THE EFFECTS OF PHYSIOLOGICAL AND GENETIC MANIPULATION ON THE ANION TRANSPORT SYSTEMS OF YEAST MITOCHONDRIA

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1. Introduction

Studies on the transport of anions by mammalian mitochondria have identified seven specific carrier-systems [1, 2]. However, attempts to isolate mitochondrial transporter proteins have so far been unsuccessful [3]. An approach to this problem is initiated herein using the yeast, *Saccharomyces cerevisiae*. The advantages of this organism in the study of mitochondrial membrane functions are well recognized (for review see [4]). In particular, the mitochondrial composition of *S. cerevisiae* is subject to physiological control, and nuclear or cytoplasmic mutants affecting mitochondrial membrane functions are readily selected. Analogous genetic and physiological manipulations have already been successfully used to study transporter proteins in bacterial systems [3, 5].

The aims of the present work are to characterize the anion transport systems of yeast mitochondria, and to determine whether these systems are susceptible to genetic or physiological manipulation. This communication reports that the transport systems for L-malate, succinate, citrate and α-oxoglutarate are present in fully functional yeast mitochondria, and are not markedly altered in organelles that are respiratory deficient due to anaerobiosis or the loss of mitochondrial DNA by mutation, the latter result indicating that these transporters are coded by nuclear genes. The uptake of ATP by fully functional mitochondria and by anaerobic mitochondrial structures is similar, but the uptake of ATP by the mitochondria of a cytoplasmic petite mutant shown to lack mitochondrial DNA [6] is radically altered, indicating that some expression of mitochondrial genes is necessary for the function of the adenine nucleotide transporter.

2. Materials and methods

2.1. *Strains*

Two haploid strains of *S. cerevisiae* were used; the respiratory competent strain L410, and the respiratory deficient petite strain E5, which contains no mitochondrial DNA and was obtained from strain L410 by ethidium bromide treatment [5].

2.2. *Growth conditions*

Cells were grown at 28° in a 1% yeast extract – *Saccharomyces* salts medium [7], supplemented as follows: Type 1 cells, strain L410 grown aerobically on ethanol (1% w/v); Type 2, strain L410 grown anaerobically on galactose (4%) supplemented with ergosterol (20 mg/l) and Tween 80 (0.5% w/v); Type 3, strain E5 grown on glucose (4%). Mitochondria prepared from Type 1, 2 and 3 cells are referred to as Type 1, 2 and 3 mitochondria.

2.3. *Assay of anion transport*

Mitochondria were prepared as described previously [8]. Mitochondria (0.5–1.0 mg protein) were incubated at 30° in 3 ml of medium containing hydroxyethylpiperazine–2 N′ethane sulphonate (10 mM), bovine serum albumin (2 mg/ml), sorbitol (0.5 M), EGTA (1 mM); final pH, 6.5. K-PO_4 (2 mM, pH 6.5) and antimycin A (1 μg/ml) were present in the incubations during the measurement of the uptake of tricarboxylate cycle anions, and oligomycin (30 μg/ml) was present in incubations for the measurement of ATP uptake. Inhibitors were added 2 min prior to the addition of ^{14}C-substrates. Anion uptake was initiated by addition of ^{14}C-substrates (0.1 μCi) and stopped by filtering the medium through a millipore filter (0.45 μm pore

size) under reduced pressure in less than 2 sec. The mitochondria were then washed on the filter with 6 ml of ice-cold incubation medium. The filters were dried, and radio-activity counted in 5 ml of toluene scintillator fluid. The shortest incubation possible by this method is approx. 5 sec; experiments of 10 sec, 20 sec and 30 sec duration were also performed, and maximal uptake was observed after 20 sec for all substrates. Initial volocities could not be determined, as even at the shortest incubation time, uptake had proceeded more than 50% towards equilibrium.

The rate V_{80} is defined as 80% of the extent of anion uptake divided by the time taken to attain this extent, and is obtained from progress curve of anion uptake. Since the concentration of anion in the medium did not decrease in any experiment by more than 2%, V_{80} values may be used to compare the properties of different types of mitochondria. Similarly, empirical K_m values for the three types of mitochondria were calculated from double reciprocal plots of V_{80} values at five different concentrations of anion (0.5–15 mM for tricarboxylate cycle anions, 1.7–100 μM for the atractylate-sensitive portion of ATP uptake, and 0.05–5 mM for atractylate-insensitive ATP uptake.

