STUDIES OF ENERGY LINKED REACTIONS: A COFACTOR FUNCTION FOR UNSATURATED FATTY ACIDS IN OXIDATIVE PHOSPHORYLATION; STUDIES WITH A YEAST AUXOTROPH

David E. Griffiths, Robert L. Hyams, Enrico Bertoli and Mark Carver

Department of Molecular Sciences, University of Warwick, Coventry CV4 7AL, U. K.

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Summary

Mitochondria from the yeast unsaturated fatty acid auxotroph KD115 when grown with a high unsaturated fatty acid supplement catalyse normal oxidative phosphorylation and dihydrolipate-dependent ATP synthesis in the absence of added cofactors. Mitochondria from unsaturated fatty acid depleted KD115 cells have half the unsaturated fatty acid content of supplemented cells and do not catalyse oxidative phosphorylation and dihydrolipoate dependent ATP synthesis. Dihydrolipoate-dependent ATP synthesis can be restored specifically by addition of cofactor amounts of oleic acid and oleoyl CoA. The results provide further evidence for a cofactor role for an unsaturated fatty acid in oxidative phosphorylation.

A role for unsaturated fatty acids (UFA) in mitochondrial oxidative phosphorylation has been indicated from a variety of studies. Nutritional studies have shown that unsaturated fatty acid deficiency in experimental animals leads to a lowered growth rate and to lowered P/O ratios in isolated mitochondria [1], while unsaturated fatty acid auxotrophs of Escherichia coli [2, 3] and yeast [4] exhibit deficiencies in aerobic growth on glucose and on oxidisable substrates. The yeast unsaturated fatty acid auxotroph KD 115 [4] has been extensively investigated and the unsaturated fatty acid level of the mitochondrial membrane lipids has been shown to determine the capacity of yeast cells to catalyse oxidative phosphorylation [5, 6]. The ability of KD115 yeast cells to grow on non-fermentable substrates is lost below a 20%

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unsaturated fatty acid content [5] and P/O ratios in isolated mitochondria drop to less than 0.1 when the unsaturated fatty acid content of mitochondrial lipids drops to 30 - 35% [5, 6]. Other studies [7, 8, 9] have shown a specificity for Δ 9, cis monoenoic acids for restoration of aerobic growth and maximal growth yield and the development of mitochondrial respiration in yeast KD115 cells. The effects of unsaturated fatty acid depletion on oxidative phosphorylation have been attributed by Linnane & coworkers [5, 6] to changes in the physical properties of mitochondrial membranes leading to altered proton permeability, loss of membrane proton gradient and collapse of the membrane potential. The studies by Lands & coworkers [7, 8, 9] while indicating a structural role for unsaturated fatty acids also point to a metabolic function as a result of growth inhibition studies with trans isomers of unsaturated fatty acids.

Studies in this laboratory [10, 11, 12] indicate a specific cofactor role for an unsaturated fatty acid in the terminal reactions of mitochondrial oxidative phosphorylation. Dihydrolipoate-dependent ATP synthesis catalysed by purified detergent dispersed preparations of ATP synthase (OS-ATPase) from heart, yeast and E. coli have been shown to have a specific requirement for oleic acid plus oleoyl CoA which can be met with varying degrees of efficiency by other cis Δ 9-unsaturated fatty acids [10, 11, 12; D. E. Griffiths, R. L. Hyams & M. Carver, unpublished work]. In addition, oleoyl-S-lipoate and oleoyl phosphate have been shown to be substrates for ATP synthesis catalysed by ATP synthase and mitochondrial preparations [10, 11, 12], thus providing further evidence for a cofactor role for an unsaturated fatty acid (oleic acid).

This paper describes studies with a yeast unsaturated fatty acid auxotroph KD115 [4] grown under conditions of low unsaturated fatty acid supplementation which lead to loss of mitochondrial oxidative phosphorylation

Table 1 Fatty acid composition of mitochondria from yeast mutant KD115 grown on low and high supplements of unsaturated fatty acid

Extraction of fatty acids and determination of mitochondrial fatty acid content was as described previously (14).

TWEEN-80 (mg/ml) in growth medium	wt. % of total mitochondrial fatty acids							
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{16:1}$	$C_{18:1}$
4.0 (high)	trace	0.4	0.9	1.5	22.5	2.8	14.6	57.3
0.4 (low)	trace	4.2	5.4	6.5	43.2	6.4	3.8	30.5

[5, 6]. Mitochondria from these cells do not catalyse dihydrolipoate dependent ATP synthesis and a specific requirement for an unsaturated fatty acid (oleic acid) for ATP synthesis is demonstrated.

