BONGKREKIC ACID RESISTANT MUTANTS OF *SACCHAROMYCES CEREVISIAE*

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1. Introduction

ATP uptake by fully functional *Saccharomyces cerevisiae* mitochondria have been investigated by us [1] and the properties of the ATP transporter closely resemble those reported for mammalian mitochondria [2].

Bongkrekic acid and atractylate have been shown to be highly specific inhibitors of adenine nucleotide exchange in mitochondria [1–5] and through interaction with the transporter, indirectly inhibit mitochondrial respiration. Accordingly the inhibition of the mitochondrial ATP transporter in vivo by either antibiotic would prevent the growth of respiratory competent *S. cerevisiae* cells on a non-fermentable substrate whereas growth on a fermentable substrate would be largely unaffected. This paper describes the isolation of three bongkrekic acid resistant nuclear mutants and the properties of their adenine nucleotide transporter. The characteristics of two of the mutants suggest that they are probably whole cell permeability mutants. However the properties of ATP uptake by mitochondria from a third mutant, denoted L440-BOA-r, are significantly different from those of the normal mitochondrial ATP transporter system. These differences are sufficient to account for the in vivo resistance of the organism to bongkrekic acid.

We have previously shown that the formation of a normal adenine nucleotide transporter requires the products of the mitochondrial protein synthesizing system probably coded by mitochondrial DNA [1]. The results of the present study on a bongkrekic acid resistant nuclear mutant L440-BOA-r suggest that the synthesis of a normal adenine nucleotide transporter requires the cooperative action of the mitochondrial and cytosolic protein synthesizing systems. Similar results have previously been reported for the synthesis of two other mitochondrial membrane-associated enzyme complexes, cytochrome oxidase [6] and oligomycin sensitive ATPase [7].

2. Materials and methods

2.1. *Strains and growth conditions*

The respiratory competent haploid strain L410 (α ura, his) was used as the parental strain and all mutants were isolated from this strain. Strain L2200 (a ade_1 lys_2 trp_1) was employed in the genetic crosses. For biochemical analyses the cells were grown aerobically at 28°C in a 1% Difco yeast extract–salts medium [8] supplemented with 1% ethanol (w/v) in fluted conical flasks to ensure adequate aeration. The solid medium used in the isolation of mutants and subsequent genetic analysis consisted of 1% Difco yeast extract, 2% peptone, 2% ethanol, and 2% agar, denoted YEPE. Drug plates containing bongkrekic acid (BOA) were prepared by adding BOA in aqueous solution to melted YEPE agar which had been previously cooled to 60°C.

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Table 1
ATP uptake in the presence of atractylate and bongkrekic acid by mitochondria from strain L410 BOA-s and mutant strain L440 BOA-r.

[ATP] and inhibitors	Initial rate of ATP uptake*		Extent of ATP uptake at equilibrium*	
	Parent	Mutant	Parent	Mutant
0.5 μM ATP				
+ None	2.0	1.0	23.5	8.9
+ 10 μM BOA	0.75	0.92	47.0	11.5
+ 50 μM BOA	0.7	0.5	47.7	17.5
+ 100 μM ATR	0.6	0.95	6.1	8.3
+ 500 μM ATR	0.6	0.62	6.0	5.5
50 μM ATP				
+ None			210	190
+ 100 μM ATR			154	193
+ 500 μM ATR			151	191

* Rate of ATP uptake (pmoles ATP/mg protein/sec) and extent of ATP uptake at equilibrium (pmoles ATP/mg protein) by mitochondria from parent strain L410 and mutant strain L440 were determined as described in Materials and methods.

At low concentrations of ATP (0.5 μM) concentrations of ATR (100 μM) and BOA (10 μM) maximally inhibit the uptake of ATP by the transporter in normal mitochondria while only slightly affecting uptake of ATP by the mutant mitochondria (less than 10% inhibition, table 1). Higher concentrations of the two inhibitors are necessary to inhibit maximally ATP uptake in the mutant; at low concentrations of ATP (0.5 μM) 500 μM ATR inhibits the rate of total ATP uptake by 40% while 50 μM BOA inhibits the rate by 50%. The extent of ATP uptake at equilibrium by normal mitochondria is 23.5 pmoles ATP/mg protein compared to 8.9 pmoles bound by mutant mitochondria. Concentrations of ATR (100 μM) and BOA (10 μM) which decrease and increase the extent of ATP uptake at equilibrium by 70% and 100% respectively by normal mitochondria, have a minimal effect on the extent of ATP uptake by mitochondria from the mutant strain. High concentrations of ATR (500 μM) and BOA (50 μM) respectively decrease and increase the extent of uptake by about 40% and 90%.

