An Oligomycin-Resistant Adenosine Triphosphatase and its Effects on Cellular Growth, Mitochondrial Oxidative Phosphorylation and Respiratory Proton Translocation in *Saccharomyces cerevisiae*

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Mutations at the OLI 1 or OLI 2 loci of mitochondrial DNA in *Saccharomyces cerevisiae* are associated with a diminished growth rate in nutritionally suboptimal cultures supplemented with an oxidizable carbon source. In the case of mutant O₁₄₆^R (OLI 1) there is a 35% loss of mitochondrial protein during fractionation *in vitro*, suggesting that the mutationally altered adenosine triphosphatase (ATPase) confers some instability on the mitochondrial membrane. The possibility is discussed that this reflects an unstable mitochondrial population *in vivo*, leading to the observed growth deficiency. Mitochondria from mutant O₁₄₆^R at the OLI 1 locus show a relatively oligomycin-resistant State-3 respiration, but the same ADP/O and respiratory-control quotients as the isonuclear wild-type. A slightly lowered Q_{O_2} with NADH-linked substrates was observed and is discussed. For both strains the apparent H⁺/O ratios were close to 4 with pyruvate, ethanol and α -oxoglutarate, but consistently lower with succinate and citrate. For each substrate a characteristic $t_{\frac{1}{2}}$ (time for half-decay of the transmembrane pH differential) range was found, consistent with the view that the substrates effectively carry the protons back across the membrane. As expected, H⁺/O ratios were independent of $t_{\frac{1}{2}}$ for all substrates, with the exception of α -oxoglutarate in the case of the wild-type, where an inverse correlation was found. The lack of this correlation in the case of the mutant was the only apparent difference in the translocation parameters observed. A hypothesis relating this to the functioning of the oligomycin-resistant ATPase is proposed.

Mutations at the OLI 1 or OLI 2 loci of mitochondrial DNA in *Saccharomyces cerevisiae* (Avner *et al.*, 1973) result in mitochondria that show partially oligomycin-resistant ATPase (adenosine triphosphatase, Mg²⁺-activated, EC 3.6.1.4) activity (Griffiths & Houghton, 1974; Somlo *et al.*, 1974). These mutants are therefore potentially useful tools for studying the mechanism of oxidative phosphorylation, particularly the nature of the postulated proton-translocating ATPase (Mitchell & Moyle, 1965, 1968; Mitchell, 1974). The highly purified ATPase complexes isolated from the mitochondria of these mutants also display oligomycin-resistance (Somlo *et al.*, 1974; Cosson & Spiridakis, 1974). This indicates that the phenomenon is a property of the complex itself rather than a feature of the membrane as a whole. [The 'ATPase complex' (Tzagoloff *et al.*, 1973) stands for F₁ (Penefsky *et al.*, 1960) associated with the 'oligomycin-sensitivity-conferring protein' (MacLennan & Tzagoloff, 1968) and the 'membrane factor' (Tzagoloff & Meagher, 1972) plus phospholipids.]

The experiments described in the present paper were undertaken to determine whether the presence of the mutationally altered ATPase introduces any measurable changes in the energy-conserving system at the level of the isolated mitochondria.

Materials and Methods

Chemicals

ADP, carbonyl cyanide *m*-chlorophenylhydrazone and respiratory substrates were obtained from Calbiochem (Los Angeles, CA, U.S.A.). Bovine serum albumin and oligomycin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Valinomycin was obtained from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). Sorbitol was provided by Roquette Frères (Lille, France).

Strains

Growth-rate experiments; diploid strains PS 211 (C^SE^SO₁₄₆^R, oligomycin-resistant mutant at the OLI 1 locus), PS 195 (E^SC^SO₁₄₄^R, oligomycin-resistant

mutant at the OLI2 locus), PS 194 ($C^S E^S O^S$, their isonuclear wild-type) (Somlo *et al.*, 1974); further, LM 878 ($C^S E^S O^S$, oligomycin-resistant mutant at the OLI4 locus) and LM 877 ($C^S E^S O^S$, its isonuclear wild-type) (Clavilier, 1976). Mitochondrial experiments: only PS 194 and PS 211 were used.