Materials and Methods

The sources of chemicals and reagents used have been described previously [10, 11] as have the preparation of solutions of lipoate and dihydrolipoate and the synthesis of oleoyl-S-lipoate and oleoyl phosphate [11]. Dihydrolipoate-dependent ATP synthesis was assayed in a glucose-hexokinase trap system at 30°, using rotenone and antimycin A inhibited mitochondria, by measurement of disappearance of inorganic phosphate and/or glucose-6-phosphate formation as described previously [10, 11] and in the legend to Table 2.

The unsaturated fatty acid auxotroph KD115 was grown aerobically on glucose with supplements of unsaturated fatty acid as described previously [13]. Unsaturated fatty acid supplements were added to the growth medium in the form of Tween 80 (polyoxyethylene sorbitan mono-oleate) as a source of oleic acid at 4 mg Tween 80/ml (high supplement) or 0.4 mg Tween 80/ml (low supplement). Mitochondria from cells grown on high unsaturated fatty acid supplement had normal P/O ratios with succinate as substrate whereas mitochondria from cells grown on low unsaturated fatty acid supplement had P/O ratios of less than 0.1 as shown by Linnane & coworkers [5, 6,]. The methods for isolation of mitochondria and the determination of mitochondrial fatty acids were as described previously (14). The fatty acid composition of mitochondria is summarised in Table 1.

Table 2 Dihydrolipoate-dependent ATP synthesis by mitochondria from strain KD115 grown on high unsaturated fatty acid supplement

ATP synthesis was estimated in a glucose-hexokinase trap system at 30° (11). Mitochondria from KD115 yeast grown on glucose with high UFA supplement were inhibted with antimycin A (2 μg/mg protein) and rotenone (2 μg/mg protein) after suspension in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA at 10 mg protein/ml. A 0.1 ml aliquot (1 mg protein) was taken into 1.0 ml of phosphorylation medium containing 250 mM sucrose, 20 mM glucose, 2 mM $MgCl_2$, 0.5 mM EDTA, 2 mM ADP, 6.6 mM potassium phosphate, 20 mM Tris-Cl buffer pH 7.3, hexokinase 25 units plus various inhibitors and additions. The reaction was initiated by addition of dihydrolipoate (2 μmol) and after shaking in air for 20 min, samples were removed for estimation of inorganic phosphate and glucose-6-phosphate (11). The fatty acid additions were 10 nmol each.

ADDITIONS	ATP synthesis/20 min	
	$\Delta\mu molP_i$	$\Delta\mu molG6P$
1. None	0.0	0.0
2. Dihydrolipoate	2.8	2.4
3. Dihydrolipoate + oligomycin (5 μg)	0.1	0.0
4. Dihydrolipoate + oleate*	2.77	2.80
5. Dihydrolipoate + linoleate	2.63	2.40
6. Dihydrolipoate + linolenate	2.54	2.40
7. Dihydrolipoate + palmitate*	2.59	2.90
8. Dihydrolipoate + arachidonate	2.64	2.75
9. Lipoic acid (2 μmol)	0.0	0.0

* Palmitoyl CoA (10 nmol) addition did not inhibit ATP synthesis.

Results and Discussion

Mitochondria isolated from KD115 yeast cells grown with a high unsaturated fatty acid supplement have a normal oxidative phosphorylation capacity [5, 6,] and catalyse dihydrolipoate-dependent ATP synthesis in a rotenone and antimycin A inhibited system, (Table 2). There is no requirement for additional cofactors and the reaction is similar to that catalysed by heart, liver and yeast mitochondria and submitochondrial particles [10, 11, 12]. The reaction is not inhibited by saturated fatty acids, saturated fatty acyl CoA's and other unsaturated fatty acids. As mitochondria from KD115 yeast

Table 3 Dihydrolipoate-dependent ATP synthesis by mitochondria from yeast mutant KD115 grown on low unsaturated fatty acid supplement

ATP synthesis was estimated in a glucose-hexokinase trap system using rotenone & antimycin A inhibited mitochondria from low-UFA supplemented yeast as described in Table 2. The reaction was initiated by addition of dihydrolipoate (1 μmol) and the fatty acid acyl CoA additions were 10 nmol each.

ADDITIONS	ATP synthesis/20 min	
	Δμmol P_i	Δμmol G6P
1. None	0.0	0.0
2. Dihydrolipoate	0.0	0.0
3. Dihydrolipoate + oleate	0.0	0.0
4. Dihydrolipoate + oleate + oleoyl CoA	1.5	1.4
5. Dihydrolipoate + oleate + oleoyl CoA + oligomycin (5 μg)	0.0	0.0
6. Dihydrolipoate + oleoyl CoA	0.0	0.0
7. Dihydrolipoate + oleate + palmitoyl CoA	0.0	0.0
8. Dihydrolipoate + oleoyl CoA + palmitate	0.0	0.0
9. Dihydrolipoate + linoleate + oleoyl CoA	0.7	0.6
10. Dihydrolipoate + linolenate + oleoyl CoA	0.0	0.0
11. Dihydrolipoate + arachidonate + oleoyl CoA	0.0	0.0

Control experiments showed no ATP synthesis with additions of: oleate, oleoyl CoA, palmitate, palmitoyl CoA, dihydrolipoate + palmitate, dihydrolipoate + palmitoyl CoA, dihydrolipoate + palmitate + palmitoyl CoA.

cells grown with a high unsaturated fatty acid supplement contain over 70% unsaturated fatty acids it is apparent that any unsaturated fatty acid requirement is met by the mitochondrial membrane pool(s) of unsaturated fatty acid.

