STUDIES OF ENERGY-LINKED REACTIONS: OLEOYL PHOSPHATE-DEPENDENT ATP SYNTHESIS (OLEOYL PHOSPHOKINASE) ACTIVITY OF MEMBRANE ATPase AND SOLUBLE ATPases FROM MITOCHONDRIA, CHLOROPLASTS, CHROMATOPHORES AND *ESCHERICHIA COLI* PLASMA MEMBRANE

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1. Introduction

A cofactor role for lipoic acid and unsaturated fatty acids in oxidative phosphorylation has been demonstrated and net synthesis of ATP catalysed by mitochondrial particles and ATP synthase preparations has been demonstrated utilising oleoyl-*S*-lipoate and oleoyl phosphate as substrates [1–5]. Oleoyl phosphate-dependent ATP synthesis by submitochondrial particles and ATP synthase preparations (Complex V) was shown to be insensitive to uncouplers such as FCCP, TTFB and '1799', and to oligomycin and triethyltin, but was sensitive to DCCD and F_1ATPase inhibitors such as efrapeptin [1]. The oleoyl phosphate-dependent ATP synthesis (oleoyl phosphokinase) reaction is thus representative of the terminal reactions of oxidative phosphorylation and provides a new experimental system for study of the capacity for ATP synthesis by a variety of soluble ATPases and coupling factors which have been isolated from bioenergetic membranes [6], including several soluble preparations with low or latent ATPase activity [7–9].

This paper demonstrates that the oleoyl phosphate-dependent ATP synthesis (oleoyl phosphokinase) reaction is a characteristic reaction of all bioenergetic membranes including chloroplasts and chromatophores. In addition, it is shown that a soluble ATPase preparation [10] isolated by chloroform extraction of all bioenergetic membranes, catalyses the oleoyl phosphokinase reaction.

Some general features of this reaction catalysed by the soluble ATPase preparation from heart mitochondria [10] are described, including the specificity for a *cis* fatty acid phosphate and the marked inhibition by elaidoyl phosphate and erucoyl phosphate. In addition, the general properties of two related reactions are described, namely, oleoyl phosphatase and an oleoyl phosphate-dependent P_i–ATP exchange reaction.

2. Materials and methods

The source of many of the chemicals and reagents used has been described [1]. Glucose-6-phosphate, NADP, hexokinase, oleoyl chloride, erucoyl chloride, palmitoyl chloride, elaidoyl chloride, erucic acid, palmitic acid and elaidic acid were all obtained from the Sigma (London) Chemical Company, Kingston-upon-Thames, Surrey.

$^{33}P_i$ was obtained as carrier-free orthophosphoric acid from New England Nuclear GmbH, Dreieichenhain, FRG.

Oleoyl phosphate, erucoyl phosphate, palmitoyl phosphate and elaidoyl phosphate were synthesized from the appropriate chlorides and anhydrous phosphoric acid by a modification of the method described by Hildebrand and Spector [11] as follows: 162.4 mg anhydrous orthophosphoric acid is dissolved in 15 ml dry acetonitrile and 250 μl triethylamine added with stirring in a large stoppered test tube. Oleoyl chloride 0.5 ml, was added and the mixture stirred in the dark for 30–60 min at room temperature. NaCl, 40–50 ml 1 M, is added to form an emulsion which is extracted

with 2 × 30 ml aliquots of chloroform. The pooled chloroform extracts are washed with salt solution to remove orthophosphate and dried over Na_2SO_4. The dried extract is concentrated to an oil in vacuo and samples of the oil were dissolved in dimethylformamide for addition to the reaction mixture. The preparation is 85–95% oleoyl phosphate.

A similar method is used for synthesis of the other acyl phosphates and the method can be scaled down for synthesis of ^{14}C- or ^{32}P-labelled oleoyl phosphate.

Fatty acyl lipoates were synthesised by modification of published procedures for the synthesis of fatty acyl CoAs, either by reaction of dihydrolipoate with the appropriate acyl chloride [1,11] or by reaction with the respective *N*-hydroxysuccinimide ester [12].

Ox heart submitochondrial particles and ATP synthase preparations (Complex V) were prepared as described [1]. *Rhodopseudomonas capsulata* chromatophores were kindly donated by Professor A. Melandri (University of Bologna, Italy). *Rhodospirillum rubrum* chromatophores were prepared as described [13]. Chloroplasts were isolated from pea seedlings (*Pisum sativum*) as described by Blair and Ellis [14]. *Escherichia coli* was grown and membrane vesicles prepared as described [4]. Membrane vesicles were prepared from cells washed twice in 0.25 M sucrose, 10 mM $MgCl_2$, 50 mM Tris–Cl, pH 8.0, and resuspended in the same buffer. Cells were broken by passage through a French pressure cell at 6000 lb/in^2. Unbroken cells and debris were removed by centrifugation at 12 000 × *g* for 10 min. Membranes were sedimented at 100 000 × *g*.