Aerobic cultures

Erlenmeyer flasks (volume 6 litres) containing 800ml of culture medium (Difco yeast extract, 1%; $(NH_4)_2SO_4$, 0.12%; ethanol, 1%) were inoculated with 4ml of aerobic stationary-phase glucose-grown preculture (Somlo, 1962). Cultures were vigorously shaken at 28°C, and harvested about 24h later at 1.6–1.8mg of cell protein/ml (stationary phase in this medium at 3mg of cell protein/ml). Immediately after harvesting, cells were washed in the following medium: sorbitol, 0.65M; EDTA (sodium salt), 0.1 mM; the pH was 6.5, and the medium was kept ice-cold until cell breaking, but not longer than 1 week.

Preparation of mitochondria (0°C)

Yeast cells were suspended in portions of 'breaking medium' (25 ml per 1.3 g of cell protein, equivalent to 800ml of culture) (breaking medium = washing medium plus 0.1% bovine serum albumin). Breakage was carried out in a Braun homogenizer (B. Braun, Melsungen, Germany) with 20g of glass beads (diam. 0.45mm) for 2min at the low speed (2000 oscillations/min). The homogenate was decanted and the beads were rinsed with 5 ml of breaking medium. Combined washings were centrifuged 10min at 1500g. The supernatant, containing about 40% of the total cell protein, was diluted twice with breaking medium to facilitate further fractionation and again centrifuged for 10min at 1500g. The resulting supernatant was centrifuged for 10min at 9500g and the abundant fluffy layer discarded. The pellet was suspended in a Potter–Elvehjem homogenizer in breaking medium and centrifuged again for 10min at 9500g. The fluffy layer was again discarded, and the mitochondria suspended in the Potter–Elvehjem homogenizer at 10–15mg of protein/ml (the total was about 25 mg/portion).

Mitochondrial respiration rates

These were measured at 30°C with a Clark electrode (Gilson Oxygraph), in a medium containing: sorbitol, 0.65M; EDTA (sodium salt), 2mM; KH_2PO_4 , 10mM; substrate, 3mM; the pH was adjusted to 6.3 with NaOH. The solubility of O_2 at 30°C was taken as 0.222mM (Chappell, 1964). ADP/O ratios were calculated by the method of Chance & Williams (1955).

Measurement of proton translocation

pH changes were recorded with a Radiometer UY GK 2322C electrode linked to a Vibron Electro-

Fig. 1. Comparison of respiratory-driven H^+ translocation at (a) 25°C and (b) 15°C

The substrate was α -oxoglutarate. The O_2 pulse was with 30 μ l of KCl (see the text for further details). Mitochondria were from the O_{146}^R mutant (at locus OLI 1). $\Delta[H^+]$ calibration: 25°C, 2.68 μ g-atoms of H^+ /1 pH unit; 15°C, 2.52 μ g-atoms of H^+ /1 pH unit.

	H^+/O	$t_{\frac{1}{2}}$
25°C	3.7	12s
15°C	4.0	32s

meter, model 33 B2 (Electronic Instruments Ltd., Richmond, Surrey, U.K.) and a Servotrace recorder (Sefram, Paris, France). The mitochondria were incubated in a water-jacketed cell designed to allow continuous N₂ gassing (Mitchell & Moyle, 1967a). The method of Mitchell & Moyle (1967b) was used earlier with some variations for *S. cerevisiae* mitochondria (Kovac *et al.*, 1972; Haslam *et al.*, 1973), but the described methods were not suitable for our purpose, which was to use a medium also supporting oxidative phosphorylation with ADP/O and respiratory-control ratios as high as those obtained with the standard respiration medium. We therefore adopted a medium similar to that used by Downie & Garland (1973) for *Candida utilis* mitochondria and that contained: sorbitol, 0.65M; KCl, 150mM; glycylglycine, 4mM; bovine serum albumin, 0.1% (pH as such about 6.2); substrates, 1mM; mitochondrial protein, 4–5mg; total volume, 3.3ml. Yeast mitochondria produce considerable quantities of acid when N₂-gassed in translocation media, as also noted by Downie & Garland (1973). The presence of 150 mM-KCl and 0.1% bovine serum albumin accelerated the attainment of the anaerobic pH equilibrium, this taking between 15 and 30min under our above-described conditions. Higher KCl and serum albumin concentrations were deleterious to oxidative phosphorylation. The actual pH-equilibrium value depended on the nature of the substrate (about 6.2 with succinate or citrate, 5.9 with α -oxoglutarate and 5.5 with ethanol or endogenous substrate).

