IRON-LIMITED GROWTH OF TORULOPSIS UTILIS, AND THE REVERSIBLE LOSS OF MITOCHONDRIAL ENERGY CONSERVATION AT SITE 1 AND OF SENSITIVITY TO ROTENONE AND PIERICIDIN A

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1. Introduction

Comparative studies of mitochondria and submitochondrial particles from various yeasts have demonstrated certain differences between those with and those without phosphorylation coupled to electron transfer from $NADH_2$ to ubiquinone [1-13]. It is evident from such studies (table 1) that the presence or absence of site 1 phosphorylation is associated with changes of several other mitochondrial properties that are related to this region of the respiratory chain, and the true significance of these variations is obscured by the possibility that they are species differences that are otherwise unrelated to the mechanism of energy conservation at site 1. This particular drawback is overcome when comparative studies are confined to a single species, the problem then being to obtain adaptations to culture conditions, or mutations, that result in appropriate changes of mitochondrial function. This report is concerned with the effects of iron-limited growth of *T. utilis* cells and their mitochondria. Iron-limited growth causes loss of mitochondrial energy conservation at site 1, loss of sensitivity to rotenone and piericidin A, loss of a mitochondrial component that binds piericidin A, and marked falls in the mitochondrial content of cytochromes and non-heme iron. The rates of mitochondrial respiration are relatively unaltered.

2. Methods

T. utilis (N.C.Y.C. 193) was grown continuously in 0.5 L working volume chemostats of the "Porton" type [14], automatically controlled at 30° and pH 5.0. The dilution rate was 0.3 hr^{-1}. The iron-limited medium contained 137 mM glycerol; 12.8 mM NaH_2PO_4; 2.8 mM Na_2HPO_4; 75 mM NH_4Cl; 11.5 mM K_2SO_4; 1.0 mM citric acid; 1.25 mM $MgCl_2$; 100 μM $CaCl_2$; 50 μM $MnCl_2$; 10 μM $CoCl_2$; 5 μM $CuCl_2$; 5 μM H_3BO_3; 10 μM Na_2MoO_4; 4 μg biotin/L; and 0.25 ml. of antifoam FG (Midland Silicones Ltd.)/L. Water was distilled twice from an all glass apparatus, and the chemostat used for iron-limited growth had non-ferrous components in contact with the culture. Iron in this medium was derived from impurities in other reagents; its concentration was 0.7 μg atom Fe/L, and control experiments demonstrated that growth on this medium was iron-limited. When 100 μM $FeSO_4$ was added to the above medium growth was glycerol-limited.

Mitochondria were prepared from cells that had been collected over 15 hr at 0° and converted into spheroplasts [15]. In some experiments the cells grown on iron-limited medium were collected, washed, suspended in 200 μM $FeSO_4$ at a concentration of 0.5 g wet wt/ml at 30°, and bubbled with air for 15 hr prior to reharvesting and the preparation of mitochondria. There were therefore three types of cell and their derived mitochondria: (i) glycerol-limited or "G", (ii) iron-limited or "F", and (iii) iron-recovered or "FR" from treatment of F-cells with $FeSO_4$.

3. Results

These are summarized in table 2. In confirmation of earlier reports [1,4], mitochondria from cells

Table 1
Differences between the $NADH_2$ → ubiquinone segments of the respiratory chain of various yeasts.

	T. utilis	*S.carlsbergensis*	*S.cerevisiae*
Site 1 phosphorylation	Present [1]	Absent [2]	Absent [3]
Rotenone and Amytal sensitivity	Sensitive [4]	Insensitive [2]	Insensitive [5,6,7]
Piericidin A sensitivity	Sensitive (this paper)	Insensitive [8]	-
Flavin of $NADH_2$ oxidase	FMN and FAD [9]	-	FAD [6,10]
Ubiquinone species	UQ_7 [9,11]	UQ_6 [4]	UQ_6 [12]
ESR signal attributed to non-heme iron specifically reduced by $NADH_2$	Present [9]	-	Absent [10,13]

Table 2
Properties of mitochondria from glycerol-limited (G), iron-limited (F) and iron-recovered (FR) *T.utilis*.