At high concentrations of ATP (50 μM) the contribution of specific ATR-sensitive uptake to the total uptake by mitochondria from the parent strain is diminished; the extent of uptake at equilibrium is inhibited 30% by 100 μM ATR. In contrast the uptake of ATP by mutant mitochondria is insensitive to 500 μM ATR (table 1). Bongkrekic acid has little influence on ATP uptake by both parent and mutant mitochondria at high concentrations of ATP since the binding sites are almost fully saturated [5].

The binding constants of specific ATR-sensitive ATP uptake by normal mitochondria of strain L410 have been investigated in detail in this laboratory and are reported elsewhere [1, 5]. Binding occurs at high and low affinity sites with dissociation constants of 1 μM (K'd) and 20 μM (K''d) respectively. The double reciprocal plots of rate of BOA- and ATR-sensitive ATP uptake by parent and mutant mitochondria are shown in fig. 1. The K_m values for the specific ATP uptake by parent and mutant mitochondria of 0.4 – 0.5 μM and 3–4 μM respectively indicate an 8-fold decrease in affinity of the specific ATP-binding site in the mutant mitochondria.

The Scatchard plot of the equilibrium extent of ATR-sensitive ATP uptake by mitochondria isolated from the mutant L440 indicates the existence of only a single binding site for the specific uptake of ATP with a dissociation constant of 5.5 μM (fig. 2). Also shown in fig. 2 is the increase in equilibrium extents of binding in the presence of BOA by the two mitochondrial types. BOA (10 μM) increases the extent of binding of ATP by the mitochondria of the parental strain and the dissociation constants of the high and low affinity binding sites now become 0.1 μM and 3.0 μM respectively, which represents about a 10-fold increase in affinity (from 1.0 μM to 0.1 μM and 20 μM to 3 μM). Significantly, the affinity of the single

2.2. *Isolation of mutants and testing for bongkrekic acid resistance*

Spontaneous BOA resistant mutants were isolated by plating out 10^8 cells on YEPE plates containing varying concentrations of the drug and incubating for 5 days at 30°C. The parent sensitive strain L410 did not grow on plates containing 0.009 μg BOA/ml. Three colonies, isolated from a plate containing 0.08 μg BOA/ml, were purified and their ability to grow on plates containing different concentrations of BOA was tested. The growth of all resistant colonies on plates unaffected by concentrations of BOA up to 0.08 μg/ml but was inhibited by 0.32 μg BOA/ml. The resistant mutants have been designated L451 BOA-r, L452 BOA-r and L440 BOA-r while the parental strain would be L410 BOA-s.

2.3. *Assay of ATP transport*

Mitochondria were prepared as described previously [9] and depleted of endogenous nucleotides by preincubation in a medium containing arsenate to minimise the contribution of ATP exchange [2]. Mitochondria (1.0–1.2 mg protein) were incubated at 30°C in 3 ml of medium containing 2-*N*-2 hydroxyethylpiperazin-*N'* ethane sulphonate (10 mM) pH 6.5, as buffer, bovine serum albumin (2 mg/ml), sorbitol (0.5 M), EGTA 1 mM, oligomycin (1 μg/ml). ATP uptake was initiated by addition of [^{14}C] ATP (0.05 μCi) and stopped by filtering the mitochondrial suspension through a Millipore filter (0.45 μm pore size) and washing with 6 ml cold incubation buffer. The filters were dried and counted in 5 ml of toluene scintillator fluid. Uptake was measured for the time intervals 5, 10, 30, 60 and 120 sec by which time equilibrium had been reached. Atractylate (ATR) and bongkrekic acid were added 2 min prior to the addition of the [^{14}C] ATP to examine their effect on the rate of ATP uptake. To investigate the effect of BOA on the equilibrium extent of binding, mitocondria were equilibrated with [^{14}C] ATP for 1 min prior to the addition of BOA and uptake was allowed to proceed for a further minute. K_m and V_{max} values for specific ATR-sensitive and BOA-sensitive ATP uptake were calculated from double reciprocal plots of the rates of uptake at seven concentrations of ATP in the range 0.5 μM–50 μM. Dissociation constants of ATP binding sites of the transporter were determined as described by Weidemann et al. [2] by extrapolations of Scatchard plots of the extent of ATR-sensitive ATP uptake. The increase in the extent of binding of ATP at equilibrium in the presence of BOA was also analysed in Scatchard plots and is referred to as BOA-sensitive binding.

3. Results

3.1. *Growth characteristics of bongkrekic acid resistant mutants*

The growth rates of resistant mutants L451 BOA-r and L452 BOA-r on ethanol are comparable to that of the wild-type parental strain. In addition the properties of ATP uptake by mitochondria isolated from these two strains, including kinetic and binding constants, sensitivity of the inhibitors ATR and BOA closely resemble those for wild-type mitochondria [1]. Hence it is tentatively concluded that their in vivo resistance to BOA is not mitochondrial in origin and that they are probably whole cell permeability mutants. The mutant L440 BOA-r is of more interest; although the growth of the parent strain L410 and this mutant strain on glucose is identical, the generation time for growth of the mutant on ethanol is 7.0 hr, more than twice that of the parent. These preliminary observations suggested that this mutation was expressed as an alteration in mitochondrial metabolism.

