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The Effects of Altered Sterol Composition on the Mitochondrial Adenine Nucleotide Transporter of *Saccharomyces cerevisiae*

By J. M. HASLAM, ANNE M. ASTIN* and WRIGHT W. NICHOLS
Department of Biochemistry, University of Liverpool, P.O. Box 147, *Liverpool L69 3BX, U.K.*

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1. The membrane sterol composition of mitochondria of the *ole-3* mutant of *Saccharomyces cerevisiae* was manipulated by growing the organism in the presence of Tween 80 (1%, w/v) plus defined supplements of δ-aminolaevulinate. 2. Changes in mitochondrial sterol content induced considerable changes in the adenine nucleotide transporter. 3. As the sterol content was decreased, the affinity of the transporter for ATP did not alter significantly, but the rate of ATP uptake was greatly decreased, the total number of atractylate-sensitive binding sites diminished, and the proportion of high-affinity binding sites was decreased. 4. Since sterol depletion also uncouples oxidative phosphorylation [Astin & Haslam (1977) *Biochem. J.*, **166**, 287–298] and prevents the intramitochondrial generation of ATP, the decrease in the rate of ATP uptake by sterol-depleted mitochondria will cause a decrease in intramitochondrial ATP concentrations *in vivo*. This probably explains the inhibition of mitochondrial macromolecular synthesis that has previously been reported in lipid-depleted yeast mitochondria.

The membrane sterol composition of the *ole-3* mutant of *Saccharomyces cerevisiae* can be manipulated by growing the organism in the presence of Tween 80 (1%, w/v) plus defined supplements of δ-aminolaevulinate (Astin *et al.*, 1977; Astin & Haslam, 1977). In the present work this system is used to investigate the effects of altered sterol composition on the function of the adenine-nucleotide transporter of isolated mitochondria. It is shown that sterol depletion inhibits mitochondrial ATP transport *in vitro*, and profoundly affects other properties of the adenine nucleotide transporter.

Experimental

Growth of cells and isolation of mitochondria

The growth of the wild-type strain S288C, and of the mutant strain *ole-3*, the isolation of mitochondria, and the determination of mitochondrial lipid composition and protein were as described previously (Proudlock *et al.*, 1971; Haslam *et al.*, 1973; Astin *et al.*, 1977; Astin & Haslam, 1977).

Assay of ATP uptake by isolated mitochondria

Kinetic and binding parameters of ATP transport were measured at 4°C by using the assay system of Weidemann *et al.* (1970) as modified by Haslam *et al.* (1973). This method uses mitochondria that have first been depleted of endogenous nucleotides by incubation with arsenate to minimize the uptake of ATP by exchange. By using high concentrations of atractylate, which inhibits the adenine nucleotide binding to the carrier (Bruni *et al.*, 1964; Groot *et al.*, 1971; Klingenberg *et al.*, 1975), the portion of ATP binding specifically to the carrier sites can be discriminated from binding to other sites. Nevertheless, atractylate-sensitive uptake of ATP still comprises two components; first binding of ATP at carrier sites in the membrane, and second exchange of ATP into the intramitochondrial pool of adenine nucleotides. The latter is decreased, but not eliminated, by preincubation with arsenate (Weidemann *et al.*, 1970). The two types of uptake were distinguished by performing experiments as follows: (A) uptake in the absence of atractylate, (B) atractylate (100 μM) was added before the [^{14}C]ATP to prevent both binding and exchange, (C) atractylate (100 μM) was added after the [^{14}C]ATP had equilibrated with the mitochondria to discharge [^{14}C]ATP from membrane carrier sites, but this has no effect on [^{14}C]ATP uptake into the intramitochondrial pool. (A) gives total ATP uptake; (A − C) gives binding to carrier sites; (C − B) gives exchange into the intramitochondrial pool. K_D values for binding and exchange of ATP were determined from Scatchard plots as described by Weidemann *et al.* (1970), and empirical kinetic parameters of ATP uptake were determined as described by Haslam *et al.* (1973). An empirical reaction rate v_{80} was defined, which is 80% of the extent of ATP uptake divided by the time taken to attain this extent. v_{80} values were obtained from the progress curves of ATP uptake. Similarly, empirical

* Present address: Department of Biochemistry, Monash University, Clayton, Vic. 3168, Australia.

