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OXIDATIVE PHOSPHORYLATION IN YEAST

IX. MODIFICATION OF THE MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSLOCATION SYSTEM IN THE OXIDATIVE PHOSPHORYLATION-DEFICIENT MUTANT op_1

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SUMMARY

The properties of intact cells and isolated mitochondria of the op_1 mutant of *Saccharomyces cerevisiae*, which had been shown previously to be deficient in oxidative phosphorylation, have been studied further.

1. When isolated mutant mitochondria were preincubated with substrate and phosphorylation was started by the addition of external ADP, low P/O ratios were obtained under standard conditions. The P/O ratios could be raised to normal values approaching 2 with citrate and succinate in the presence of unusually high concentrations of ADP. Under these conditions the Michaelis constant for ADP of respiration and phosphorylation was found to be 2.9 mM. When isolated mitochondria were added to a medium containing substrate and adenine nucleotide, the Michaelis constant for ADP was found to be lower, about 0.5 mM and maximal P/O ratios of only 0.8 were achieved.

2. Adenine nucleotide translocation across the membrane of the mutant mitochondria was found to be different from that in wild-type mitochondria and dependent on the energy level in mitochondria. When the intramitochondrial nucleotide pool consisted mostly of ADP and AMP, the rate of adenine nucleotide translocation was approx. 30 times lower than in wild-type mitochondria and the Michaelis constants for ADP of the translocation process were similar in the two types of mitochondria, being lower than 10 μM. When the nucleotide pool was enriched in ATP, the translocation rate in mutant mitochondria was as high as in wild-type mitochondria but the Michaelis constant for external adenine nucleotide was more than 100 times higher in the former than in the latter.

3. An examination of the effects of the uncoupler, oligomycin, valinomycin and nigericin on the translocation process in the mutant mitochondria provided additional evidence that varying energization of mutant mitochondria was responsible for the variations of the translocation rates and the Michaelis constants under different experimental conditions.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine.

4. The properties of the adenine nucleotide carrier of mutant mitochondria were studied and found to be different from those of wild-type mitochondria.

5. It has been concluded that the modification of adenine nucleotide translocation across the mitochondrial membrane is responsible for oxidative phosphorylation deficiency in the op_1 mutant. The implications of these findings for the understanding of the adenine nucleotide translocation mechanism and the role of the translocation system in the control of cellular syntheses and growth are discussed.

INTRODUCTION

Since the first description of the oxidative phosphorylation-deficient op_1 mutant in 1967 (ref.1) a number of reports have been published devoted to the study of this mutant[1–10]. The mutant cells are unable to grow substantially on oxidizable, non-fermentable substrates, in spite of the presence of normal amounts of cytochromes and an apparently normal respiration. A detailed genetic analysis confirmed that a single nuclear gene was responsible for the lesion[4] and demonstrated its independence from the other nuclear genes affecting mitochondrial functions in yeast[5]. It has been shown that the superimposition of a cytoplasmic mutation to respiratory deficiency of the nuclear op_1 mutation resulted in cells being unable to multiply[6].

In confirmation of the first report[1], it has been shown that mitochondria isolated from the op_1 mutant exhibited a low P/O ratio when measured under the usual conditions[2,3]; the P/O ratios were normalized, however, if high concentrations of ADP were included in the glucose-hexokinase trap[3]. The coupling step of the oxidative phosphorylation machinery has been shown to be preserved since uncoupling agents activated respiration in the mutant mitochondria in a similar fashion as in wild-type mitochondria[2]. However, according to one report, ADP must be present in the suspending medium in order to observe the stimulation of respiration by uncouplers[3]. With respect to mitochondrial phospholipid and "structural protein", the mutant does not differ from wild-type yeast[7].

Determination of molar growth yield[8] as well as spectral data[9] have indicated that the oxidative phosphorylation system is ineffective not only in isolated mitochondria but also *in vivo*. This, however, has been questioned on the basis of experiments in which the intracellular adenine nucleotides and changes in their distribution by addition of oxidizable substrates to intact op_1 cells were measured[10].

Several hypotheses have been put forward to explain the inefficiency of oxidative phosphorylation in the op_1 mutant[2,3,10] but experimental evidence has not been sufficient to discriminate between them. This study shows that the system of adenine nucleotide translocation across the mitochondrial membrane is modified in the op_1 mutant and the modification can account for the lesion in its oxidative phosphorylation. A preliminary account of this work has been published[11].

EXPERIMENTAL

Cells and mitochondria

The diploid mutant strain *Saccharomyces cerevisiae* DH1 was used as in previous

studies[2,6,8,9]. The cells were cultured as described previously and mitochondria were isolated from protoplasts[12] by a slightly modified procedure[13,14].

