STUDIES OF ENERGY-LINKED REACTIONS: DIHYDROLIPOATE- AND OLEATE-DEPENDENT ATP SYNTHESIS IN YEAST PROMITOCHONDRIA

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1. Introduction

A role for lipoic acid residues in oxidative phosphorylation has been demonstrated in yeast and heart mitochondrial preparations and a requirement for a unsaturated fatty acid as a cofactor has been demonstrated in ATP synthase preparations [1–4]. It is proposed that the terminal reactions of oxidative phosphorylation are analogous to substrate-level phosphorylation, with lipoic acid residues providing a functional link (energy-transfer system) between the respiratory chain and the ATP synthase complex. In these experiments the respiratory chain was inhibited by rotenone and antimycin A which apparently inhibit the contribution of the respiratory chain. However, in a complex membrane preparation it may not be possible to inhibit all the functional capacity of the respiratory chain and thus evaluate the role of all respiratory chain components.

Yeast promitochondria from anaerobically grown cells do not possess a functional respiratory chain and lack the cytochrome system and ubiquinone as shown by Criddle and Schatz [5]. However, promitochondria still retain a functional energy-transfer system as evidenced by an oligomycin sensitive ATPase and an uncoupler-sensitive P_i–ATP exchange reaction [6] and thus provides an experimental system for evaluating the role of lipoic acid in the energy-transfer system which is still present in these organelles. In addition, the unsaturated fatty acid (UFA) composition of promitochondria can be manipulated by adjustment of the UFA content of the growth medium and thus provides a system for evaluation of the role of unsaturated fatty acids.

2. Materials and methods

The source of many of the chemicals and reagents used have been described previously [7]. DL-α-lipoic acid, DL-dihydrolipoic acid, oleic acid, oleoyl CoA, palmitic acid, palmitoyl CoA, Tween-80, ADP and hexokinase were all obtained from Sigma Chemical Co. (London). DL-dihydrolipoic acid was dissolved in 0.25 M sucrose, 10 mM Tris–Cl pH 7.5, 1 mM EDTA titrated with 1 M Tris base to pH 7.5–8.0. DL-α-lipoic acid was dissolved in methanol. Oleoyl-*S*-lipoate was prepared from dihydrolipoate and oleoyl chloride as described previously [3] and dissolved in dimethylformamide. The preparation is approx. 70% pure and contains oleic acid and lipoic acid.

A haploid strain (D22) of *Saccharomyces cerevisiae* was used and its anaerobic growth with or without UFA supplements, the isolation of promitochondria in the presence of cycloheximide [5] and gradient purification of promitochondria, were as previously described [8]. UFA supplements were added in the form of Tween-80 (polyoxyethylene sorbitan monooleate) as a source of oleic acid. Total promitochondrial fatty acids were determined by gas chromatography of the methyl esters, methylation being carried out in 0.5 M HCl–methanol at 60°C for 2 h. The methyl esters were analysed on a Perkin-Elmer F-11

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instrument with a polyethylene glycol succinate column operating at 180°C.

ATP synthesis was assayed in a glucose-hexokinase trap system by the disappearance of inorganic phosphate [3]. The reaction medium contained 1 mg promitochondrial protein, 250 mM sucrose, 22 mM glucose, 2 mM $MgCl_2$, 0.2 mM EDTA, 2 mM ADP, 20 mM Tris–Cl, pH 7.3 and 25 units of hexokinase. Total vol. 1.1 ml. The promitochondria were preincubated for 5 min with the inhibitors and cofactors as indicated, prior to initiation of the assay by addition of dihydrolipoate. Incubation was for 20 min at 30°C by shaking in air. Disappearance of inorganic phosphate was shown to correlate with glucose-6-phosphate formation.

3. Results

Yeast promitochondria do not contain the cytochrome and ubiquinone components of the respiratory chain and so cannot respire or phosphorylate with pyruvate/malate, succinate or ascorbate/TMPD as substrates as shown previously [5,6]. Table 1 shows that ATP synthesis driven by dihydrolipoate occurs in promitochondria from UFA-supplemented cells without any requirement for additional added cofactors. The reaction is sensitive to oligomycin and uncoupling agents such as FCCP and 1799, thus demonstrating that a typical mitochondrial energy-transfer system is still present in yeast promitochondria as indicated in previous studies [5,6].