Although valinomycin was not essential with mammalian mitochondria (Mitchell & Moyle, 1967b), this specific K⁺ ionophore was necessary in our experimental conditions (30ng/mg of protein); in its absence very low H⁺ ejection occurred. However, if valinomycin was added before the attainment of anaerobic pH equilibrium, the magnitude of H⁺ ejections induced by successive identical O₂ pulses increased for up to 40min after valinomycin addition. Therefore we chose to add valinomycin only after spontaneous acidification had ceased completely. Even then, maximal and constant H⁺-ejection responses occurred only after valinomycin had been present for a few minutes. The responses then remained stable for at least 1h. After the addition of valinomycin, cycles of very slow pH micro-oscillations started that did not perturb the rapid H⁺-ejection response to the O₂ pulses.

Measurements of H⁺ translocation were carried out at 15°C to obtain higher H⁺ peaks, a slower H⁺ backflow and better precision. In comparison with translocation at 25°C (Fig. 1), H⁺ ejection is slowed down as well as H⁺ backflow. The time for the half-decay of the transmembrane pH differential (Δ pH), referred to as $t_{\frac{1}{2}}$ (Mitchell & Moyle, 1967b) was 3–4 times greater at 15°C, whereas the H⁺/O ratios were practically unaffected. O₂ pulses were

administered as additions (30 and 50 μ l) of KCl (30mM), air-saturated at 15°C (0.287mM; Chappell, 1964). In this range the H⁺/O values at 15°C were independent of the size of the pulse, i.e. the quantity of H⁺ ejected was proportional to the O₂ content of the pulse. The H⁺/O ratios were calculated by extrapolating the H⁺-decay curve back to the time when half of the added O₂ was consumed, assuming State-3 respiration rates (Mitchell & Moyle, 1967b), usually between 1 and 5s after O₂ addition. The endogenous State-3 rate was near zero; in this case, the decay curve was extrapolated to zero time. The H⁺/O ratios for endogenous substrate are therefore probably slightly overestimated.

Mitochondrial protein was determined (by the

Fig. 2. Comparative effect of oligomycin on respiratory control

(a) Wild-type; (b) O_{LI} 1 mutant (locus OLI 1). The controls (no oligomycin) are run in 'respiration medium'; the same results are obtained in the translocation medium supplemented with 10mM-KH₂PO₄ (see the Materials and Methods section). Medium (1.5ml) + 3mM- α -oxoglutarate (control) $\pm$ oligomycin to give 7.5 μ g/mg of mitochondrial protein were incubated; mitochondria (1.0mg of protein, wild-type or mutant) were added last. Successive arrows indicate addition of 300ng-atoms of ADP. The total volume was 1.7ml. Oligomycin was added as 3 μ l of a 2.5 mg/ml solution in methanol. The same amount of methanol alone did not modify the traces. Energy parameters for the controls (no oligomycin):

	ADP/O	Respiratory control
Wild-type	2.1	2.6
	2.3	3.3
Mutant	2.2	2.3
	2.4	2.0

method of Lowry *et al.*, 1951) before the start of each translocation experiment, and identical amounts were incubated for mutant and wild-type. After each series of O₂ pulses, titration was carried out with 3 μ l of 20 mM-HCl in the presence of 5 μ M-carbonyl cyanide *m*-chlorophenylhydrazone. There was no statistically significant difference between the titration of mutant and wild-type mitochondria.

The translocation parameters were calculated with the aid of a program based on the law equation of Mitchell & Moyle (1967a), elaborated by Dr. J. L. Risler (Centre de Génétique Moléculaire, Gif). The program is available on request.

Results

Disadvantages of the mutation at the OLI1 locus

(1) *Decreased growth rate under suboptimal culture conditions.* The point mutations in the OLI1 or OLI2 loci of mitochondrial DNA (Avner *et al.*, 1973) confer oligomycin-resistance on whole cells growing on oxidizable carbon sources. It was initially thought that the ensuing partial oligomycin-resistance of the mitochondrial ATPase (Griffiths & Houghton, 1974; Somlo *et al.*, 1974) produced no energetic drawback; in fact the mutants and the wild-type had the same growth rate and yield on nutritionally rich, chemically undefined, medium supplemented with an oxidizable carbon source. However, on nutritionally suboptimal, chemically defined, medium (Difco Yeast Nitrogen Base, 0.7%) supplemented with an oxidizable carbon source (ethanol, 1%) the growth rate of the mutants (but not their growth yield) was decreased in proportion to the increase of oligo-

mycin-resistance of the respective mitochondrial ATPase:

Oligomycin resistance: Wild-type = OLI 1 < 2 < 4
Mean generation time: 5h 5h 6h 7h

No growth rate difference was found with a fermentable carbon source.