Michaelis constants (K_m) and $V_{80,max.}$ values were determined from double-reciprocal plots of v_{80} values at different concentrations of ATP. The mitochondria were energized owing to the presence of the oxidizable substrate ethanol (52mM) plus oligomycin (50μg/mg of protein). The oligomycin also prevented hydrolysis of the ATP.

Criteria of intactness of the mitochondria

(*a*) *Cytochrome spectra.* Reduced minus oxidized cytochrome spectra were determined in an SP.1800 double-beam spectrophotometer (Pye-Unicam, Cambridge, U.K.). One cuvette contained in 1.0ml, mitochondria (10–12mg of protein) that had been reduced by addition of a few grains of sodium dithionite, and the other cuvette contained mitochondria at an identical concentration that had been oxidized by the addition of 10μl of a saturated solution of potassium ferricyanide.

(*b*) *Sucrose-impermeable space.* The sucrose-impermeable space of mitochondria was determined as described by Perkins *et al.* (1973), except that radioactivity was counted in the Intertechnique SL40 counter.

Materials

Materials were obtained from the same sources as previously (Haslam *et al.*, 1973; Perkins *et al.*, 1973; Astin *et al.*, 1977).

Results

Kinetic and binding parameters of atractylate-sensitive ATP uptake were measured for mitochondria from the wild-type strain S288C, and were compared with those of mitochondria from the mutant strain *ole-3* which had been grown in media containing various supplements of δ-aminolaevulinate to manipulate membrane sterol composition, as described by Astin *et al.* (1977). Fig. 1 shows that the rate and extent of atractylate-sensitive ATP uptake are diminished in sterol-depleted mitochondria from strain *ole-3* compared with wild-type mitochondria. Increasing the concentration of δ-aminolaevulinate in the growth medium to 100mg/litre restores both sterol content and ATP uptake to normal values.

The effects of sterol depletion on Scatchard plots of ATP uptake at equilibrium are shown in Fig. 2. The K_D values for atractylate-sensitive binding of ATP are not significantly affected by sterol depletion, but the number of specific binding sites and the percentage of high-affinity sites are greatly decreased in the sterol-depleted organelles.

Kinetic parameters and binding constants for atractylate-sensitive uptake of ATP by mitochondria with different contents of sterols are summarized in Table 1. In mitochondria from *ole-3* cells with high sterol contents (10μg/mg of protein) the K_m and $V_{80,max.}$ values for the rate of uptake, the K_D values for atractylate-sensitive binding and the number of specific ATP-binding sites were similar to those of mitochondria from the wild-type strain S288C. Sterol-depleted mitochondria from strain *ole-3*, containing 1.7μg of sterol/mg of protein, have high- and low-affinity binding sites whose K_D values do not differ, but the number of binding sites decreases 3-fold and the percentage of high-affinity sites decreases from 25% to 10% in the sterol-depleted organelles.

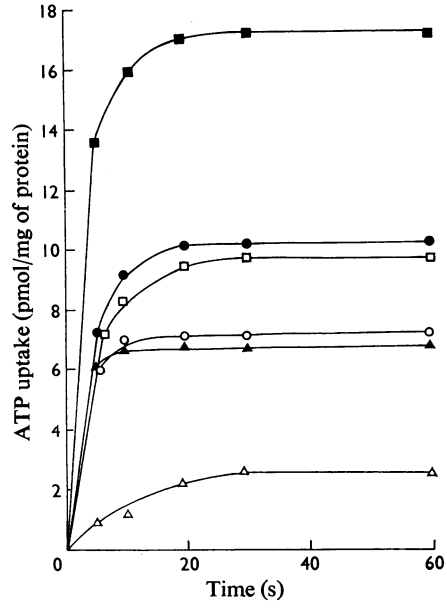

Fig. 1. *Time course of ATP uptake by wild-type and by sterol-depleted mitochondria*