A requirement for an unsaturated fatty acid in the energy-transfer reactions of promitochondria can be demonstrated by manipulating the UFA composition of promitochondria from anaerobically grown cells.

Table 2 shows the fatty acid composition of promitochondria from UFA-supplemented cells (grown in the presence of 4 mg/ml Tween-80). These promitochondria contain 68% unsaturated fatty acids and are capable of dihydrolipoate-dependent ATP synthesis in the absence of added cofactors as shown in table 1. UFA-depleted promitochondria which contain only 8% unsaturated fatty acids do not synthesise ATP with dihydrolipoate alone (table 3). This result is similar to that obtained with ATP synthase preparations from heart and yeast mitochondria

Table 1
ATP synthesis by promitochondria isolated from UFA-supplemented anaerobically grown yeast

Additions	Δ μmol P_i/20 min
1. None	0.00
2. Succinate (15 μmol)	0.00
3. Dihydrolipoate (2.0 μmol)[a]	1.99
4. Dihydrolipoate + oligomycin (2 μg)	0.00
5. Dihydrolipoate + oleate (10 nmol) + oleoyl CoA (10 nmol)	1.96
6. Dihydrolipoate + palmitate (10 nmol) + palmitoyl CoA (10 nmol)	1.95
7. Oleoyl-*S*-lipoate (1 μmol)	1.05
8. Oleoyl-*S*-lipoate + oligomycin	0.00

[a] In the presence of uncouplers (5 μg FCCP or 5 μg 1799) no phosphorylation was observed

Control experiments with additions of oleate, oleoyl CoA, palmitate and palmitoyl CoA showed no phosphorylation.

ATP synthesis was assayed in a glucose–hexokinase trap system as described in Materials and methods. Similar results are obtained in the presence of antimycin A and rotenone. Promitochondria were suspended in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.5, at 10 mg protein/ml. An aliquot (1 mg protein) was added to the phosphorylation medium and the reaction initiated by addition of dihydrolipoate. The concentrations of various additions are those which are first indicated.

Table 2
Composition of membrane lipids of promitochondria from anaerobically grown yeast

UFA addition Tween-80 (mg/ml)	Wt. % of total fatty acids								
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{16:1}$	$C_{18:1}$	Total UFA (%)
0	1.8	16.5	15.2	13.6	38.3	6.7	4.8	3.1	7.9
4	trace	0.5	0.5	2.0	23.7	5.6	14.5	53.2	67.7

Estimation of fatty acid content of promitochondria was as described in Materials and methods.

where a specific requirement for unsaturated fatty acids has been demonstrated [3]. ATP synthesis in promitochondria from UFA-depleted yeast cells can be restored by addition of oleic acid and oleoyl CoA (table 3) as is found in the case of detergent-dispersed ATP synthase preparations.

It should be noted that ATP synthesis occurs in both UFA-depleted and UFA-supplemented promitochondria when oleoyl-*S*-lipoate is used as the substrate for the reaction. This reaction is also sensitive to oligomycin and uncoupling agents as found in other mitochondrial systems. Saturated fatty acids and acyl CoA derivatives are potent inhibitors of ATP synthesis in UFA-depleted promitochondria (table 3) but they have little or no effect on the reaction catalysed by UFA-supplemented promitochondria (table 1). A similar pattern of inhibition is observed in respiratory-competent mitochondria and submitochondrial particles where the reaction is not inhibited by palmitate or palmitoyl CoA but marked inhibition of ATP synthesis catalysed by ATP synthase (OS–ATPase) preparation by palmitate and palmitoyl CoA is observed. It is apparent that in any system where there is a requirement for an added unsaturat-

Table 3
ATP synthesis by promitochondria isolated from UFA-depleted anaerobically grown yeast

Additions	Δ μmol P_i/20 min
1. None	0.00
2. Succinate (15 μmol)	0.00
3. Dihydrolipoate (1 μmol)	0.00
4. Dihydrolipoate + oleate (10 nmol)	0.00
5. Dihydrolipoate + oleate + oleoyl CoA(10 nmol)[a]	1.48
6. Dihydrolipoate + oleate + oleoylCoA + oligomycin (2 μg)	0.00
7. Dihydrolipoate + palmitate (10 nmol) + oleoyl CoA(10 nmol)	0.00
8. Dihydrolipoate + oleate + palmitoyl CoA	0.00
9. Oleoyl-*S*-lipoate (1 μmol)	1.05
10. Oleoyl-*S*-lipoate + oligomycin	0.00

[a] In the presence of uncouplers (5 μg FCCP or 5 μg 1799) no phosphorylation was observed

Control experiments with additions of oleate, palmitate, palmitoyl CoA, dihydrolipoate + oleoyl CoA, dihydrolipoate + palmitate, dihydrolipoate + palmitate + palmitoyl CoA, dihydrolipoate + palmitoyl CoA were inactive.