		Mitochondria		
State 3 oxygen uptake (μg atom O/min/mg) with P/O ratios in parentheses (a)		G	F	FR
2-oxoglutarate		170 (3.7)	125 (2.9)	92 (3.8)
Malate with pyruvate		110 (2.6)	275 (1.9)	283 (3.0)
L-glycerol-3-phosphate		520 (1.5)	352 (1.9)	128 (1.9)
Inhibition of respiration of NAD-linked substrates (a) by piericidin A (100 μμmole/mg)		> 90%	zero	> 90%
rotenone (10 μM)		> 90%	zero	60%
Reduction of flavoprotein (b) or NAD (c) by glycerophosphate in state 4. Results are expressed as % of the extent in state 5, or as the time for half-completion in state 4.		130% $T½ < 1$ sec	10% = 30 sec	130% = 2 sec
High-affinity binding site for piericidin A (d)		Present	Absent	-
Cytochrome content (e) (mμmole/mg)	cyt. a, a_3	0.26	0.03	0.04
	cyt. C, C_1	0.83	0.08	0.04
	cyt. b	0.30	0.06	0.04
Non-heme iron content (f) (mμg atom Fe/mg)				
Released on sonication		21.0	1.8	-
Membrane bound		8.2	2.6	-
Acid-extractable flavin, membrane bound (g) (mμmole/mg)	FMN	0.018		0.017
	FAD	0.079		0.052

Experimental procedures were (a) Polarographic assay [21], with 5 mM substrates in the media of ref. 2. (b) measured fluorimetrically [23]. Most of the mitochondrial flavoprotein fluorescence is due to flavoprotein(s) at the redox level of NAD [19,20]. (c) Fluorimetric assay [24]. (d) Data of fig. 1. (e) Measured by split– or double-beam spectroscopy, using the wavelength pairs and extinction coefficients of ref. [22] for the aerobic minus anaerobic difference. (f) Non-heme iron was measured [27] in extracts [28] of the soluble and membranes mitochondrial fractions obtained by ultrasonic disruption and centrifugation. (g) Fluorimetric assay [29]. The values for oxygen uptake, P/O ratios and energy-dependent reduction of NAD were taken in each case from one preparation of mitochondria. Similar observations were made on at least four other preparations.

grown under "normal" conditions (i.e. G-cells and mitochondria) exhibited P/O ratios of approximately 4, 3 and 2 respectively for the oxidation of 2-oxoglutarate, pyruvate with malate, and L-glycerol-3-phosphate. The oxidation of NAD-linked substrates but not succinate, glycerophosphate or added $NADH_2$ was 90-95% inhibited by 10 μM rotenone. Piericidin A, considered to have a common site of action with rotenone [16,17,18] caused 90-95% inhibition of the oxidation of NAD-linked substrates at a concentration of 90 $\mu\mu$mole/mg protein. The small percentage of the respiration rate that was insensitive to piericidin A (but was sensitive to antimycin A), was due to the oxidation of endogenous substrate, and was absent in some preparations. Energy conservation at site 1 was also demonstrated by means of the energy-dependent reduction of mitochondrial NAD and flavoprotein during glycerophosphate oxidation. Further fluorimetric and spectrophotometric studies located the site of action of piericidin A in a similar region to that of rotenone [19,20], i.e. between the $NADH_2$ dehydrogenase flavoprotein and the group cytochrome b, ubiquinone and further flavoprotein [20].

By contrast, F-mitochondria had P/O ratios of approximately 3, 2 and 2 respectively for the oxidation of 2-oxoglutarate, pyruvate with malate, and glycerophosphate. Neither rotenone (10 μM) nor piericidin A (0.1 – 10 μM) inhibited the respiration of the above substrates. Reversal of electron transport at site 1 during state 4 glycerophosphate oxidation did not occur. Mitochondria from FR-cells resembled those from G-cells; both had energy conservation at site 1. Attempts to convert directly F-mitochondria into FR-mitochondria by incubation of F-mitochondria with $FeSO_4$ were unsuccessful.

The cytochrome and non-heme iron contents of F-mitochondria were several-fold lower than those of G-mitochondria. The ubiquinone species of F-mitochondria was UQ_7 with a trace of a higher homologue, and did not differ from that reported for normal *T.utilis* [9,11]. The acid-extractable FAD and FMN contents of F- and G-mitochondria were similar, as were the fluorescent characteristics of flavoproteins studied in the intact mitochondria.

Fig. 1 demonstrates the titration of G-mitochondria with piericidin A. The addition of bovine plasma albumin shifts the titration curve in a manner that is a measure of the binding of piericidin A to albumin.

