CHANGES OF MITOCHONDRIAL $NADH_2$ OXIDATION PATHWAYS IN *TORULOPSIS UTILIS* GROWN ON ACETATE

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1. Introduction

Mitochondria from *Torulopsis utilis* oxidize $NADH_2$ by two distinct routes: an "internal" $NADH_2$ dehydrogenase effects reduction of the respiratory chain cytochromes with intramitochondrially generated $NADH_2$, whereas an "external" dehydrogenase utilises added or extramitochondrial $NADH_2$ [1]. Oxidation of $NADH_2$ by the internal dehydrogenase involves both site I phosphorylation and the rotenone or piericidin A sensitive site, whereas these features are not involved in oxidation by the external dehydrogenase [1, 2]. A combination of the external dehydrogenase with an appropriate carbon shuttle for the exchange of reducing equivalents between intra- and extramitochondrial $NADH_2$ has been implicated [3] in the failure of rotenone or piericidin A to inhibit the growth of *T. utilis* (fig. 1). We wish to report that acetate-limited growth of *T. utilis* results in the loss of the external rotenone-insensitive $NADH_2$ dehydrogenase. In keeping with the scheme of fig. 1, cell growth on acetate can be completely inhibited by rotenone or piericidin A. A basis for screening for mutants resistant to these inhibitors is thereby established.

2. Methods

The growth of *T. utilis* (N.C.Y.C. 193), the medium for glycerol-limited growth, the preparation of mitochondria and polarographic measurements of oxygen uptake have been previously described [2]. For acetate-limited growth 240 mM sodium acetate replaced glycerol as the carbon source, and automatic control of the culture at pH 5.0 was effected with 2 M HCl. The dilution rate was $0.2\ hr^{-1}$. The yield of cells in the chemostat with acetate limitation was 2.5 mg dry wt/ml culture, and an overnight (12 hr) collection finally yielded about 60 mg mitochondrial protein. Growth of *T. utilis* on solid media was investigated with 2% (w/v) agar that contained the components of the chemostat medium at twice its concentration except that of the carbon source, which was unchanged. The pH was adjusted to 5.0. The inoculum for plates was prepared by inoculating 100 ml of a liquid medium (1% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract (w/v) at pH 6.0) with the stock culture of *T. utilis* N.C.Y.C. 193 (maintained on slopes at 0°), incubating at 30° for 24 hr, and diluting 2.0 ml of the resulting culture in 100 ml sterile water. The agar plates were inoculated with 0.4 ml of the diluted culture and inspected for growth after 48 hr at 30°. When inhibitors were included in the solid medium, they were added as methanolic solutions to the molten agar giving concentrations of 0–0.13 mM rotenone or 0–30 μM piericidin A.

3. Results

3.1. *Mitochondrial respiration*

Table 1 shows that mitochondria from cells grown on acetate as opposed to glycerol exhibit [1] a four-fold drop in the rate of L-glycerol-3-phosphate oxidation [2] oxidation of added $NADH_2$ with a similar P/O ratio and inhibitor sensitivity to that observed with $NADH_2$ generated intramitochondrially with pyruvate and malate.

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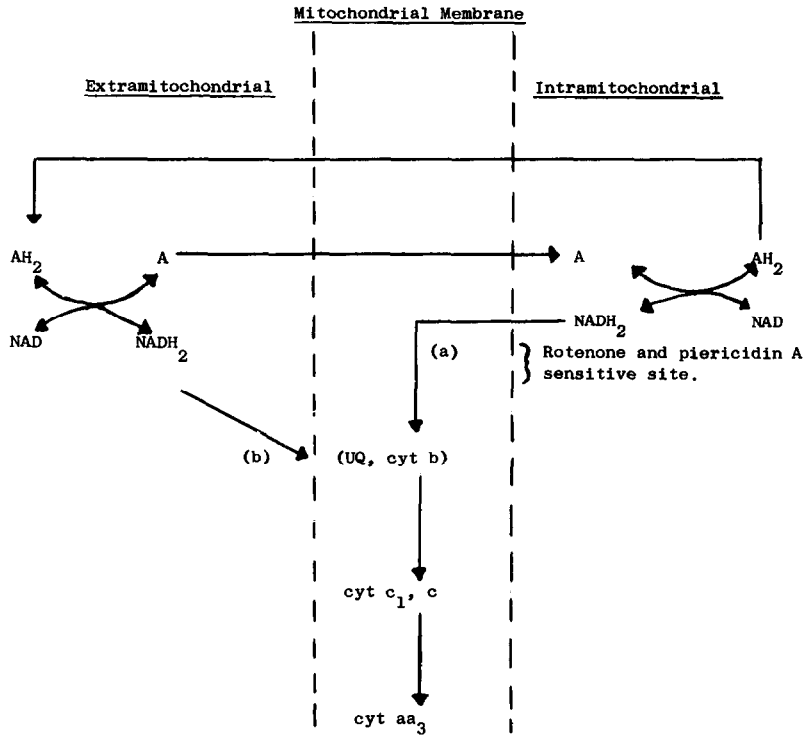