Determination of adenine nucleotides in intact cells

A procedure of Peña *et al.*[15] was employed to extract nucleotides from the cells. 2.5 ml of yeast suspension (about 70 mg dry wt/ml) were rapidly added to 1.25 ml of ice-cold 60% $HClO_4$. After 20 min the suspension was centrifugated and the pH of the supernatant was adjusted with 5 M K_2CO_3 to 7.5. After separating potassium perchlorate by centrifugation, adenine nucleotides present in the supernatant were determined spectrophotometrically at 366 nm by appropriate Boehringer kits.

Other analytical procedures

These were described in previous papers[2,12,14].

Materials

The source of chemicals was indicated in a previous paper[14]. In addition, hexokinase (Type S-300, Sigma), oligomycin (Sigma), NADH (VEB Arzneimittelwerk-Dresden) and nigericin (Reanal) were employed in this study.

RESULTS

Respiration and phosphorylation of mutant mitochondria under various conditions

In agreement with previous results[2], low P/O ratios of mutant mitochondria were found when measured manometrically with a glucose–hexokinase trap. Under the same conditions, maximal phosphorylation efficiency was obtained with wild-type

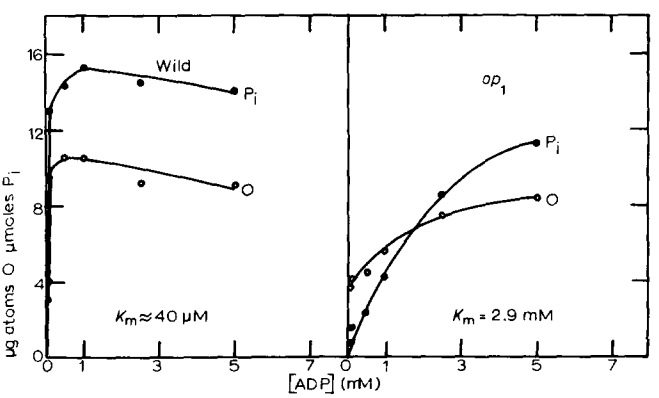


Fig. 1. Oxidation and phosphorylation of wild-type and op_1 mutant mitochondria as a function of ADP concentration. The main compartment of the Warburg flasks contained in 2.0 ml: 0.48 M mannitol, 0.5 mM EDTA, 10 mM Tris–maleate buffer, 10 mM phosphate, 7.5 mM $MgCl_2$, 10 mM potassium citrate, 0.15% bovine serum albumin, 25 mM glucose, 5 units hexokinase, 1.1 mg protein of wild-type mitochondria or 1.6 mg protein of op_1 mutant mitochondria, and ADP in concentrations indicated on abscissa, final pH was 6.4. The central well contained 0.2 ml 2 M NaOH and a piece of fluted paper. Measurements were started after 7 min of thermal equilibration by the addition of the glucose and hexokinase from the side arms and after 20 min of additional incubation were terminated by adding 2 ml 10% trichloroacetic acid. P_i in the supernatant was determined by the procedure of Sumner[33].

mitochondria. However, when the ADP concentration in the reaction medium was raised to unusually high levels, both the oxidation and phosphorylation rates were increased such that at 5 mM ADP, P/O ratios comparable to those of wild-type mitochondria were found. Increase in phosphorylation efficiency at high concentrations of ADP was also observed by Beck *et al.*[3]. Fig. 1 shows the results of an experiment with wild-type and mutant mitochondria using citrate as substrate; similar results were obtained with succinate. When the results of Fig. 1 were plotted according to Lineweaver and Burk, an apparent K_m of about 40 μM for ADP was calculated for wild-type mitochondria, the corresponding value for mutant mitochondria being 2.9 mM. Identical K_m values were calculated both for the increment of O_2 uptake and for P_i uptake.

When measured polarographically, respiration of the mutant mitochondria in State 4 was not stimulated by addition of a small amount of ADP, unlike that of wild-type mitochondria. The respiration was enhanced if the ADP was successively added to respiring mitochondria up to unusually high levels; the apparent K_m for ADP was found to be 1.9 mM. However, if ADP was present in the original incubation medium

TABLE I

PHOSPHORYLATION BY MUTANT MITOCHONDRIA IN POLAROGRAPHIC EXPERIMENTS

The polarographic vessels contained in 2.0 ml: 0.6 M mannitol, 20 mM KCl, 1.5 mM EDTA, 10 mM Tris–maleate, 0.1% bovine serum albumin, 10 mM potassium phosphate labelled with ^{32}P ($9 \cdot 10^5$ cpm·μmole^{-1} P_i), ADP in varying concentrations (0.5 to 5.0 mM) and substrate as indicated in the table; final pH 6.4. Mutant mitochondria were added at concentrations of 0.42 mg protein·ml^{-1} at 30 °C and 1.8 mg protein·ml^{-1} at 10 °C; incubation time was 2 min at 30 °C and 3.5 min at 10 °C. The reaction was terminated by adding 0.1 ml 100% (w/v) trichloroacetic acid and esterified phosphate was determined as described previously[12].