The rate of uptake of ATP (1μM) at 4°C by arsenate-depleted mitochondria from wild-type and sterol-depleted mutant mitochondria was measured as described in the Experimental section. The lipid composition of the wild-type mitochondria was: 70% of the total fatty acids were unsaturated; sterols were 11.7μg/mg of protein, of which 90% was ergosterol. The lipid composition of the mutant mitochondria was: 70% of the total fatty acids were unsaturated; sterols were 1.7μg/mg of protein, of which 35% was ergosterol. Solid symbols indicate ATP uptake by wild-type mitochondria, and hollow symbols indicate ATP uptake by sterol-depleted mutant mitochondria. ■, □, Total ATP uptake in the absence of atractylate. ▲, △, ATP uptake in the presence of atractylate (0.1mM) (atractylate-insensitive uptake). ●, ○, Atractylate-sensitive ATP uptake (obtained by subtraction).

$V_{80,max.}$ values for the rate of ATP uptake were also decreased to 1.3 pmol/s per mg of protein in the sterol-depleted organelles, compared with 6 pmol/s per mg of protein in wild-type and *ole-3* mitochondia with high sterol contents. The atractylate-insensitive portion of uptake remained non-saturable when sterol contents were low, and the sensitivity to atractylate was diminished. This indicates that the sterol content of membranes is important for the normal operation of the adenine nucleotide transporter.

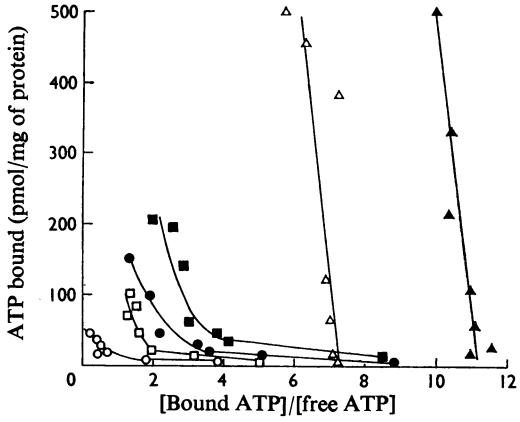

Fig. 2. *Scatchard plots of ATP uptake by mutant mitochondria containing high and low contents of sterols*
The extents of uptake of ATP (0.5–50 μM) at equilibrium in the presence and absence of atractylate (0.1 mM) were determined at 4°C as described in the Experimental section. Solid symbols indicate ATP uptake by mitochondria of the following lipid composition; 72% of the total fatty acids were unsaturated; sterols were 10.4 μg/mg of protein. Hollow symbols indicate ATP uptake by mitochondria of the following lipid composition: 62% of the total fatty acids were unsaturated; sterols were 1.2 μg/mg of protein. ▲, △, Atractylate-insensitive uptake; ■, □, uptake of ATP by exchange; ●, ○, atractylate-sensitive binding to membrane sites. The biphasic curves for atractylate-sensitive binding to membrane sites were resolved into high- and low-affinity sites by using the extrapolations of Weidemann *et al.* (1970).

Criteria of intactness of the mitochondria

In view of the changes observed in ATP uptake by mitochondria with low sterol contents, it was important to establish that the alteration in the properties of the ATP transporter was not simply due to damaged mitochondria. Reduced-minus-oxidized cytochrome spectra were determined, and the sucrose-impermeable space of the organelles was measured as a criterion of mitochondrial intactness. Breakage of the outer membrane will cause loss of cytochrome *c*, whereas disruption of the inner mitochondrial membrane will lower the specific impermeability to sucrose and diminish the sucrose-impermeable space. The reduced-minus-oxidized cytochrome spectra were similar for all types of mitochondria irrespective of sterol composition, and characteristic absorption bands of all the respiratory cytochromes were clearly identifiable. Table 2 gives the sucrose-impermeable spaces of the mitochondrial preparations, and confirms that the inner membranes of the sterol-depleted mitochondria were intact with respect to their permeability functions.