(2) *Decreased mitochondrial yield during preparation.* For experiments with mitochondria, the wild-type strain and the isonuclear mutant PS 211 (locus OLI1), showing the highest oligomycin-resistance of the mitochondrially integrated ATPase, were grown in a natural medium with ethanol as carbon source, where their generation times were identical (about 3h). Surprisingly, in the course of many carefully quantified preparations, there was a systematically decreased yield of mitochondrial protein in the case of the mutant. Both strains were harvested at the same growth stage (about one generation before the end of growth) and handled under the same conditions. In the washed mitochondrial pellet 34 $\pm$ 9% less protein was recovered (standard error; mean of 22 preparations from 11 cultures) from the mutant than from the wild-type. Sucrose-density-gradient analyses (Somlo & Krupa, 1974) on the 'low-speed' supernatants and on the mitochondrial pellets showed that the amounts of mitochondrial protein and ATPase were identical for both strains in the low-speed supernatant; the deficit appeared only after sedimentation of the mitochondrial pellet. In every case there was a single mitochondrial peak at $d = 1.19$. This observation excludes the interpretation that the mutant cells contain less mitochondria or are more difficult to extract.

Table 1. *Respiratory characteristics of mutant and wild-type mitochondria*

Mutant: O₂^R_{4.5} (locus OLI 1). Q_{O₂} (μ l/h per mg of protein): Ethanol: ratio of wild-type/mutant in five pairs of experiments; 1.4 $\pm$ 0.2 (s.e.). α -Oxoglutarate: ratio of wild-type/mutant in 14 pairs of experiments; 1.2 $\pm$ 0.1 (s.e.). Succinate: ratio of wild-type/mutant in ten pairs of experiments; 1.0 $\pm$ 0.1 (s.e.). For the other substrates, means of two different experiments are indicated. ADP/O and respiratory-control quotient: Where the standard error is not indicated (numbers of different experiments in parentheses), means of two experiments are given. By 'different experiments' we mean different mitochondrial preparations.

		Q _{O₂}				
		NADH-linked substrates				
		α -Oxoglutarate	Ethanol	Citrate	Pyruvate + L-malate	Succinate
Wild-type		88	100	76	53	158
Mutant		74	65	61	53	164
		ADP/O quotient				
Wild-type		2.2 $\pm$ 0.2 (11)	1.6	1.6	1.7	1.5 $\pm$ 0.2 (9)
Mutant		2.2 $\pm$ 0.3 (12)	1.6	1.6	1.5	1.4 $\pm$ 0.1 (9)
		Respiratory-control quotient				
Wild-type		3.0 $\pm$ 0.6 (11)	1.7	1.5	1.4	1.7 $\pm$ 0.2 (9)
Mutant		2.7 $\pm$ 0.5 (12)	1.5	1.5	1.5	1.7 $\pm$ 0.2 (9)

Fig. 3. H^+/O ratios plotted against $t_{1/2}$. (a) Wild-type; (b) O_{LI1} mutant (locus OLI 1). Abbreviations for substrates: C, citrate; S, succinate; P+M, pyruvate+L-malate; OG, α -oxoglutarate; Eth, ethanol; End, endogenous substrate; P, pyruvate. Correlation coefficients (r) for H^+/O - $t_{1/2}$ pairs were calculated for each substrate, as well as Student's (t) test for a 5% confidence limit:

	Wild-type			Mutant		
	r	t	5% limit	r	t	5% limit
α -Oxoglutarate	-0.89	8.9	2.05	-0.27	1.4	2.07
Citrate	-0.55	2.4	2.23	—	—	—
Ethanol	0.30	1.0	2.23	-0.45	1.1	2.30
Succinate	-0.06	0.24	2.10	0.50	2.0	2.25
Endogenous	0.06	0.16	2.40	0.26	0.7	2.40

Each H^+/O - $t_{1/2}$ pair represents one O_2 pulse, and, in general, four successive pulses were given per mitochondrial preparation.

Table 2. H^+/O and t_4 values

Mutant: $O_2^F_{46}$ (locus OLI 1). Standard errors are indicated for the H^+/O values. The number of O_2 pulses, in general four per mitochondrial preparation, are given in parentheses. All results were pooled to calculate the means.