Temp. (°C)	*Substrate*	*Phosphorylation rate at 5 mM ADP* (*nmoles P_i esterified·min^{-1}·mg^{-1} protein*)	*K_m for ADP* (*mM*)	*P/O ratio*
30	NADH, 1 mM	154.0	0.4	0.46–0.60
30	Succinate, 20 mM	220.0	0.6	0.50–0.76
10	NADH, 2 mM	22.0	0.5	0.40–0.65
10	TMPD, 200 μM + ascorbate, 3 mM	25.9	0.6	0.20–0.24

TABLE II

ADENINE NUCLEOTIDE CONTENT IN WILD-TYPE AND op_1 MUTANT MITOCHONDRIA AND THE EFFECT OF INCUBATION WITH SUBSTRATE

Stock suspension of mitochondria (5.8 mg protein from wild-type strain and 8.6 mg protein from op_1 mutant) was directly analysed (first line in the table) or preincubated for 2 min at 30 °C with 0.6 M mannitol, 20 mM potassium phosphate and 20 mM α-ketoglutarate, final pH 6.4.

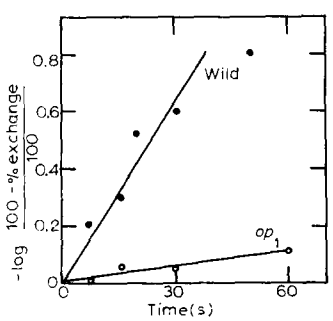
Substrate	*Adenine nucleotides* (*nmoles·mg^{-1} protein*)					
	Wild-type strain			*op_1 mutant*		
	ATP	*ADP*	*AMP*	*ATP*	*ADP*	*AMP*
None	3.71	5.25	1.27	1.31	5.62	0.81
α-Ketoglutarate	7.06	1.31	1.42	4.27	1.58	0.62

prior to addition of mitochondria, a K_m for ADP about 5 times lower was found (0.3–0.7 mM in different experiments). The latter arrangement allowed us to measure phosphorylation in the polarographic experiments. Under these conditions, the K_m for ADP phosphorylation was lower than in the manometric experiment and maximal P/O ratios were also considerably diminished (Table I).

These data show that, if mitochondria had not been preincubated with substrate prior to ADP addition, they exhibited a higher apparent affinity for ADP and a lower phosphorylation efficiency than mitochondria preincubated with substrate. The mitochondria under the two conditions should have differed in the composition of their adenine nucleotide pool. Mitochondria in the dense stock suspension were essentially anaerobic and devoid of ATP, while those preincubated with substrate in the absence of ADP should have been enriched in ATP. This is shown by the analytical data presented in Table II. The results suggested that the composition of the endogenous nucleotide pool may have altered the affinity of the mutant mitochondria for external ADP. As several authors have presented evidence[16–20] that the ADP affinity of mitochondrial oxidative phosphorylation correlates with the ADP affinity of the mitochondrial adenine nucelotide translocation system, the translocation of adenine nucleotides across the membrane of op_1 mitochondria was studied under different incubation conditions.

Properties of the adenine nucleotide translocation system of mutant mitochondria

As shown in Fig. 2, the rate of adenine nucleotide exchange was considerably lower in op_1 mitochondria than in wild-type mitochondria. In this procedure[14], mitochondria were the last addition to a reaction mixture which contained no substrate. Affinities of the translocation process for ATP and ADP in op_1 mitochondria, measured



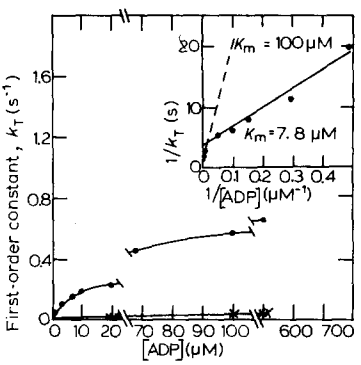


Fig. 2. Time course of adenine nucleotide exchange in wild-type and op_1 mitochondria. Mitochondria from wild-type yeast (2.2 mg protein·ml^{-1}) or from op_1 mutant (1.8 mg protein·ml^{-1}) were prelabelled with [^{14}C]ATP and then incubated at 2 °C in a medium containing 200 μM ADP, 0.4 M sorbitol, 10 mM Tris–maleate buffer and 1 mM EDTA; final pH was 6.4. Incubation was arrested by rapid filtration and "back exchange" was determined as described previously[14].

Fig. 3. Concentration dependence of ADP translocation in mutant mitochondria. Mitochondria from op_1 mutant (0.07 mg protein·ml^{-1}) prelabelled with [^{14}C]ATP were incubated for 20 s at 10 °C in a medium containing 0.4 M sorbitol, 1 mM EDTA, 10 mM Tris–maleate buffer and ADP at concentrations indicated on the abscissa; final pH was 6.4. Incubation was arrested by 200 μM atractyloside and the mitochondria were separated by centrifugation. The lower curve shows translocation in the presence of 200 μM atractyloside.