3.2. *Parameters of ATP uptake by mitochondria from strains L410 and L440 BOA-r*

The activity and sensitivity to ATR and BOA of ATP uptake by mitochondria isolated from L410 and L440 are summarized in table 1. The mutant L440 BOA-r exhibits properties significantly different from those of the uptake of ATP by strain L410 mitochondria. At low concentrations of ATP (0.5 μM) the rate of total uptake of ATP by normal mitochondria is 2 pmoles ATP/mg protein/sec, twice that by the mutant mitochondria. As we have established previously for yeast mitochondria [1] the total uptake of ATP is composed of a non-specific ATR-insensitive portion in addition to high affinity ATR-sensitive uptake by the transporter. In considering the effect of inhibitors on the transporter, it is necessary to appreciate that the higher the ATP concentration, the larger is the contribution that passive ATP uptake will make to total ATP uptake.

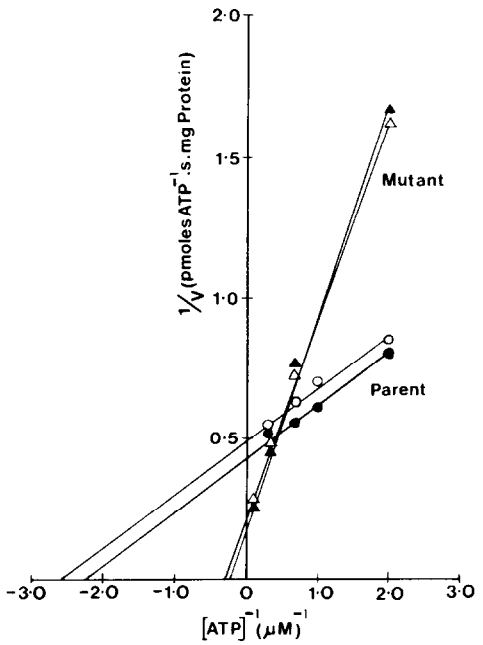

Fig. 1. Double reciprocal plot of the concentration dependence of the role of specific ATP uptake by mitochondria from strains L410 BOA-s and L440 BOA-r. The rate of uptake of ATP by mitochondria from strain L410 was determined in the presence of 10 μM BOA (○–○–○) and 100 μM ATR (●–●–●), as described in Materials and methods. The uptake of ATP by mitochondria from the mutant strain L440 was determined in the presence of 50 μM BOA (△–△–△) and 500 μM ATR (▲–▲–▲).

From the double-reciprocal plots the kinetic constants (K_m) for ATR-sensitive rate of uptake were calculated to be 0.4 μM for normal mitochondria and 3μM for the mutant mitochondria. The K_m values for ATR-sensitive uptake were 0.5 μM and 4.0 μM for the normal and mutant mitochondria respectively.

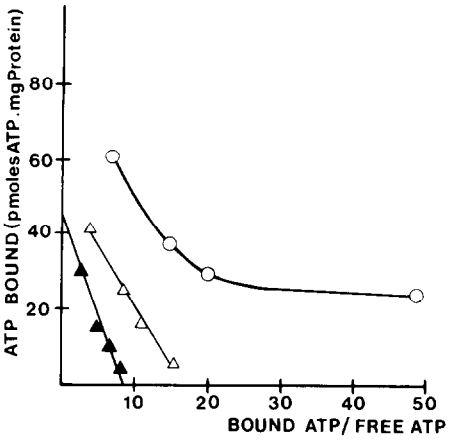

Fig. 2. Scatchard plot of ATP uptake by mitochondria from parent strain L410 and mutant strain L440. The equilibrium extents of uptake [^{14}] ATP were determined as described in Materials and methods. Concentrations of ATR (100 μM) and BOA (10 μM) used in experiments on normal mitochondria did not fully inhibit uptake of ATP by mutant mitochondria. Equilibrium binding by the mitochondria of the mutant was determined in the presence and absence of ATR (500 μM) and BOA (50 μM). The increased uptake of ATP by normal mitochondria in the presence of 10 μM BOA (○–○–○) occurs at high (K'd = 0.1 μM) and low (K''d = 3.0 μM) affinity binding sites. In mutant mitochondria atractylate sensitive binding (▲–▲–▲) occurs at a single site which has a K'd of 5.5 μM. The K'd of the single binding site in the presence of 50 μM BOA (△–△–△) is 3 μM. The dissociation constants were calculated as described by Weidemann et al. [2] from the gradient of the linear regions of the curves.

