [^{14}C]lysine, and stopped after 5 min. Similar results were obtained in 3 different experiments.

3. Results and discussion

3.1. Bacterial expression of the ARG-11 protein

The ARG-11 protein over-expressed in *E. coli* C0214(DE3) (see Fig. 1, lane 2) had an apparent molecular mass of about 32 kDa (the calculated molecular mass including the initiator methionine is 31 580). It was not detected in bacteria harvested immediately before induction of expression (not shown) nor in cells harvested after induction but lacking the ARG-11 coding sequence in the expression vector (lane 1). The N-terminal sequence of the protein, MEDSKKKGLIEG, was identical to the predicted sequence. The protein accumulated in the bacteria as inclusion bodies. About 50 mg of purified protein (see Fig. 1, lane 3) were obtained from a 1 litre culture.

3.2. Substrate specificity and inhibitor sensitivity

The ARG-11 protein catalysed a [^{3}H]ornithine/ornithine ex-

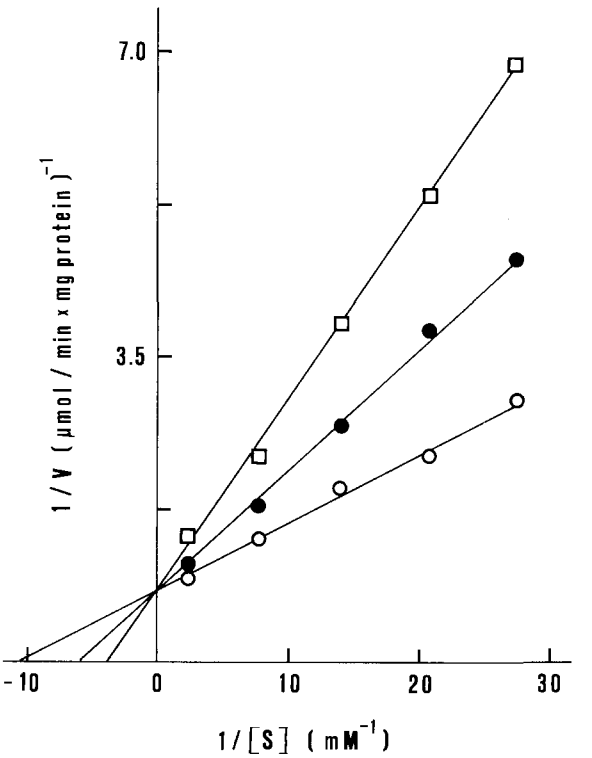

Fig. 2. Dependence of the rate of ornithine/ornithine exchange in reconstituted liposomes on the external ornithine concentration and competitive inhibition by arginine and lysine. [^{3}H]Ornithine and 5 mM DTE were added to proteoliposomes containing 20 mM ornithine. Except in the controls (○), 1 mM lysine (●) or 1 mM arginine (□) was added simultaneously with the [^{3}H]ornithine.

change, which was inhibited by *p*-hydroxy-mercuri-benzoate, a known inhibitor of the ornithine carrier purified from rat liver mitochondria [18], but not the homo-exchanges of phosphate, pyruvate, malate, oxoglutarate, citrate, glutamate, aspartate, adenosine diphosphate, carnitine and glutamine (external concentrations 0.5 mM, transport 10 min reaction). No such activity was found by reconstitution of sarkosyl-solubilised inclusion bodies from bacterial cells either lacking the expression vector for ARG-11, or harvested immediately before induction of over-expression.

The substrate specificity of the ARG-11 protein was investigated by measuring the uptake of [^{3}H]ornithine and of [^{14}C]lysine into proteoliposomes which had been pre-loaded with a variety of substrates (see Table 1). The highest activities were observed in the presence of internal ornithine, arginine and lysine. There was also significant activity with D-ornithine and D-lysine, and α-methyl-L-ornithine, but not with internal histidine or citrulline. The residual values with internal glutamate, threonine, proline, glutamine, oxoglutarate, phosphate, ADP, and (not shown) alanine, leucine, aspartate and malate were approximately the same as the value with internal sucrose. Therefore, the substrate specificity of the ARG-11 protein is confined to a narrow range of basic amino acids, and is similar to that of the rat ornithine carrier [18], except that citrulline is not transported by the yeast protein.

Ornithine transport by the ARG-11 protein was inhibited by sulphydryl reagents (mersalyl, *p*-hydroxy-mercuri-benzoate and *p*-chloro-mercuri-benzene sulphonate at 5 μM, *N*-ethylmaleimide and eosin 5-maleimide at higher concentrations), and various cations, that inhibit the rat ornithine carrier [18,19], also inhibited the yeast protein (see Table 2). As with the rat ornithine carrier, divalent cations were more effective inhibitors than monovalent cations. The most effective cation inhibitor was Pr^{3+}, but its inhibition is probably unspecific and due to its binding strongly to lipid head groups. Carboxyatractyloside, 1,2,3-benzenetricarboxylate, butylmalonate and phthalonate (inhibitors of the ADP/ATP, citrate, dicarboxylate and oxoglutarate transporters [20–23], respectively) did not inhibit the ARG-11 protein.