	Wild-type		Mutant	
	H^+/O	t_4 (s)	H^+/O	t_4 (s)
Citrate	2.0 ± 0.5 (12)	13	2.1 ± 0.6 (5)	10
Pyruvate+L-malate	2.8 ± 0.3 (4)	19	—	—
Succinate	2.4 ± 0.3 (18)	21	2.2 ± 0.2 (11)	17
α -Oxoglutarate	4.1 ± 0.6 (24)	27	3.4 ± 0.5 (25)	27
Ethanol	4.5 ± 0.4 (12)	48	4.0 ± 0.3 (10)	44
Endogenous substrate	4.1 ± 0.6 (9)	53	4.1 ± 0.6 (9)	56
Pyruvate	2.9 ± 0.2 (4)	61	—	—

Characteristics of isolated mitochondria

Electron micrographs of isolated mitochondria (Plate 1) show relatively pure preparations and no obvious difference in size distribution or integrity between wild-type and mutant.

Fig. 2 shows typical traces that illustrate respiratory control and the effects of oligomycin. It is clear that the mitochondria from wild-type and mutant have very similar energy parameters (see also Table 1). By contrast, the coupling of mutant mitochondria is clearly more resistant to oligomycin than in case of the wild-type. Higher concentrations of oligomycin (e.g. $20 \mu\text{g}/\text{mg}$) completely abolish respiratory control for both strains. Thus, although the mutation implies a significant loss of mutant mitochondria during the preparation, the mitochondria recovered from the mutant have conserved a coupling integrity similar to the wild-type, as well as the property of partial oligomycin-resistance previously established at the level of the hydrolytic ATPase reaction. These conditions were necessary prerequisites for the respiratory-chain-driven H^+ -translocation studies.

Fig. 3 shows H^+/O as a function of t_4 for various substrates, although it is unusual to plot these two parameters on the same graph. We found it necessary to use this representation because it appeared clearly, in the case of the wild-type (Fig. 3a), in particular for α -oxoglutarate, that there was an inverse correlation between H^+/O and t_4 . As t_4 was found to be quite variable in different experiments, a meaningful comparison between substrates, and particularly between wild-type and mutant mitochondria had to be carried out by considering both parameters simultaneously. Table 2 shows the mean values calculated for the translocation parameters.

Discussion

Mutants affected at the OLI1 or OLI2 locus of mitochondrial DNA have an oligomycin-resistant mitochondrial ATPase. This raises several questions: for example, does the presence of such mitochondria

affect cell growth and metabolism?, are there problems in isolating the mitochondria?, and what are the bioenergetic characteristics of the mitochondria once isolated?

The fact that there is a disadvantage at the cellular level is demonstrated by the decreased growth rate under suboptimal culture conditions with an oxidizable but not with a fermentable carbon source. Isolation problems arise in the case of the mitochondria from the mutant at locus OLI 1, even when the cells were grown in rich culture conditions where there is no difference in growth rate; there is a systematic decrease of some 35% in the mitochondrial yield, as well as a corresponding increase in the proportion of mitochondrial phospholipids, indicating a preferential loss of protein (J. C. Kader, M. Krupa & M. Somlo, unpublished work; Somlo & Cosson, 1976).

In spite of the decreased yield, the mitochondria isolated from the mutant had bioenergetic characteristics very similar to those of the isonuclear wild-type, no significant difference being found in either the ADP/O or the respiratory-control ratios for several substrates. However, there was a slightly decreased respiration rate with NADH-mediated substrates with the possible exception of pyruvate, but clearly no decrease with substrates such as succinate and D-lactate that are not NADH-linked (Table 1). The decreased respiration rates with NADH-linked substrates for the mutant mitochondria suggest the interesting possibility that the NADH dehydrogenase is located in the coupling membrane sufficiently near to the ATPase 'membrane factor' to be functionally influenced by the alteration in the ATPase. Another possibility would be that the NADH dehydrogenase is inhibited by the excess of phospholipid present in the mutant mitochondria (see above). It may be significant that the only exception to the lower respiration rate in the NADH-linked substrate series was pyruvate, respired at a lower rate than the others, implying that in this case the rate-limiting step was not the NADH dehydrogenase.