Thus the changes in the properties of the adenine nucleotide carrier in the mutant mitochondria with lower sterol contents was not simply due to damage to the membranes, and could be directly attributed to changes in membrane sterol composition.

Table 1. *Kinetic parameters and binding constants for atractylate-sensitive uptake of ATP by mitochondria with different sterol contents*
The kinetic values and binding constants were determined from double-reciprocal plots of the v_{80} rates of uptake obtained from curves such as Fig. 1 and Scatchard plots of the extents of uptake at equilibrium such as Fig. 2. The temperature was 4°C .The units of K_D are μM, and the values in parentheses indicate the percentage of ATP bound at that site. Units of $V_{max.}$ are pmol/s per mg of protein, and for total sites, pmol/mg of protein. The results are the average of two experiments. K'_D refers to the high-affinity binding site and K''_D to the low-affinity binding site for ATP.

Mitochondrial origin	δ-Aminolaevulinate supplement (mg/l)	Kinetic parameters		Binding Constants			Unsaturated fatty acid (%)	Total sterol (μg/mg of protein)
		K_m (μM)	$V_{max.}$	K'_D (μM)	K''_D (μM)	Total sites		
S288C	None	2.9	6.5	1 (22)	20 (78)	152	70	11.8
ole-3	50	3.5	5.9	2 (27)	27 (73)	150	74	10.4
ole-3	20	Not determined		2 (10)	27 (90)	95	70	3.3
ole-3	10	3.4	1.3	2 (10)	29 (90)	55	66	1.7

Table 2. *Sucrose-impermeable spaces of arsenate-depleted S288C and ole-3 mitochondria*
Mitochondria were preincubated with arsenate, then incubated in the assay medium for ATP uptake together with ^3H_2O and [^{14}C]sucrose to determine the sucrose-impermeable space as described by Perkins *et al.* (1973). Values for spaces are given as μl/mg of protein.

Mitochondrial origin	δ-Aminolaevulinate supplement (mg/l)	Water space	Sucrose space	Sucrose-impermeable space
ole-3	50	8.4	6.4	2.0
ole-3	20	8.5	6.6	1.9
ole-3	10	7.9	6.3	1.6
S288C	None	8.6	6.7	1.9

Discussion

The results reported here for ATP uptake by yeast mitochondria of normal lipid composition are similar to those obtained for mitochondria of other strains of *S. cerevisiae* (Kolarov *et al.*, 1972; Haslam *et al.*, 1973; Lauquin *et al.*, 1976). In sterol-depleted mitochondria of the mutant *ole-3* the apparent K_m of the adenine nucleotide transporter for ATP did not change significantly, but the rate of uptake of ATP was decreased to 22% of normal as the sterol content was lowered from 10.4 to 1.7 μg/mg of protein. A similar effect on the properties of the adenine nucleotide transporter of yeast mitochondria has been reported under conditions of unsaturated fatty acid depletion (Haslam & Fellows, 1975).

There is considerable evidence that the components of the transporter are located in the inner mitochondrial membrane in such a way that lipids provide both the necessary micro-environment to permit mobility of the carrier (Lauquin & Vignais, 1973; Scherer & Klingenberg, 1974), as well as specific sites at which effector ions such as Ca^{2+} and Mg^{2+} can interact so as to modify such mobility (Spencer & Bygrave, 1973, 1974). Klingenberg *et al.* (1975) found that if ox heart mitochondria are depleted of lipid by extraction *in vitro*, less ATP is bound and the sensitivity of ATP uptake to atractylate is decreased. More specifically, Spencer *et al.* (1976) showed that the removal of 1% of the phospholipids from rat liver mitochondrial membranes leads to decreases of 50% in ADP-translocation activity and 25% in ATP-translocation activity. However, Lauquin *et al.* (1976) found that changing the type of unsaturated fatty acid in the mitochondrial lipids did not greatly affect the transporter. In sterol-depleted mitochondria the lack of sterol will cause a reorganization of the membrane lipids which would be expected to affect the interaction of the transporter with lipids. In particular, a more rigid organization of the lipids could explain the decrease in the rate of ATP translocation.