In addition, the ability of unlabelled potential substrates to inhibit the reconstituted [^{3}H]ornithine/ornithine exchange was examined. Ornithine, lysine, arginine and, to a lesser extent, the D-stereoisomers of the first two amino acids inhibited the uptake of radiolabelled ornithine (Table 2), whereas citrulline, histidine and (not shown) threonine, glutamate, glutamine, phosphate, malate, oxoglutarate and ADP did not. Therefore, the substrate specificity and inhibition characteristics of the ARG-11 protein are very similar to those of the rat ornithine carrier, and they differ only in their behaviour with citrulline [18,19].

3.3. Kinetic characteristics of the ARG-11 protein

The K_m and V_{max} values for ornithine exchange at 25°C, from a typical experiment (see Fig. 2), were 0.094 mM and 1.2 mmol/min per g protein, respectively, and the average values from six experiments were 0.11 ± 0.01 mM and 1.1 ± 0.3 mmol/min per g protein, respectively. The K_m for ornithine uptake is close to that of the rat ornithine carrier [19], but the V_{max} is lower than that of the rat protein (3.2 ± 0.7 mmol/min

Table 2
Effect of inhibitors and externally added substrates on the uptake of [^{3}H]ornithine into proteoliposomes containing the ARG-11 protein

Reagents	Ornithine transport (μmol/min/g protein)	Inhibition (%)
Experiment 1		
None	986	–
N-Ethylmaleimide	20	98
Eosin 5′-maleimide	47	95
Mersalyl	0	101
p-HMB	128	87
p-CMBS	0	100
Bathophenanthroline+pyridoxal 5′-phosphate	0	102
Na^+	700	29
K^+	631	36
Ca^{2+}	384	61
Mg^{2+}	296	70
Pr^{3+}	168	83
Carboxyatractyloside	907	8
1,2,3-Benzenetricarboxylate	887	10
Butylmalonate	917	7
Phthalonate	927	6
Experiment 2		
None	1060	–
L-Ornithine	32	97
L-Lysine	509	52
L-Arginine	233	78
D-Ornithine	572	46
D-Lysine	773	27
L-Histidine	1018	4
L-Citrulline	1028	3

Proteoliposomes were loaded with 30 mM ornithine and transport was started by adding 0.4 mM [^{3}H]ornithine and 5 mM DTE. 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 [^{3}H]ornithine. All inhibitors and substrates were used at a concentration of 8 mM, except for *N*-ethylmaleimide and eosin 5′-maleimide (1 mM), organic mercurials (5 μM), cations (added as Cl^- salts at 15 mM), carboxyatractyloside (0.15 mM), bathophenanthroline (10 mM) and pyridoxal 5′-phosphate (30 mM). Similar results were obtained in three independent experiments.