Fig. 1. Pathways of $NADH_2$ oxidation in *T. utilis*. A and AH_2 refer to the oxidized and reduced substrates of an NAD-dependent dehydrogenase, (e.g. alcohol dehydrogenase) which is located both intra- and extramitochondrially. Other enzymes or carriers are (a)-internal NADH dehydrogenase; (b)-external NADH dehydrogenase; UQ-ubiquinine, cyt *c*, *c*, *a*, a_3 - the respective cytochromes.

Table 1
Oxidative behaviour of mitochondria from acetate- or glycerol- grown *T. utilis*.

Carbon source	Substrate	Oxidation rate (natom/min/mg)	P/O ratio	% Inhibition of oxidation by rotenone (10 μM) or piericidin A (0.1 μM)
Glycerol	Pyruvate (5 mM) L-Malate (5 mM)	117 ± 22 (5)	3.2 ± 0.2 (5)	80–90%
Acetate	Pyruvate (5 mM) L-Malate (5 mM)	110 ± 34 (6)	3.2 ± 0.4 (5)	80–90%
Glycerol	L-Glycerol-3-phosphate (5 mM)	348 ± 73 (4)	1.77 ± 0.20 (4)	zero
Acetate	L-Glycerol-3-phosphate (5 mM)	97 ± 11 (4)	not measured	zero
Glycerol	$NADH_2$ (0.5 mM)	373 ± 32 (4)	2.0 ± 0.2 (3)	zero
Acetate	$NADH_2$ (0.5 mM)	207 ± 32 (6)	2.8 ± 0.6 (5)	80–90%

The polarographic technique and composition of the incubation medium have been described elsewhere [2]. Results are expressed as the mean of several separate preparations (number in parentheses) ± S.E.M. In the case of mitochondria from acetate grown cells the respiratory control ratio for L-glycerol-3-phosphate oxidation was too low to calculate the P/O ratio.

3.2. *Cell growth on solid media*

When glycerol was the carbon source, cell growth at 48 hr was not visibly altered by the presence of 0.26 mM rotenone or 60 μM piericidin A. With acetate as the carbon source, growth was completely inhibited by 0.13 mM rotenone or 12 μM piericidin A and about 50% inhibited by 13 μM rotenone or 1.2 μM piericidin A.

4. Discussion

The failure of rotenone or piericidin A to inhibit growth of *T. utilis* on solid media has been an obstacle in the development of procedures that screen for mutants resistant to these inhibitors. Clearly, such mutants would be of value in characterizing the as yet unidentified inhibitor-sensitive site and its relationship to other features of the $NADH_2$ to cytochrome segment of the respiratory chain. However, the introduction of acetate as the carbon source permits detection of inhibitor resistant mutants on solid growth media [4].

The loss of the rotenone insensitive "external" dehydrogenase from acetate grown cells and the emergence of cell growth sensitivity to the inhibitors are consistent with the rotenone inhibition by-pass of fig. 1. This by-pass allows intramitochondrial $NADH_2$ to be oxidized by the respiratory chain in the presence of rotenone, and it also by-passes the energy conservation process at site 1. Evidence against this pathway operating in the absence of rotenone comes from the observation that the presence of site 1 phosphorylation in isolated mitochondria is correlated with a 25–30% increased growth yield of cells in continuous culture, when compared to that of iron deficient cells that lack site 1 [5]. It seems likely, therefore, that controls normally exist to inhibit the by-pass.

The mechanism whereby mitochondria from acetate grown cells oxidize $NADH_2$ in a piericidin A sensitive manner is unknown. Indeed, the observations are surprising since they imply either that added $NADH_2$ penetrates the mitochondrial membrane (which is apparently not the case with glycerol grown cells) or that a novel route of hydrogen transfer has emerged.

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References

[1] T.Ohnishi, G.Sottocasa and L.Ernster, Bull. Soc. Chim. Biol. 48 (1966) 1189.
[2] P.Ann Light, C.I.Ragan, R.A.Clegg and P.B.Garland, FENS Letters 1 (1968) 4.
[3] P.B.Garland, R.A.Clegg, P.Ann Light and C.I.Ragan In:Inhibitors. Tools in cell research, eds. Th.Bücher and H.Sies, 20th Mosbacher Colloquium (Springer, Berlin, Heidelberg, New York, 1969) p. 217.
[4] P.Ann Light, unpublished observations.
[5] R.A.Clegg, C.I.Ragan, B.A.Haddock, P.Ann Light, P.B. Garland, J.A.Swann and R.C.Bray, FEBS Letters 5 (1969) 207.