Weidemann *et al.* (1970) proposed that the two types of binding site observed in mammalian and yeast mitochondria have different affinities for adenine nucleotides because of their different locations on the inner and outer surfaces of the membrane. They proposed that the high-affinity sites are located on the inner surface in contact with the residual endogenous adenine nucleotide pool, and therefore are fully saturated at low external concentrations of ATP, giving an apparent low K_D value ($\leq 1\ \mu$M). The low-affinity sites may represent binding at sites on the outer surface of the membrane, and thus exhibit the true dissociation constant for binding of ATP ($K_D = 20\ \mu$M). In sterol-depleted mitochondria the total number of binding sites is decreased by two-thirds, and there is a predominance of low-affinity sites, suggesting that either the internally facing sites are not fully saturated because of a low endogenous nucleotide content, or that the lack of sterols affects the spatial arrangement of the carrier across the membrane.

The sterol-depleted organelles also lack coupled oxidative phosphorylation, and will have a low internal (ATP+ADP) concentration, even before the arsenate preincubation, which will lead to further depletion. A further explanation is that the ATP required for the maintenance of basic mitochondrial functions will have to be transported into the sterol-depleted mitochondria from the cytosol, where the higher concentrations of (ATP+ADP) will ensure that most of the transporter-binding sites are oriented externally. The kinetics of ATP uptake by mitochondria are affected by the energy state of the mitochondria (Klingenberg, 1970; Souverijn *et al.*, 1973). The uptake of ATP^{4-} in exchange for endogenous ADP^{3-} is electrogenic (Klingenberg, 1970), and is opposed by the membrane potential of energized mitochondria. Thus uncouplers of oxidative phosphorylation stimulate the rate of uptake of ATP in yeast mitochondria of normal lipid composition (Kolarov *et al.*, 1972; Haslam & Fellows, 1975). The sterol-depleted organelles are also uncoupled, and this would be expected to stimulate the rate of ATP uptake. The fact that sterol depletion inhibits ATP uptake indicates that the properties of the transporter have been radically altered.

The changes in the adenine nucleotide transporter do not account for the loss of ATP synthesis, because

chemical uncouplers fail to stimulate respiration by sterol-depleted mitochondria in the presence of ADP (Astin & Haslam, 1977). Thus the lesions in oxidative phosphorylation and in adenine nucleotide transport are distinct phenomena. Both types of effect are also observed in mitochondria of the fatty acid desaturase mutant *ole-1* which have been depleted of unsaturated fatty acids (Haslam *et al.*, 1971; Haslam & Fellows, 1975).

The lack of coupled oxidative phosphorylation and the decreased transport of adenine nucleotides will effectively deplete the organelles of all intramitochondrial ATP. The combination of these two effects will indirectly cause an inhibition of those processes requiring ATP within the mitochondria. One such process is the mitochondrial protein-synthesizing system, which is absent for lipid-depleted organelles (Watson *et al.*, 1971; Marzuki *et al.*, 1975). Furthermore, mitochondrial DNA replication is inhibited, leading to an induction of the cytoplasmic *petite* mutation (Marzuki *et al.*, 1974), and Subik *et al.* (1972) have shown that when ATP production within the mitochondria as well as its transport across the membrane is prevented there is an increase in the proportion of cytoplasmic *petite* mutants, indicating an inhibition of mitochondrial DNA synthesis.

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