Soluble ATPase was prepared by treatment of membranes with chloroform according to the procedure of Beechey et al. [10], except that the concentration of submitochondrial particles, chloroplasts, chromatophores or membrane vesicles was 25 mg protein/ml, and that the emulsion formed after 20 s mixing with chloroform using a vortex mixer was broken by centrifuging at 5000 × *g* for 10 min. The aqueous layer was removed and centrifuged at 100 000 × *g* for 30 min and the supernatant used directly without further purification.

Oxidative phosphorylation was measured using a glucose-hexokinase trap system as described previously [1]. ATPase activity, protein and inorganic phosphate was assayed as described by Griffiths and Houghton [15]. P_i–ATP exchange activity was measured as described by Hatefi et al. [16] except that bovine serum albumen was omitted from the reaction medium.

3. Results

Membrane-bound preparations of ATP synthase present in membrane preparations from heart and yeast mitochondria, *E. coli* plasma membrane, chloroplasts and chromatophores catalyse the net synthesis of ATP from oleoyl phosphate (table 1, column A). A stoicheiometric conversion of oleoyl phosphate to ATP is shown by all systems. In many reactions shown in table 1, equilibrium is reached but parallel experiments at shorter reaction periods indicate that the rate of the transphosphorylation reaction is in the range of 100–200 nmol/mg/min, a rate which is equivalent to that of oxidative phosphorylation catalysed by these membrane preparations, but is lower than the maximal rates of photophosphorylation catalysed by chloroplast and chromatophore preparations. In all cases the reaction is sensitive to DCCD at levels below 5 nmol/mg protein and to a F_1-ATPase inhibitor, efrapeptin. Examination of the sensitivity of the reaction catalysed by mitochondrial membranes shows that it is insensitive to oligomycin, ionophores and uncoupling agents such as FCCP, S-13, '1799' and TTFB. However, the oleoyl phosphokinase reaction catalysed by membrane-bound ATP synthase is sensitive to dinitrophenol (100% inhibition at 30 μM). These findings regarding inhibitor sensitivity are essentially similar to those previously described [1] for heart sub-mitochondrial particles and a solubilised ATP synthase preparation (Complex V).

A number of soluble ATPase preparations were examined to establish the minimal structure requirements for catalysis of the oleoyl phosphokinase reaction. Preparations of heart F_1-ATPase with spec. act. 70–80 purified by the method of Knowles and Penevsky [17] were shown to be inactive, or to have very low oleoyl phosphokinase activity. However, the soluble ATPase preparation of Beechey et al. [10] obtained by chloroform extraction of mitochondrial particles was shown to catalyse ATP synthesis from oleoyl phosphate at a high rate. Table 1, column B shows that soluble ATPases obtained from all bioenergetic membranes catalyse the oleoyl phospho-

Table 1
Oleoyl phosphate dependent ATP synthesis by bioenergetic membrane preparations and derivative chloroform extracted ATPase preparations

Preparation membrane or ATPase			Oleoyl phosphate (μmol added)	ATP synthesis (μmol G6P/20 min) A[a]	B[b]
Heart and liver mitochondria			1	1.02	–
			2	2.07	–
	+ DCCD	(1 μg)	1	0	–
Escherichia coli plasma membrane			1	1.05	1.1
			2	2.07	2.1
	+ DCCD	(1 μg)	1	0	0
Chloroplast membranes			1	1.01	1.07
			2	2.1	2.1
	+ DCCD	(1 μg)	1	0	0
Rhodospeudomonas capsulata			1	1.01	1.1
			2	2.05	2.05
	+ DCCD	(1 μg)	1	0	0
Rhodospirillum rubrum			1	1.1	1.0
			2	2.1	2.1
	+ DCCD	(1 μg)	1	0	0
Heart SMP			1	1.02	1.07
			2	2.1	2.11
			3	n.t.	3.11
	+ DCCD	(1 μg)	1	0	0
	+ oligomycin	(2 μg)	1	1.05	1.1
	+ efrapeptin	(1 μg)	1	0	0
	+ TTFB	(1 μg)	1	1.01	1.01
	+ FCCP	(1 μg)	1	1.02	1.02
	+ valinomycin	(2 μg)	1	1.08	1.12
	+ nigericin	(2 μg)			
	+ DNP	(5 μg)	1	0.18	0.19
	+ DNP	(10 μg)	1	0.05	0
	+ '1799'	(1 μg)	1	0.98	0.05