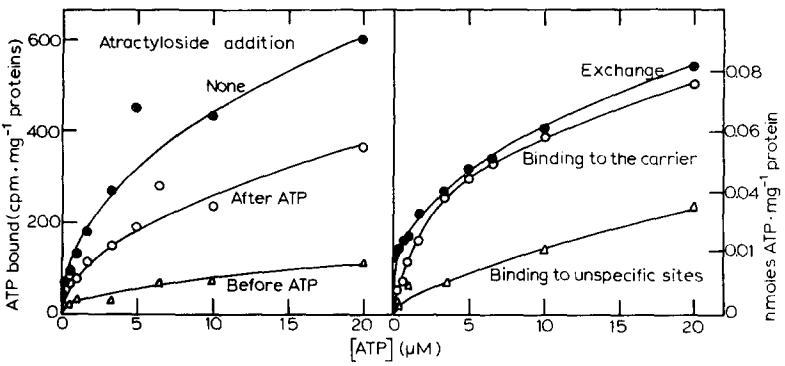


Fig. 4. Concentration dependence of [^{14}C]ATP uptake by op_1 mutant mitochondria and differentiation of the uptake into binding and exchange. Mitochondria (3.1 mg protein·ml^{-1}) were depleted of endogenous adenine nucleotide by preincubation for 15 min at 30 °C in an isotonic medium enriched with 10 mM phosphate buffer and 5 mM $MgCl_2$ (refs 14, 34) and incubated at 2 °C for 1.5 min in a medium containing 0.4 M sorbitol, 1 mM EDTA, 10 mM Tris–maleate buffer and [^{14}C]ATP at concentration indicated on abscissa; final pH was 6.4. Atractyloside was either omitted from the medium (●—●) or added before (△—△) or 1.5 min after ATP (○—○). The uptake measured under the three conditions was used in evaluation of the exchange and of the binding to unspecific sites and to the carrier sites, respectively[34]. Adenine nucleotide content after depletion (in nmoles·mg^{-1} protein): total, 2.03; ATP, 0.41; ADP, 1.04; AMP, 0.58.

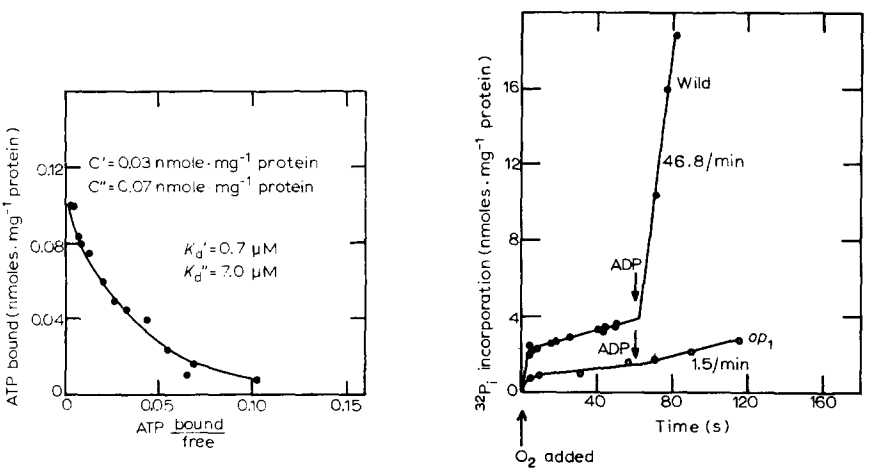


Fig. 5. Scatchard plot of the data of Fig. 4. The total atractyloside-removable binding was plotted against the individual bound/free ratios of [^{14}C]ATP.

Fig. 6. Phosphorylation of endogenous and exogenous ADP by mitochondria from wild-type yeast and op_1 mutant. Mitochondria (3.75 mg protein·ml^{-1} of wild type, 6.3 mg protein·ml^{-1} of op_1 mutant) were preincubated anaerobically at 2 °C for 10 min in 8 ml of a medium containing 0.6 M mannitol, 1.5 mM EDTA, 10 mM Tris–maleate buffer, 20 mM KCl, 200 μM TMPD and 3 mM ascorbate, final pH 6.4. One mM potassium phosphate labelled with ^{32}P ($9 \cdot 10^6$ cpm·μmole^{-1} P_i) was then added and 3 min later, phosphorylation was started by bubbling air through the medium. At the arrow, 1 mM ADP was introduced. At various time intervals, 0.4-ml aliquots were taken, deproteinized with 0.1 ml 100 (w/v) trichloroacetic acid and the esterified phosphate was determined as in Table I. The smaller extent of endogenous phosphorylation in op_1 mutant was probably due to the lower content of adenine nucleotides (10.1 nmoles·mg^{-1} protein in wild type; 6.4 nmoles·mg^{-1} protein in op_1 mutant).

under these conditions, were not substantially different from those of wild-type mitochondria; the Michaelis constants being in the order of 10 μM. However, in addition to the low Michaelis constant, one and sometimes two larger Michaelis constants could be distinguished in reciprocal plots of translocation *versus* nucleotide concentration. This phenomenon was more distinct when translocation was measured at higher temperatures (*e.g.* Fig. 3).