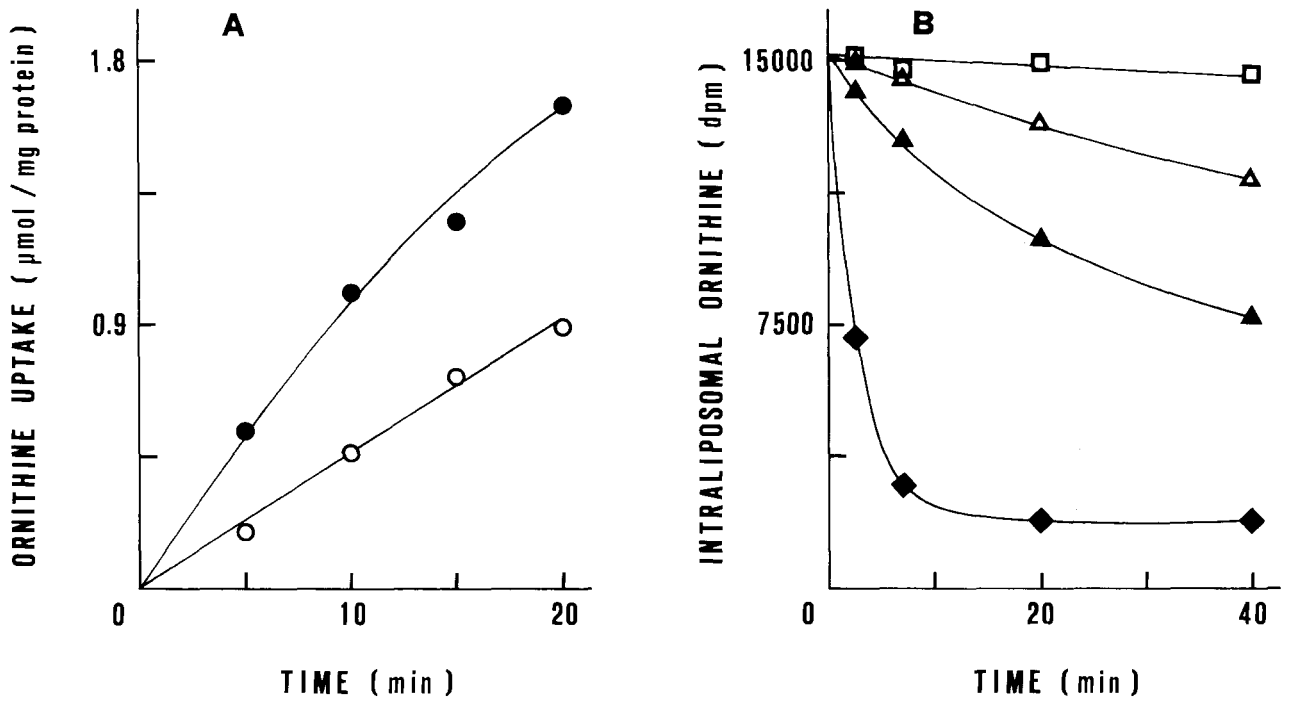

Fig. 3. Dependence of ornithine uniport on the trans-membrane pH gradient. The reconstitution mixture contained 15 mM Pipes and 15 mM Hepes at pH 6.0 (●) or pH 8.0 (○, □, △, ▲, ◆). After reconstitution of the carrier into liposomes, 30 mM unbuffered sucrose was used to equilibrate and to elute the Sephadex G-75 columns. In (A), transport was started by adding mixtures of 5 mM DTE, 0.4 mM [^{3}H]ornithine and 15 mM Hepes at pH 8.0. In (B), transport was started by adding (△) 15 mM Pipes/15 mM Hepes pH 8.0 and 5 mM DTE, (▲) 15 mM Pipes/15 mM Hepes pH 6.0 and 5 mM DTE, (□) 15 mM Pipes/15 mM Hepes pH 6.0, 5 mM DTE, 30 mM pyridoxal 5′-phosphate and 10 mM bathophenanthroline, and (◆) 15 mM Pipes/15 mM Hepes pH 8.0, 5 mM DTE and 10 mM ornithine.

per g protein) [19]. The other two substrates, arginine and lysine, inhibited ornithine uptake competitively. The K_i values of arginine and lysine for [^{3}H]ornithine uptake were 0.6 ± 0.1 mM and 1.3 ± 0.2 mM, respectively (average of four experiments). The K_i value for lysine is close to that of the rat protein [19].

3.4. Dependence of ornithine uniport on the transmembrane pH gradient

The influence of intraliposomal pH (at fixed external pH) on the uptake of [^{3}H]ornithine as uniport (in the absence of internal ornithine) was analyzed. The amount of substrate taken up by uniport increased markedly with decreasing internal pH from 8.0 to 6.0 (see Fig. 3A), whereas the rate of ornithine/ornithine exchange (out 0.4 mM; in 20 mM) was unaffected (not shown). Therefore, the ARG-11 protein catalyses both ornithine/ornithine and ornithine/H^+ exchanges.

The ornithine/H^+ exchange activity was confirmed by measuring the efflux of [^{3}H]ornithine from proteoliposomes. The addition of pH 6.0 buffer to proteoliposomes with an internal pH of 8.0 induced greater efflux of ornithine than an external pH of 8.0, and the efflux was prevented by inhibitors of ornithine/ornithine exchange (see Fig. 3B). Over the first 40 min, the data fitted a single-exponential decay equation. The transport rates were 103 and 375 dpm/min per g protein for the unidirectional efflux at external pH 8.0 and 6.0, respectively, and 4970 dpm/min per g protein for the ornithine/ornithine exchange. Therefore, the exchange reaction is much faster than the uniport, and in this respect, the ARG-11 protein is similar to the mitochondrial phosphate and carnitine carriers [24,25].

3.5. Conclusions

The main physiological function of the yeast ornithine carrier is in the export of ornithine at the expense of the proton motive force generated across the mitochondrial membrane by electron transport. The inner membranes of rat liver mitochondria were suggested to have two transport systems for ornithine, one for ornithine (and lysine) flux via an ornithine/H^+ antiport [26–28], and the second for ornithine import in exchange for citrulline [29]. The ornithine/citrulline carrier (purified but not sequenced from rat liver mitochondria [18]) transports lysine, arginine, ornithine and citrulline by an electroneutral antiport mechanism [18,19,30]. In addition to the ornithine/citrulline exchange, the purified protein catalyses less efficiently an ornithine/H^+ exchange [30]. Therefore, the mammalian carrier seems to have evolved to accept citrulline as substrate, and fulfills the important function of exchanging cytosolic ornithine and intramitochondrial citrulline, an essential step in the urea cycle.

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