[a] Parallel determinations of the rate of ATP synthesis by membrane preparations gave values in the range 110–200 nmol/min/mg protein

[b] Parallel determinations of the rate of ATP synthesis by soluble ATPase using shorter incubation periods gave values in the range 1400–2600 nmol/min/mg protein

ATP synthesis was estimated in a glucose-hexokinase trap system at 30°C [1]. A 0.05 ml aliquot (1 mg membrane protein or 100 μg ATPase protein) was taken into 1 ml of phosphorylation medium containing 250 mM sucrose, 22 mM glucose, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.2 mM ADP, 20 mM Tris–Cl buffer, pH 7.3, hexokinase 50 units, plus various inhibitors and additions. The reaction was initiated by addition of oleoyl-phosphate as listed in the table. After 20 min, samples were removed for estimation of glucose 6-phosphate [1]. The results for membrane preparations are listed under column A and the results for the soluble ATPase preparations are listed under column B

kinase reaction as shown for the membrane bound enzyme. Rates of 1500–3000 nmol/min/mg protein have been obtained with the unpurified preparations. The chloroform extraction method [10] is the method of choice for obtaining a membrane free, soluble preparation of minimal complexity which retains the capacity for ATP synthesis from oleoyl phosphate.

Table 1 shows that the oleoyl phosphokinase reaction catalysed by soluble ATPase is insensitive to oligomycin and uncouplers such as FCCP and TTFB, but is sensitive to DCCD, efrapeptin and dinitrophenol. Of particular interest is the finding that the soluble enzyme is inhibited by '1799,' whereas the membrane bound enzyme is not. The oleoyl phosphokinase reaction is magnesium dependent, a common feature of phosphoryl group transfer reactions.

The specificity for the acyl phosphate in the oleoylphosphokinase reaction is illustrated in table 2. The *trans* isomer, elaidoylphosphate is inactive and is a potent inhibitor, as is erucoyl phosphate, the acyl phosphate of erucic acid (C 22 : 1, *cis*Δ13), a toxic unsaturated fatty acid [18]. Palmitoyl phosphate is inactive and is a relatively poor inhibitor of oleoyl phosphokinase activity as compared with elaidoyl phosphate and erucoyl phosphate. Oleoyl phosphate, elaidoyl phosphate and erucoyl phosphate are also effective inhibitors of the ATPase reaction catalysed by the soluble ATPase preparation (I_{50} values 3.5, 7 and 0.8 nmol/mg protein, respectively). Palmitoyl phosphate, in contrast, does not inhibit the ATPase reaction even at 100 nmol/mg protein.

The soluble ATPase preparation [10] exhibits zero or very low levels of P_i–ATP exchange activity, but the reaction is markedly enhanced by addition of oleoyl phosphate (table 3). The oleoyl phosphate-dependent P_i–ATP exchange reaction exhibits the same range of inhibitor sensitivities and substrate specificities obtained in studies of the oleoyl phosphokinase reaction. The insensitivity to oligomycin and FCCP indicates that the observed P_i–ATP exchange activity does not involve the whole reaction sequence involved in oxidative phosphorylation, but only the terminal reactions which are still retained by the soluble ATPase preparation. The possible participation of a P_i–oleoyl phosphate exchange reaction in the overall oleoyl phosphate-dependent P_i–ATP exchange reaction remains to be evaluated. Stimulation by oleoyl phosphate of P_i–ATP exchange activity of heart submitochondrial particles has also been demonstrated (Hyams, R. L. and Griffiths, D. E., unpublished work) and the sensitivities to inhibitors and uncouplers parallel those observed with the

Table 2
Acyl phosphate specificity of the oleoylphosphokinase reaction

Additions				ATP synthase (μmol G6P / 10 min)
None				0
Oleoyl phosphate	(1 μmol);	C18 : 1, *cis*Δ 9		1.15
Elaidoyl phosphate	(1 μmol);	C18 : 1, *trans*Δ 9		0
Palmitoyl phosphate	(1 μmol);	C16 : 0		0
Erucoyl phosphate	(1 μmol);	C22 : 1, *cis*Δ 13		0
Oleoyl phosphate	(1 μmol)	+ elaidoyl phosphate	(10 pmol)	0.03
		+ elaidic acid	(24 pmol)	0
		+ palmitoyl phosphate	(500 nmol)	0.23
		+ palmitic acid	(500 nmol)	1.15
		+ erucoyl phosphate	(10 pmol)	0.01