Fig. 4 depicts a concentration dependence of ATP uptake measured by a procedure[21] enabling differentiation between exchange and the binding of ATP to non-specific sites and to the nucleotide carrier. Similar measurements with wild-type and respiration-deficient mitochondria had shown[14] that both types of mitochondria contained one group of sites at about 0.1 nmol·mg^{-1} mitochondrial protein with a dissociation constant of about 0.4 μM. When the results of Fig. 4 were evaluated in a Scatchard plot[22], the binding data obtained with the op_1 mutant mitochondria did not fit a straight line, unlike wild-type and respiration-deficient mitochondria (Fig. 5). The plot suggested the existence of two binding sites with respective dissociation constants 2 and 20 times higher than those in wild-type mitochondria.

The low rate of adenine nucleotide translocation may partly account for the low phosphorylation efficiency of op_1 mitochondria. With the mitochondria being loosely coupled, the rate of respiration would considerably exceed the rate of phosphorylation if slow translocation became the rate-limiting step. As shown in Fig. 6, the rate of endogenous phosphorylation, measured by a procedure of Heldt and Klingenberg[23], was too high to be determined properly but it was probably not much different in op_1 and wild-type mitochondria. On the other hand, the rate of phosphorylation of exogenous ADP was markedly lower in the mutant. The rate of phosphorylation of exogenous ADP by op_1 mitochondria (1.5 nmoles·min^{-1}·mg^{-1} at 2 °C) correlates with the rate of translocation (1.2 nmoles·min^{-1}·mg^{-1}) determined at the same temperature. However, the translocation data presented so far do not explain the high Michaelis constant of respiration and phosphorylation for ADP and the restoration of normal P/O ratios with high concentrations of ADP.

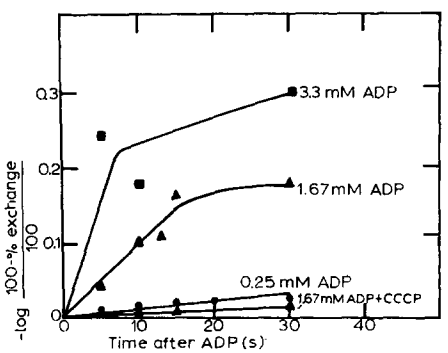


Fig. 7. Logarithmic plot of the exchange of ADP at different ADP concentrations and in the presence of CCCP. Mitochondria from op_1 mutant (2.0 mg protein·ml^{-1}), prelabelled with [^{14}C]ATP for 45 min, were preincubated with 2 mM NADH at 2 °C for 90 s in the medium containing 0.4 M sorbitol, 1 mM EDTA, 10 mM potassium phosphate, 15 mM KCl and 10 mM Tris–maleate buffer, final pH 6.4. The exchange was started with ADP and arrested by 200 μM atractyloside. Mitochondria were separated by filtration. CCCP concentration, when present, was 10 μM.

An explanation is provided by measurements of adenine nucleotide translocation under conditions which are not usually employed in translocation studies but which resemble conditions under which oxidative phosphorylation is being studied. In this experiment, mitochondria preloaded with radioactive adenine nucleotide are incubated with substrate and the measurement of translocation is commenced by adding external non-labelled adenine nucleotide. As shown in Fig. 7, under these conditions the translocation rate is not maximal at relatively low concentrations of external adenine nucleotide but can be raised to the rate found with wild-type mitochondria by increasing the external adenine nucleotide concentration. The Michaelis constant for ADP, determined under these conditions in three independent experiments, was found to be 1.4, 2.1 and 3.3 mM which correlates with the values found in the determination of oxidative phosphorylation.

When uncoupler was included in the reaction medium, the translocation rate was again low and could not be raised by increasing the concentration of external adenine nucleotide (Fig. 7). In fact, the translocation activity of mitochondria preincubated with substrate in the presence of uncoupler was similar to that found previously in the experiments with non-preincubated mitochondria. The translocation activities and Michaelis constants were similar when either ADP or ATP was used as external adenine nucleotide.

In mitochondria preincubated with substrate in the absence of exogenous adenine nucleotide, the endogenous nucleotide pool apparently consisted mostly of ATP while if uncoupler was present or in non-preincubated mitochondria the pool was largely converted to ADP. It is conceivable that the properties of the translocation system were modified when ATP was the predominant nucleotide loaded on the carrier from the inner side of the mitochondrial membrane. Another possibility is that the mitochondrial membrane was energized by intramitochondrial ATP and that this energization of the membrane was the direct cause of the modification of the translocation activity. In the latter case, the translocation system would have been changed in the same fashion independent of whether the energization was achieved by ATP, by respiration or reversal of energy-driven ion fluxes across the membrane. More specifically, the determining component of the energized state may have been represented by a pH difference or by an electric potential difference across the membrane[24].