Table 1
Uptake of tricarboxylate cycle anions by type 1 mitochondria.

Substrate	Other additions	Apparent K_m (mM)	V_{80}
L-malate	None	6.3	2.0
	n-Butyl malonate (6 mM)		1.0
	Phosphate omitted		1.8
Succinate	None	6.8	75
	n-Butyl malonate (6 mM)		57
	Methyl succinate (7 mM)		31
	Phosphate omitted		70
Citrate	None	5.5	40
	L-malate (10 μM)		67
	D-malate (10 μM)		57
	L-malate (10 μM) plus n-Butyl malonate (6 mM)		34
	Phosphate omitted		36
α-oxoglutarate	None	3.3	1.5
	D,L-Threo hydroxyaspartate (4 mM)		0.8
	Phosphate omitted		1.3

Apparent K_m values were determined as described in Methods. V_{80} rates of uptake (nmoles/mg protein/sec) are defined in Methods, and in order to clearly demonstrate the action of inhibitors were determined at concentrations of substrates equal to the apparent K_m values. Thus V_{max} values are twice the V_{80} values observed in the absence of inhibitors.

3. Results and discussion

3.1. *Uptake of tricarboxylate cycle anions by fully functional mitochondria*

The most important criteria that establish the identity of a transporter system are i) saturation kinetics of the concentration dependence, ii) substrate specificity, iii) the existence of specific inhibitors of transport [2], iv) in the specific case of the mitochondrial transporters for di- and tricarboxylate anions a fourth criterion has also been established, namely a requirement for the concurrent presence of other anions. Chappell [1] has shown that the dicarboxylate transporter requires phosphate for optimal activity, whilst that for tricarboxylate anions requires both phosphate and a suitable dicarboxylate anion. Table 1 shows that the uptakes of L-malate, succinate, citrate and α-oxoglutarate by fully functional yeast mitochondria (Type 1) obey the above criteria, and are similar to those observed with mammalian mitochondria. In all cases uptake shows saturation kinetics, and there is a small but reproducible stimulation by phosphate. The uptake of L-malate is inhibited by n-butyl malonate, uptake of succinate is inhibited by n-butyl malonate or methyl succinate, and the uptake of α-oxoglutarate is inhibited by D, L-threo hydroxyaspartate. The uptake of citrate is stimulated by phosphate and D- or L-malate, and the stimulation by malate is prevented by n-butyl malonate.

3.2. *Uptake of tricarboxylate cycle anions by mitochondria from anaerobically grown cells and petite cells*

The kinetic parameters of uptake and the effects of inhibitors on the transport of tricarboxylate cycle anions by Type 2 and 3 mitochondria are summarized in table 2. Although both types of organelle lack cyto-

Table 2
The uptake of tricarboxylate cycle anions by mitochondria from anaerobically grown cells and petite cells of *S. cerevisiae*.

Substrate	Additions	Type 2 mitochondria K_m (mM)	Type 2 mitochondria V_{80}	Type 3 mitochondria K_m (mM)	Type 3 mitochondria V_{80}
L-Malate	None	12	2.5	5.5	0.83
	n-Butyl malonate (6 mM)		1.7		0.51
Succinate	None	11	100	3.3	77
	Methyl succinate (7 mM)		75		60
Citrate	None	5	50	4.3	45
	L-Malate (10 μM)		58		48
α-Oxoglutarate	None	16	2.0	1.0	1.1
	D, L-Threo hydroxy-aspartate (4 mM)		1.5		0.7

Apparent K_m values and V_{80} rates were determined as described in table 1.

chromes and a functional respiratory chain, and are greatly changed in membrane morphology, the transporter systems for L-malate, succinate, citrate and α-oxoglutarate are all present. The presence of the transporters in petite mitochondria that lack mitochondrial DNA indicates that these systems are coded by nuclear genes.

However, minor changes in the properties of certain transporters are evidenced by the apparent K_m values for anion uptake. In particular, the K_m for α-oxoglutarate uptake increases 5-fold in anaerobic mitochondria, but decreases 3-fold in petite mitochondria relative to fully functional mitochondria. The K_m values for the uptakes of L-malate and succinate increase 2-fold in anaerobic mitochondria, but are unaltered in petite mitochondria. In contrast, K_m values for uptake of citrate are similar in all three types of organelle. These relatively small changes in K_m values probably reflect the extensive changes in membrane environment that have occurred in anaerobic and petite mitochondria rather than indicate changes in the composition of transporters themselves.