Phosphorylation was assayed as described in table 1 by uptake of inorganic phosphate in a glucose-hexokinase trap system. Similar results are obtained in the presence of antimycin A and rotenone.

ed fatty acid (UFA-depleted promitochondria and ATP synthase) due to depletion of the normal UFA content that saturated fatty acids and acyl CoA derivatives are potent inhibitors.

4. Discussion

The demonstration of dihydrolipoate dependent ATP synthesis in promitochondria supports the previous findings by Schatz and coworkers [5,6] that these organelles possess the energy transfer system of oxidative phosphorylation, despite the absence of a functional respiratory chain. It is significant that all energy conservation systems which have been examined, even those which previously have been demonstrated to retain only functions such as oligomycin sensitive ATPase activity and/or P_i–ATP exchange activity, catalyse inhibitor and uncoupler sensitive dihydrolipoate dependent ATP synthesis [2–4].

Previous experiments indicate that the respiratory chain is not necessary for dihydrolipoate-dependent ATP synthesis [3,4] as the reaction occurs in the presence of rotenone and antimycin A and the present experiments with promitochondria appear to confirm that the cytochromes and ubiquinone are not involved. However, the redox reactions involved in dihydrolipoate-dependent ATP synthesis have not been characterised nor has the redox couple which is the electron acceptor in this reaction. As promitochondria contain flavoprotein and dehydrogenase systems and it is possible that non-cytochrome components of complex III and complex IV may exhibit redox functions it cannot be assumed that all redox functions of the respiratory chain have been eliminated. Furhter investigation is required before it can be established that all the components required are contained in the ATP synthase (OS–ATPase) complex or whether relevant reactions such as disulphide reduction or reductive acylation of disulphides are constitutive reactions of respiratory chain complexes such as complex III and complex IV.

The demonstration of dihydrolipoate dependent ATP synthesis by promitochondria raises the question as to whether the reaction is of metabolic significance in the anaerobic yeast cell. Reduction of lipoate by a suitable dehydrogenase or flavoprotein-linked dehydrogenase coupled to an acceptor such as fumarate is a possible ATP generating system and similar reactions are of major metabolic significance in anaerobic bacteria where the membrane ATPase has been assumed to have a proton pumping function only [9]. A possible role for ATP-generating reactions in promitochondria and in yeast 'petite' mitochondria has been discussed by Kovac [10] on the basis of sensitivity to inhibitors and studies of cytochrome deficient mutants. Detailed examination of dihydrolipoate-dependent, oleoyl-*S*-lipoate-dependent and oleoyl phosphate-dependent ATP synthesis [3,4] in mitochondrial 'petite' mutants, nuclear 'petite' mutants and mitochondrial *mit*$^-$ mutants with specific deletions in cytochrome content and ATPase subunits should be of value in determining the components involved as well as establishing their biogenetic origin.

The results in tables 1 and 3 relating to promitochondria from UFA-supplemented and UFA-depleted cells provide further support for a specific cofactor role for an unsaturated fatty acid in the terminal reactions of oxidative phosphorylation. A detailed investigation of the specificity of the requirement for unsaturated fatty acids in UFA-depleted promitochondria has not been made but studies with ATP synthase preparations from heart mitochondria and *E.coli* indicate a high degree of specificity for *cis*,Δ-9-monoenoic acids (D. E. Griffiths, R. L. Hyams and M. Carver, unpublished studies). A similar specificity has been reported by Walenga and Lands [11] in their studies of the growth yield of an unsaturated fatty acid auxotroph [12] on a variety of unsaturated fatty acids. The specific requirement for an unsaturated fatty acid for ATP synthesis in promitochondria reported here and the previous demonstration of a requirement for an unsaturated fatty acid for ATP synthesis by ATP synthase preparations [3,4] provide an explanation for the inability of UFA auxotrophs of *E.coli* [13] and yeast [11] to grow on oxidisable substrates as well as the effects of UFA depletion on oxidative phosphorylation in mitochondria from the UFA auxotroph KD115 [14].

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