As shown in Table III, the translocation properties of mutant mitochondria preincubated with substrate in a complex medium were essentially the same in the presence or absence of oligomycin in the medium. Oligomycin prevented respiration-linked synthesis of intramitochondiral ATP but not energization of mitochondria by respiration[25]. Thus, energization of mitochondrial membrane, not the intramitochondrial ATP itself, was the factor modifying the translocation properties.

This was also borne out by the observation that mitochondria energized by valinomycin-induced efflux of K^+ (refs 26 and 27) exhibited similar translocation characteristics to those energized by respiration (Table III). On the other hand, preincubation of mitochondria with substrate was made ineffective when K^+, valinomycin and a "permeant" anion were included in the preincubation medium. Under these conditions, energization due to the respiration-driven formation of a membrane potential was prevented[24].

Nigericin is known to abolish the pH difference across the mitochondrial membrane leaving the membrane potential undisturbed[28]. As shown in Table III, nigericin

TABLE III

TRANSLOCATION OF ADENINE NUCLEOTIDE IN MUTANT MITOCHONDRIA UNDER VARIOUS CONDITIONS

The process was measured at 2 °C. Translocation activity (V_t) was measured with 1.5 mM external ADP. Medium without substrate contained 0.4 M sorbitol, 1 mM EDTA, 10 mM Tris–maleate buffer, 1.5 mM ADP, final pH 6.4. Measurements were taken on addition of mitochondria (0.7 mg protein·ml^{-1}) prelabelled with [^{14}C]ATP. Medium with substrate contained in addition, 2 mM NADH, 15 mM KCl and 10 mM potassium phosphate. Mitochondria (0.7 mg protein·ml^{-1}) prelabelled with [^{14}C]ATP were preincubated in the medium for 1.5 min and measurement was started by adding 1.5 mM ADP. When K^+ was omitted from the medium, Tris salts were used instead. Inhibitors, when used, were included in the medium so that mitochondria were preincubated in the presence of the inhibitors.

Medium and inhibitor	*Energy condition in mitochondria*	V_t (*nmoles·min^{-1}·mg^{-1} protein*)	K_m (*mM*)
Medium without substrate	Non-energized	1.5	0.01
Medium with substrate			
no other addition	Energized by respiration or by ATP	11.0	1.4
+ oligomycin (5 μg·ml^{-1})	Energized only by respiration	9.5	0.9
+ nigericin (1 μg·ml^{-1})	Electric component of the energized state preserved	13.8	1.25
+ valinomycin (1 μg·ml^{-1}) + 10 mM potassium acetate	Electric component of the energized state abolished	5.9	0.15
+ CCCP (10 μM)	De-energized	2.9	0.03
Medium without K^+ and without substrate;			
+ valinomycin (1 μg·ml^{-1})	Energized by K^+ efflux	5.2	1.25

TABLE IV

DATA ON ADENINE NUCLEOTIDE TRANSLOCATION AND THE PROPERTIES OF THE CARRIER IN MITOCHONDRIA FROM WILD-TYPE YEAST AND *op*$_1$ MUTANT

Data	*Source of mitochondria:*	
	Wild type	*op*$_1$ *mutant*
Number of binding sites (nmoles·mg^{-1} protein)	0.1	0.1
Types of sites (%) (high:low affinity)	100:0	40:60
Dissociation constants (μM)	0.40	0.70; 7.00
*Translocation in non-energized mitohondria**		
Translocation activity $V_{t(max)}$ (nmoles·min^{-1}·mg^{-1} protein)		
at 2 °C	32	1.2
at 10 °C	***	5.1
K_m for ADP (μM)	4.75	8.0; 100.0
*Translocation in energized mitochondria***		
$V_{t(max)}$ at 2 °C	***	34.0
$V_{t(max)}$ at 10 °C	***	50.0
K_m for ADP (mM)	***	1.4; 2.1; 3.3

* Simple medium, measurement started by addition of mitochondria; conditions as in Fig. 2.
** Complex medium, mitochondria preincubated with substrate, measurement started by addition of adenine nucleotides; conditions as in Fig. 7.
*** Not measured.

had little effect on the properties of the translocation system of mutant mitochondria, indicating that the electrical not the chemical component of the mitochondrial high-energy state[24] was important in affecting the adenine nucleotide translocation in the mutant mitochondria.