EXPLANATION OF PLATE I

Electron micrographs of isolated mitochondria

Isolated mitochondria from (a) wild-type, magnification 20000 $\times$, and from (b) oligomycin-resistant mutant (O_{146}^r , locus OLI 1), magnification 30000 $\times$. Pellets were fixed in 1% OsO_4 in 0.1 M- Na_2HPO_4 , pH 7.2, dehydrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

Notwithstanding these minor differences in respiratory rates, the fact that the ADP/O and respiratory-control quotients are similar for all substrates tested, emphasizes that the bioenergetic integrity of the mitochondria as isolated is comparable with that of the wild-type. However, this integrity is unlikely to extend to the total mitochondrial population in the mutant cell, since there is a characteristic loss of mitochondria at a precise point during the fractionation procedure. Consequently we suppose that cells of the mutant at locus OLI 1, carrying an oligomycin-resistant mitochondrial ATPase, may contain an unstable mitochondrial population. Under nutritionally rich growth conditions, no obvious energetic deficiency appears at the cellular level, but the mitochondrial instability is reflected by the partial loss during fractionation. However, under suboptimal nutritional growth conditions, where there are greater demands on the energy-conserving system, the mitochondrial instability results in a decreased growth rate. It is noteworthy that this 'mitochondrial instability' is characteristic of the mutation at the OLI 1 locus; no decreased mitochondrial yield was found for the mutant at the OLI 2 locus (O₁₄₄^R). Studies by Tzagoloff *et al.* (1976) concerning the 'spontaneous' dissociation of a hydrophobic subunit in the case of mutants at the OLI 1 locus may be relevant to the general instability that we observed with the same mutant at the mitochondrial level (Somlo & Cosson, 1976).

As mentioned above, the isolated mitochondria are reasonably competent in the bioenergetic sense. Further, they have retained their characteristic oligomycin resistance, not only with respect to the ATPase as previously shown, but also at the level of respiratory control. This opens up the possibility of their use to investigate the mechanism of oxidative phosphorylation, particularly in relation to the postulated proton-translocating nature of the ATPase (Mitchell & Moyle, 1968).

As a prerequisite to such studies, the experiments on respiratory-driven H⁺ translocation described in the present paper were carried out to determine whether the kinetics of the H⁺ backflow after O₂ pulsing were affected by the mutationally altered ATPase 'membrane factor', such as might occur if the membrane permeability was significantly different.

On the basis of the chemiosmotic hypothesis the similar ADP/O ratios in the mitochondria from mutant and wild-type should be reflected in similar stoichiometries for respiratory-driven H⁺ translocation (Mitchell & Moyle, 1967*b*), and it seemed important to determine whether this was so. Since the first coupling site is usually missing from *S. cerevisiae* mitochondria (Ohnishi *et al.*, 1966), the theoretical maximum ADP/O ratio for NADH-linked substrates as well as for succinate is 2. The

experimental ADP/O ratios for all these substrates were in fact close to 1.5, the higher ratio in the case of α -oxoglutarate being ascribed to substrate-linked phosphorylation. The chemiosmotic hypothesis based on a stoichiometry of 2H⁺/site would thus predict experimental H⁺/O values between 3 and 4.

We obtained mean H⁺/O values ranging between 2.3 and 4.7, depending on the substrates (Fig. 3 and Table 2). The mean results for ethanol, pyruvate and α -oxoglutarate are compatible with the predicted values, whereas those for citrate and succinate are lower than expected.

Further, it is clear from Fig. 3(*a*) that the mean H⁺/O values for α -oxoglutarate do not have the same significance as those calculated for the other substrates, since there is an inverse relationship between H⁺/O and *t*_½ values. By contrast, H⁺/O ratios are independent of *t*_½ values in the cases of the other substrates, as required for reliable measurement of H⁺-translocation stoichiometry (for correlation coefficients, see Fig. 3).

Before discussing this point further, two features of *t*_½ should be noted, one concerning the variability, the other relating to the characteristic *t*_½ associated with particular substrates.