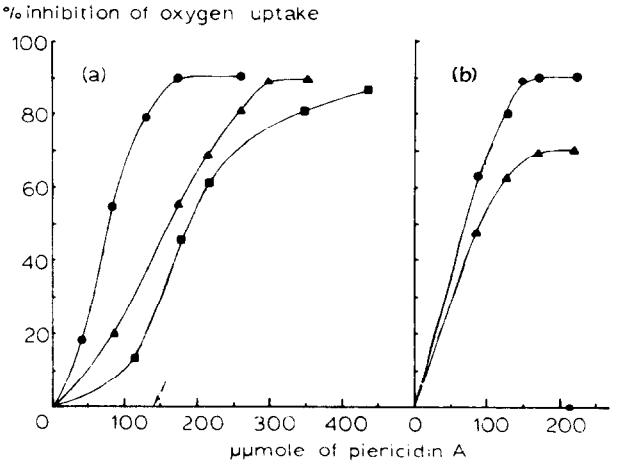

Fig.1. *Inhibition of oxygen uptake by piericidin A.* Mitochondrial oxygen uptake was measured polarographically in the presence of 5 mM pyruvate, 5 mM malate, 2 mM ADP and the phosphate buffer of ref. [2] in a final volume of 2.5 ml at 30°C. (a) ● 1.6 mg G mitochondria; ▲ 3.2 mg G mitochondria; ■ 1.6 mg G mitochondria with 20 mg bovine plasma albumin. (b) ● 1.3 mg G mitochondria; ▲ 1.3 mg G mitochondria with 1.1 mg F mitochondria; ■ 1.1 mg F mitochondria. *Abscissa:* $\mu\mu$mole of piericidin A. *Ordinate:* % inhibition of oxygen uptake.

The addition of F-mitochondria (which are insensitive to piericidin A) to G-mitochondria did not move the titration curve, although the apparent degree of inhibition was of course lowered. It can therefore be concluded that F-mitochondria do not compete with G-mitochondria for piericidin A. The interpretation is that a piericidin A binding site is present in G-mitochondria but absent in F-mitochondria.

Studies with whole cells demonstrated that whereas the oxygen uptake of G- and FR-cells was 50-70% inhibited by 0.3 mM rotenone, that of F-cells was insensitive. The cytochrome content of F-cells was approximately fivefold lower than that of G-cells.

Two important technical points must be mentioned. Firstly, the concentration of iron in the iron-limited growth medium is critical for obtaining these results. An increase from 0.7 μg atom up to 1.7 μg atom Fe/L resulted in a partial return of mitochondrial sensitivity to piericidin A. Secondly, the omission of automatic control at pH 5.0 results in growth at pH 1.0 and the production of cells that resist the spheroplast preparation procedure.

4. Discussion

These observations demonstrate that energy conservation but not electron transfer at site 1 is absent from F-mitochondria, and that this change is associated with the loss of sensitivity to rotenone and piericidin A. The recovery of these features following incubation of F-cells with $FeSO_4$ in the absence of an added carbon source makes it likely that the changes of the F-cells and their mitochondria are adaptive and not due to a genetically different cell population selected by the culture conditions. The loss and recovery of rotenone sensitivity by whole cells indicates that the changes observed in isolated mitochondria represent the *in vivo* situation rather than a preparative artefact. More detailed studies of FR mitochondria are deferred until the time course and optimal conditions for the recovery process have been established.

The simplest hypothesis to explain these observations is that there is normally a mitochondrial component that (i) is essential for energy conservation but not electron flow in the region between $NADH_2$ and ubiquinone, (ii) is responsible for the inhibition of respiration by piericidin A or rotenone, (iii) binds piericidin A, and has 90 $\mu\mu$mole of binding site per mg mitochondrial protein, (iv) is functionless and probably not synthesized under conditions of iron-limited growth, and (v) is a non-heme iron protein. It is apparent that these interpretations lean heavily on the studies of others with mammalian systems [25,26]. However, the novel experimental advantages offered by the iron-limited and iron-recovered *T.utilis* systems provide independent and otherwise unobtainable support for the tentative idea of a rotenone and piericidin A sensitive non-heme (and non-flavin) iron protein component in the segment $NADH_2 \rightarrow$ ubiquinone [26].

It has been proposed that the absence of energy conservation at site 1 in *S.carlsbergensis* or *S.cerevisiae* is due to the substitution of UQ_6 for UQ_7 [4], or FAD for FMN in the $NADH_2$ dehydrogenase [6,10]. Neither mechanism appears likely in the case of iron-limited *T.utilis*. The behaviour of G-, F- and FR-mitochondria towards piericidin A is also shown by their respective submitochondrial particles, and further studies should elucidate the relationship between the piericidin A sensitive component and the $g = 1.94$ ESR signal attributed to the $NADH_2$ dehydrogenase [9,10,13].

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