These experiments show that the adenine nucleotide translocation system in the op_1 mitochondria exhibits abnormal properties. The properties are summarized in Table IV and compared with those of wild-type mitochondria. It may be seen that, while the translocation activity of op_1 mitochondria depleted of endogenous ATP is very low, the translocation activity of mutant mitochondria enriched in endogenous ATP is similar to that of wild-type mitochondria. A translocation activity of 34 nmoles $ADP \cdot min^{-1} \cdot mg^{-1}$ at 2 °C may be compared with a respiration rate of 67 ngatoms $O \cdot min^{-1} \cdot mg^{-1}$ in the presence of TMPD and ascorbate at 2 °C.

Changes in adenine nucleotides of intact cells by addition of oxidizable substrate

The results presented above indicated that, depending upon incubation conditions, isolated mutant mitochondria could display either low or normal P/O ratios. The P/O ratios of mitochondria *in situ* would depend on the concentrations of cytosolic ADP and mitochondrial ATP, if it is assumed that they are rather loosely coupled. If tightly coupled, efficiency of their oxidative phosphorylation would be normal and only the rate of the process would have an unusual dependence on cellular adenine nucleotide concentration.

Determination of cellular adenine nucleotide under various conditions in the op_1 mutant was previously carried out by Somlo[10]. According to her results, oxidation of ethanol resulted in a high steady-state concentration of ATP which was only 20–30% lower than in a wild-type strain.

As the procedure of Somlo would not be expected to extract adenine nucleotides quantitatively from yeast[15] and as Somlo herself had difficulties in her extraction procedure, we repeated the determination of adenine nucleotides in the mutant cells by using a different extraction method and placed particular emphasis on the kinetics of nucleotide changes upon substrate addition.

Fig. 8 shows changes in cellular levels of ATP, ADP and AMP upon adding ethanol to aerobic suspensions of wild-type and op_1 mutant cells. In both types of cells, addition of ethanol was followed by a rise in the ATP level, mostly at the expense of AMP; ADP showed only minute changes, and the sum of adenine nucleotides remained essentially unchanged. The rate of the nucleotide transformation was lower in the op_1 mutant. While Somlo[10] found an elevated amount of AMP in the op_1 mutant, the steady-state levels of AMP under our conditions were essentially identical in the op_1 mutant and the wild-type strain. The steady-state concentrations of the three nucleotides before and after ethanol addition in the two strains were quite close to the adenylate kinase equilibrium[29].

Thus, in agreement with the results of Somlo, ATP is formed *in vivo* in the op_1 mutant in the course of ethanol oxidation. From these observations, however, no inference about the efficiency of oxidative phosphorylation *in vivo* can be made. If the rate of phosphorylation is calculated from the initial slope of the ATP rise upon addition of ethanol and compared with the rate of ethanol oxidation measured separately, P/O ratios as low as 0.03 were obtained for both the wild-type and mutant strain. Similar low values can be computed from the data of Somlo[10].

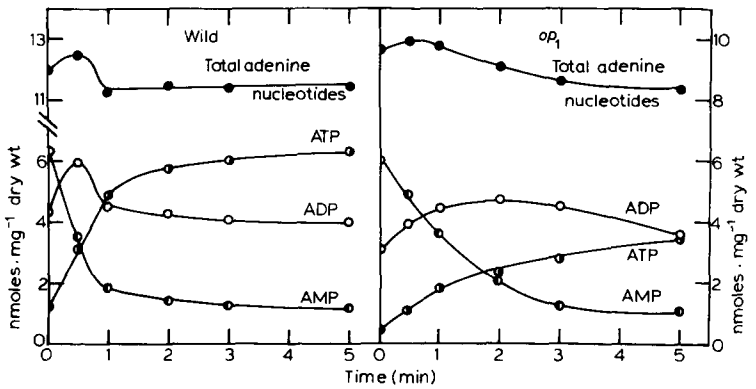


Fig. 8. Time course of changes of adenine nucleotides in wild-type yeast and op_1 mutant on ethanol addition. The cells were starved by aeration in water for 24 h at 30 °C to reduce endogenous respiration. Then they were suspended in 50 mM Tris–HCl buffer (pH 7.2) to give about 70 mg dry wt/ml and aerated by passing air through the suspension. The experiment was started by rapid addition of ethanol to final concentration of 50 mM. Aliquots of cells were taken at intervals for analysis of cellular adenine nucleotides as described in Experimental.

DISCUSSION

The present investigation has established that the nuclear op_1 gene controls the adenine nucleotide translocation in mitochondria and that the modification of the translocation system can account for the oxidative phosphorylation deficiency in the op_1 mutant. The adenine nucleotide translocation system of the mutant mitochondria depends on the actual energy state of the mitochondria, exhibiting a high rate and high Michaelis constants for nucleotides in energized mitochondria and a low rate and low Michaelis constants in de-energized mitochondria.

Since the extent of energization of the mitochondrial membrane can vary under different conditions and also depends on the quality of mitochondrial preparations, it is not surprising that values of the Michaelis constant for external adenine nucleotide were found to vary in an almost continuous range from 8 μM to 3.3 mM The lowest and the highest values apparently represent the limits reached under the entirely de-energized and fully energized conditions, respectively. This may also partly explain why several Michaelis constants could be inferred from the data of a single experiment (see *e.g.* Fig. 3), since the energy state of mitochondria may change in the course of translocation of adenine nucleotide.