First, the *t*_½ for each particular substrate shows considerable variability, not only between different mitochondrial preparations, but also within the same preparation. Although our method has been devised to allow measurement of H⁺ translocation under as stable pH conditions as possible, there remained a micro-oscillation of pH about an apparent steady state that might contribute to the *t*_½ variability without notably influencing the H⁺/O values. For instance if the mitochondria are slowly alkalinizing the medium, the H⁺ produced during an O₂ pulse should be carried back faster across the mitochondrial membrane than if the mitochondria were acidifying, owing to the greater transmembrane pH differential in the former case. Another, and probably related, source of *t*_½ variability might be a changing internal anionic composition of the mitochondria. Secondly, the above-described variability does not obscure a characteristic range of *t*_½ values associated with each substrate. In rat liver mitochondria *t*_½ values are strongly influenced by the composition of the medium, permeant anions apparently increasing the rate of Δ pH decay (Mitchell & Moyle, 1967*b*) by their translocation through the membrane in association with H⁺ ions or in exchange against OH⁻ ions (Chappell & Haahrhoff, 1967). It is significant that in the present work the shortest *t*_½ occurs with the tri- and di-carboxylic acids and the longest *t*_½ with the monocarboxylic acid pyruvate, with the endogenous substrate, which does not have to cross the membrane, and with ethanol, which has no H⁺-carrying capacity. In the case of pyruvate we have demonstrated (Fig. 3*a*) that the addition of L-malate considerably decreases

t_4 without changing the H^+/O . These results indicate that after each O_2 pulse the substrate anions, even at as low a concentration as 1 mM, are the principal effectors of H^+ backflow; the t_4 thus reflects the permeability characteristics of the membrane towards the particular substrate and the substrate's proton-carrying ability.

Both citrate and succinate rapidly permeate the membrane by specific exchange diffusion carriers and probably also by other mechanisms (Harris, 1968; Kolarov *et al.*, 1972). This, together with their high H^+ -carrying capacity, can account for the short t_4 observed for these substrates and suggests that the extrapolated H^+/O values may be an underestimate of the real value, owing to the fast H^+ backflow. (The particularly short t_4 for citrate presumably accounts for the considerable scatter of H^+/O values.) Although succinate has been reported to be a suitable substrate for O_2 -pulse experiments with mammalian mitochondria at pH 7 (Mitchell & Moyle, 1967b), it should be used with caution in yeast mitochondria; here, in fact, the entry appears to be less specific (Harris, 1968), and the lower equilibrium pH (6–6.2) would also facilitate entry as the undissociated form.

In the case of α -oxoglutarate the H^+/O values range from as high as 5.6 to as low as 2.9 in inverse correlation to t_4 (15–38 s), as shown in Fig. 3(a) for the wild-type. A tentative scheme is proposed, taking into account the following facts: the entry of α -oxoglutarate is generally considered to depend on exchange diffusion with internal malate (De Haan & Tager, 1968). Further, as mentioned above, the mitochondrial anionic matrix composition presumably varies, for example with the observed micro-oscillations of pH. If there is a high internal malate concentration, fast penetration of α -oxoglutarate will occur, and the t_4 of H^+ backflow will be short. Under these conditions the internal α -oxoglutarate concentration will be high enough to allow substrate-level phosphorylation via the oxoglutarate dehydrogenase complex; consequently the resulting ATP will be present in a high-enough concentration at the site of the H^+ -translocating ATPase to be rapidly hydrolysed, resulting in an H^+ efflux that supplements the protons ejected by the H^+ -translocating respiratory chain, giving an artificially high H^+/O value. If, on the other hand, there is a low internal malate concentration, the penetration of α -oxoglutarate will be slower and the t_4 of H^+ backflow longer. Under these conditions, substrate-level phosphorylation will not result in high-enough local concentrations of ATP to allow significant H^+ translocation through the ATPase. In contrast with the wild-type, the mitochondria from the mutant show no apparent correlation between H^+/O and t_4 values for α -oxoglutarate. In fact, this is the only notable difference in the respiratory-chain-driven translocation pattern observed (Figs. 3a and 3b, and Table 2).

In terms of the above hypothesis this would suggest that the mutationally altered ATPase is not contributing to the apparent H^+/O ratio with α -oxoglutarate as substrate because, for example, it is not translocating the protons outwards, or because it is not hydrolysing the ATP.

The H^+ -translocation experiments described in the present paper underline the variables that influence the results, such as the particular properties of the biological material used, the methodology adapted to this material and the nature of the substrates. Consequently, there is a degree of uncertainty about the absolute stoichiometries associated with some of the substrates. Nevertheless the results show that, despite these limitations, the technique is a powerful analytical tool for the comparison of normal mitochondria with those mutationally altered at various points of the energy-conserving system. In the present case it has been shown that the mitochondria from an oligomycin-resistant mutant and from its isonuclear wild-type are sufficiently similar in their membrane integrity and bioenergetic characteristics to encourage further studies more directly associated with the proton-translocating ATPase.

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