3.3. *Uptake of ATP*

Weidemann et al. [9] have shown that the uptake of ADP and ATP by rat liver mitochondria can be divided into three processes (i) high affinity binding to the adenine nucleotide transporter, that is displaced by atractylate added before or after the adenine nucleotide (ii) exchange with the internal pool of mitochondrial adenine nucleotides, that is facilitated by the transporter and is prevented by atractylate only if the inhibitor is added before the adenine nucleotide (iii) low affinity binding, that is unaffected by atractylate and is thought to represent non-specific binding of ADP and ATP to mitochondrial membranes [2]. In order to simplify the analysis of our results, yeast mitochondria were preincubated with arsenate as described by Weidemann et al. [9] to eliminate most of the uptake due to process (ii), and under these conditions uptake largely consists of binding to mitochondrial membranes. The results of these experiments are summarized in table 3. The uptake of ATP by arsenate-depleted Type 1 yeast mitochondria occurs by at least two processes that are readily distinguishable on the basis of K_m and sensitivity to atractylate. The uptake of ATP at low concentrations (< 100 μM) is extensively inhibited by atractylate, and double reciprocal plots of the atractylate-sensitive portion of uptake gives an ap-

Table 3
The uptake of [^{14}C]ATP by *S. cerevisiae* mitochondria.

Mitochondria	V_{80} Atractylate absent	V_{80} Atractylate present	Apparent K_m (μM) Atractylate sensitive	Apparent K_m (μM) Atractylate insensitive
Type 1	2.1	1.0	2	>2000
Type 2	1.4	0.6	9	>2000
Type 3	1.3	1.3	Not present	>2000

Mitochondria were depleted of endogenous adenine nucleotides by incubation with arsenate as described by Weidemann et al. [9]. Apparent K_m values and V_{80} rates were measured as described in Methods. V_{80} rates of uptake of ATP were determined at an ATP concentration of 2 μM (pmoles/mg protein/sec); atractylate was used where indicated at a concentration of 0.1 mM. The uptake of ATP at concentrations of 2 μM and 2000 μM by Type 1 mitochondria was inhibited 53% and 25%, respectively, by atractylate (0.1 mM).

parent K_m of 2 μM. The uptake of ATP at high concentrations (< 500 μM) is largely insensitive to atractylate, and double reciprocal plots of the atractylate-insensitive portion of ATP uptake over the concentration range 0.1–5 mM give an approximate K_m of 2000 μM. The uptake of ATP by anaerobic mitochondrial structures shows similar characteristics to that of fully functional mitochondria, although the K_m for atractylate-sensitive uptake is higher. The increase in K_m is similar to that obtained for the tricarboxylate cycle anion transporters, and possibly results from the modification of the inner mitochondrial membrane by anaerobiosis. In contrast, the uptake of ATP by petite mitochondria is totally insensitive to atractyloside, suggesting that petite mitochondria at low concentrations (2 μM) is somewhat higher than the atractylate-insensitive, and presumably non-specific, uptake by fully functional mitochondria. Further experiments are necessary to determine whether this residual activity is due purely to non-specific binding or also represents uptake by a modified transporter system.

There are two possible explanations for the loss of actractylate-sensitivity in ATP uptake by petite mitochondria. Firstly, mitochondrial DNA may code for the complete transport system or for the component of the transporter that binds atractylate. Such proteins would be absent from petite cells that lack mitochondrial DNA. Alternatively, mitochondrial DNA does not genetically determine any of the components of the adenine nucleotide transporter. However, since the mitochondrial genome codes for other inner membrane proteins that are consequently absent from petite mitochondria (see [10] for review), an extensive reorganization of the inner mitochondrial membrane occurs in the petite and may result in a complete alteration of the properties of the adenine nucleotide transporter, including the loss of atractylate sensitivity. Experiments are currently in progress to determine ATP binding constants for the different types of mitochondria, and should definitively reveal the extent to which the adenine nucleotide transporter has been lost or altered in petite mitochondria.

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