A number of investigators have speculated about the nature of the carrier system for adenine nucleotides in the inner mitochondrial membrane[20,21,30,31]. On the basis of extensive studies, Weidemann *et al.*[21] devised two carrier models compatible with some of the results, but they had to admit that no full agreement with the experimental data was achieved. The observations on the properties of op_1 mitochondria may help to elucidate the carrier mechanism operating in normal mitochondria.

Briggs-Haldane kinetics may be applied in an attempt to explain the properties of the translocation system in mutant mitochondria. The first property of the adenine nucleotide carrier to be modified by the op_1 mutation would be its turnover rate or the speed at which it shuttles across the mitochondrial membrane.

If the activity of the carrier is formally written as

$$A_{ext} + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AC \xrightarrow{k_3} A_{int} + C$$

where A_{ext} stands for exogenous adenine nucleotide, A_{int} for the nucleotide transferred inside mitochondria and C for the carrier; the dissociation constant of the carrier–nucleotide complex as measured in this work is given by

$$K_{diss} = \frac{k_2}{k_1}$$

and the Michaelis constant of overall translocation by

$$K_m = \frac{k_2 + k_3}{k_1}$$

In non-energized op_1 mitochondria, k_3 is low when compared to k_2 and k_1, and K_m approaches K_{diss}. Upon energization, k_3 increases markedly and this is reflected by an increase in K_m.

To bring this explanation into quantitative agreement with the data obtained, it was assumed that k_2 and k_3 are also slightly affected by the energization of mitochondria. In addition, since k_3 in wild-type mitochondria under energized and non-energized conditions is at least as high as in the energized op_1 mitochondria and yet the K_m for external nucleotide of wild-type mitochondria is much lower than in the mutant, both k_1 and k_2 should have different values in the two yeast types. In fact, dissociation constants were found to be different in wild-type and op_1 mitochondria.

The experimental results indicate that it is the electric component of the mitochondrial energized state, *i.e.* the membrane potential, which affects the translocation system and in the model proposed would increase k_3, that is the shuttling rate of the carrier in the membrane of mutant mitochondria. To make this possible, and in view of the orientation of the membrane potential in mitochondria, the carrier should carry excess positive charge when moving from the outer to the inner side of the membrane while the charges should be inverted when moving in the opposite direction. The character of the charges would not be dependent on whether ATP or ADP were carried but on different chemical groups being exposed at the surface of the carrier when moving inwards and outwards respectively. This would imply that the carrier has a different conformation when moving in one direction than when moving in the opposite direction in the membrane.

Similar conformational transitions may be envisaged in normal mitochondria except that they would not be accompanied by the charge redistribution on the surface of the carrier. Accordingly, k_3 of wild-type mitochondria would not depend on the extent of mitochondrial energization.

From values of intracellular adenine nucleotide content presented in Fig. 8, intracellular concentrations of ATP, ADP and AMP in the mutant cells can be calculated. Assuming that the nucleotides are distributed homogeneously within the cell, the respective concentrations of ATP, ADP and AMP in the cells of the op_1 mutant were 0.2, 1.1 and 2.1 mM before addition of oxidizable substrate and 1.2, 1.2 and 0.3 mM after the establishment new steady-state in cells oxidizing ethanol. If these

figures hold for intramitochondrial concentrations as well, mitochondria oxidizing ethanol can be considered as partly energized by intramitochondrial ATP. In view of results obtained with isolated mitochondria, the rates of adenine nucleotide translocation and of oxidative phosphorylation of mitochondria in the mutant cells would be determined by cytosolic concentration of ADP. The concentration of ADP were 1.2 mM and the Michaelis constant 1.4–3.3 mM, the rate of adenine nucleotide translocation across the mitochondrial membrane in the mutant would be approx. 2–4 times lower than in wild-type yeast.

It is remarkable that such a relatively mild reduction of the translocation rate in op_1 mitochondria is sufficient to prevent growth on non-fermentable substrates and the utilization of energy of substrate oxidation for cellular synthesis[1,8,10]. The appropriately adjusted rate of mitochondrial adenine nucleotide translocation in normal yeast may be of crucial importance for triggering extramitochondrial synthesis and cell growth when non-fermentable substrates serve as the energy source.

An alternative explanation is provided by the results of Klingenberg *et al.*[32] that the translocation device of mammalian mitochondria enables it to maintain a phosphorylation potential 50 times higher in the cytosol than in mitochondria. It is conceivable that, due to defective translocation machinery, op_1 mutant cells cannot sustain such a difference in the two potentials. The high cytosolic phosphorylation potential, built up by the normal translocation system, may be prerequisite for the operation of extramitochondrial assimilatory processes.

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