Mitochondria from KD115 cells grown on a low unsaturated fatty acid supplement have a lower content of unsaturated fatty acid (34 - 35%) and have lost the capacity for oxidative phosphorylation as shown by Linnane & coworkers [5, 6,]. These mitochondria also do not catalyse dihydrolipoate-dependent ATP synthesis (Table 3) but phosphorylation is restored by addition of oleate and oleoyl CoA to the incubation medium as has been shown previously with purified detergent dispersed preparations of ATP synthase (OS-ATPase) from heart, yeast and E. coli [10, 11, 12; & unpublished work].

Table 4 ATP synthesis by mitochondria from yeast mutant KD115 utilising oleoyl-S-lipoate and oleoyl phosphate

ATP synthesis was estimated as described in Table 2 by estimation of glucose-6-phosphate formation except that oleoyl-S-lipoate and oleoyl phosphate were added in dimethylformamide in place of dihydrolipoate. The concentrations refer to the approximate concentrations by weight and the preparations of oleoyl-S-lipoate and oleoyl phosphate are approximately 70% and 85% pure, respectively (11). 1 mg of mitochondrial protein per assay.

ADDITIONS	ATP SYNTHESIS (Δμmol G6P/20 min) A*	B*
1. None	0.0	0.0
2. Oleoyl-S-lipoate (1 μmol)	1.2	1.4
3. Oleoyl-S-lipoate (2 μmol)	2.7	2.7
4. Oleoyl-S-lipoate (1 μmol) + oligomycin (5 μg)	0.1	0.0
5. Oleoyl phosphate (1 μmol)	0.8	0.8
6. Oleoyl phosphate (2 μmol)	1.4	1.5
7. Oleoyl phosphate (1 μmol) + oligomycin (5 μg)	0.7	0.8
8. Oleoyl phosphate (1 μmol) + DCCD (5 μg)	0.0	0.0

*A Mitochondria from KD115 cells grown on high-UFA supplement.
B Mitochondria from KD115 cells grown on low-UFA supplement.

Oleate cannot be replaced by saturated fatty acids nor can oleoyl CoA be replaced by saturated fatty acyl CoA's. Palmitoyl CoA and palmitate are inhibitors of the complete system containing oleate and oleoyl CoA. Oleate can be replaced by Δ 9, cis monoenoic acids of similar structure but cannot be replaced by the trans isomer, elaidic acid. Linoleic acid can replace oleic acid but linolenic acid and arachidonic acid are inactive in this system. A detailed study of the specificity for unsaturated fatty acids has not been made but studies with ATP synthase preparations from heart mitochondria and E. coli indicate a specificity for Δ 9, cis monoenoic acids (D. E. Griffiths, R. L. Hyams & M. Carver, unpublished studies). A similar specificity has been reported by Walenga & Lands [7, 8, 9] in their studies of the growth yield of KD115 on testing a variety of unsaturated fatty acids. Table 4 shows that the capacity to synthesise ATP from oleoyl-S-lipoate and oleoyl phosphate

(10) is still retained by both types of mitochondrial preparations (depleted and supplemented) indicating that the enzyme systems which utilise these fatty acid derivatives are not modified as a result of unsaturated fatty acid depletion.

These studies show that partial depletion of mitochondrial unsaturated fatty acids which leads to loss of oxidative phosphorylation capacity [5, 6, 15] also leads to the loss of a partial reaction of oxidative phosphorylation - dihydrolipoate dependent ATP synthesis. The restoration of this reaction in depleted mitochondria by oleate plus oleoyl CoA supports other studies (10, 11, 12) which indicate a cofactor function for an unsaturated fatty acid in oxidative phosphorylation. The unsaturated fatty acid species involved probably represent a pool of mitochondrial unsaturated fatty acids which are not incorporated into structural membrane phospholipids but are "free" or specifically associated with binding sites on hydrophobic components of the mitochondrial inner membrane involved in oxidative phosphorylation.

This cofactor pool of unsaturated fatty acids is responsive to the nutritional status of the cell with consequent effects on oxidative phosphorylation [15]. Regulation of this membrane pool of unsaturated fatty acids together with regulation of the lipoic acid content is a possible mechanism for control of oxidative metabolism and the mechanism of glucose repression.

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