ATP binding site present in the mitochondria from the mutant strain is unaffected by 10 μM BOA and indeed only slightly affected by 50 μM BOA, the dissociation constant of the binding site changing from 5.5 μM to 3 μM.

3.3. *Genetic analysis*

In initial experiments to determine the nature of the mutation conferring BOA resistance in strain L440 BOA-r, the organism was crossed with strain L2200 BOA-s (growth inhibited by 0.009 μg BOA/ml of medium). The resistant diploid clones were completely resistant to 0.04 μg BOA/ml and showed some limited growth on 0.08 μg BOA/ml. On sporulation of the diploids the ability of the spores to grow on 0.08 μg BOA/ml segregated 2:2 for growth and non-growth, demonstrating that in strain L440 bongkrekic acid resistance is controlled by at least one nuclear gene.

4. Discussion

Table 2 summarises the results. In mitochondria from the normal strain L410 BOA-s bongkrekic acid increases the affinity of the specific high and low affinity ATP binding sites such that ATP becomes tightly bound to the mitochondrial membranes. In mitochondria from the derived bongkrekic acid-resistant mutant L440, the properties of the adenine nucleotide transporter are considerably altered; there is apparently only a single binding site and it interacts with atractylate and bongkrekic acid in a different manner from

Table 2
Parameters of ATP uptake by mitochondria from the strains L410 BOA-s and L440 BOA-r.

	ATR-sensitive ATP uptake		BOA-sensitive ATP uptake	
	Parent mitochondria	Mutant mitochondria	Parent mitochondria	Mutant mitochondria
K_m (μM)	0.5	4.0	0.4	3.0
K'd (μM)	1.0	–	0.1	–
K''d (μM)	20	5.5	3.0	3.0

The dissociation constants of ATR sensitive ATP uptake by strain L410 mitochondria were obtained from earlier work [1], other binding parameters and kinetic constants of ATP uptake by mitochondria from strains L410 BOA-s and L440 BOA-r were obtained from the data in figs. 1 and 2.

strain L410. The affinity of the binding site in mutant mitochondria for bongkrekic acid is reduced; concentrations of bongkrekic acid required to inhibit maximally the specific uptake of low concentrations of ATP (0.5 μM) are five times greater than concentrations which effect maximally the ATP uptake by mitochondria from the parent strain L410. Although ATP binding by the single site is sensitive to bongkrekic acid, the increase in the affinity of the site for ATP in the presence of bongkrekic acid is small; the K'd of the site being 3.0 μM and 5.5 μM in the presence and absence of 50 μM bongkrekic acid respectively.

At high concentrations of ATP (50 μM) the difference in rate and extent of uptake by mitochondria from parent strain L410 and mutant strain L440 is less marked since the contribution of non-specific atractylate insensitive to ATP uptake to the total uptake is predominant [1]. However some differences between mitochondria of strain L410 and L440 are still apparent. Thus ATP uptake by normal mitochondria at 50 μM ATP is inhibited 30% by 100 μM atractylate whereas uptake of ATP by mitochondria of the mutant at 50 μM is almost insensitive to 500 μM ATR indicating that in mutant mitochondria the affinity of the single binding site for atractylate as well as BOA is considerably reduced.

The inhibition of the growth on ethanol of the sensitive parental strain L410 by bongkrekic acid is a consequence of the fixation of adenine nucleotides to the inner mitochondrial membrane resulting in inhibition of adenine nucleotide exchange between the mitochondria and cytosol [4]. Thus respiratory energy would not be available to the cytosol for cell growth. The decrease in the affinity of ATP uptake by mitochondria of strain L440 as evidenced by a tenfold increase in the K_m value in vitro is probably sufficient to account for the slow growth of the organism on ethanol.

In vitro the ATP transporter of mutant strain L440-BOA-r is only slightly sensitive to 10 μM bongkrekic acid whereas 50 μM bongkrekic acid inhibits the rate of uptake by 50%. In addition the extent of ATP fixation to the binding sites is lower in mitochondria from strain L440 than in strain L410 mitochondria at equivalent concentrations of bongkrekic acid. These changes would both account for the fact that in vivo the exchange of adenine nucleotides is not totally inhibited by low concentrations of bongkrekic acid and also explain the resistance of the mutant to concentrations of bongkrekic acid which inhibit growth of strain L410 on ethanol.

We have described a nuclear mutation which affects specifically the mitochondrial adenine nucleotide transporter. Previously we have reported that the transporter is modified in cells lacking mitochondrial DNA the system becoming insensitive to ATR [1]. It appears therefore that the components of the adenine nucleotide transporter are synthesised by both the mitochondrial and cytosolic protein synthesising systems and probably it is coded for by both mitochondrial and nuclear genes.

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