Oleoyl phosphokinase activity of the chloroform-extracted ATPase [10] was assayed in the incubation system described in table 1. Oleoyl phosphate was replaced by other acyl phosphates to initiate the reaction. 100 μg soluble ATPase. Reaction period 10 min at 30°C. Inhibitory acyl phosphates were incubated with enzyme for 5 min prior to initiation of the reaction with oleoyl phosphate

Table 3
Oleoyl phosphate-dependent P_i–ATP exchange activity of soluble ATPase from heart mitochondria

Additions				P_i–ATP exchange activity (nmol/mg/min)
None				0.16 (~ 3)[a]
Oleoyl phosphate	(200 nmol)			620
Oleoyl phosphate	(600 nmol)			1691
Oleoyl phosphate	(1 μmol)			2790
Palmitoyl phosphate	(1 μmol)			0
Elaidoyl phosphate	(1 μmol)			0
Erucoyl phosphate	(1 μmol)			0
Oleoyl phosphate	(1 μmol)	+ oligomycin	(2 μg)	2818
		+ FCCP	(2 μg)	2481
		+ TTFB	(2 μg)	2865
		+ valinomycin	(1 μg)	
		+ nigericin	(1 μg)	2591
		+ DCCD	(1 μg)	1.5
		+ efrapeptin	(2 μg)	11.3
		+ '1799'	(2 μg)	0
		+ DNP	(5 μg)	0
		– ATP		0

[a] The value in brackets was obtained under conditions designed to minimise ATP hydrolysis (50 μg enzyme, 2 min incubation)

Exchange was measured essentially as described by Hatefi et al. [16] and assayed at 30°C for 5 min with 150 μg soluble ATPase from heart mitochondria [10]. ATPase spec. act. 23 μmol/mg/min

Table 4
Oleoyl phosphatase activity of ox heart soluble extracted ATPase

Additions		P_i released (μmol)
Oleoyl phosphate (1 μmol)		0.3
	+ ADP (200 nmol)	0.72
	+ '1799' (5 μg)	0.9
	+ DNP (5 μg)	1.05
	+ ADP + '1799'	0.99
	+ ADP + DNP	0.90
Oleoyl phosphate (2 μmol)		0.51
	+ ADP (200 nmol)	1.23
	+ '1799' (5 μg)	2.04
	+ DNP (5 μg)	2.07
	+ ADP + '1799'	2.13
	+ ADP + DNP	2.07
Oleoyl phosphate (2 μmol)	+ DCCD (2 μg)	0.0
	+ efrapeptin (2 μg)	0.0

Oleoyl phosphatase activity was measured by the release of inorganic phosphate [1] in a medium containing 1 ml of Sucrose 0.25 M; Tris-HCl 10 mM pH 7.5; $MgCl_2$, 5 mM; EDTA 1 mM; by 40 μg of enzyme. The assay was initiated by addition of oleoyl phosphate and after five minutes at 30°C terminated by addition of 0.5 ml 30% perchloric acid

soluble ATPase. The oleoylphosphate stimulated P_i–ATP exchange reaction has not been demonstrated with highly purified preparations of heart F_1-ATPase [17] as also found for oleoyl phosphate-dependent ATP synthesis.

In the absence of a glucose-hexokinase trap system submitochondrial particles and the soluble ATPase catalyse hydrolysis of oleoyl phosphate. However, the reaction is not catalysed by purified F_1-ATPase preparations. Oleoyl phosphatase activity is stimulated by ADP and by the uncoupler dinitrophenol and '1799' (table 4). The oleoyl phosphatase reaction is sensitive to DCCD and to efrapeptin, both of which exhibit the oleoyl phosphokinase reaction and the oleoyl phosphate-dependent P_i–ATP exchange reaction. The marked stimulation of oleoyl phosphatase activity by dinitrophenol and by '1799' and the high specific activity of the resultant oleoyl phosphatase reaction (> 10 μmol/mg protein/min) provides an explanation for inhibition of the oleoyl phosphokinase reaction and the oleoyl phosphate-dependent P_i–ATP exchange reaction (tables 1 and 3) by dinitrophenol and '1799'.

4. Discussion

The demonstration that all bioenergetic membranes catalyse a DCCD-sensitive oleoyl phosphokinase reaction provides strong evidence in support of a proposed 'oleoyl cycle' in oxidative phosphorylation and photophosphorylation involving lipoic acid and a *cis* unsaturated fatty acid such as oleic acid [1,19]. The specific cofactor requirement for a *cis* unsaturated fatty acid [4,19] is also demonstrable at the acyl phosphate level as the *trans* isomer, elaidoyl phosphate and the saturated fatty acid derivative, palmitoyl phosphate, are inactive as substrates and are inhibitors of the oleoyl phosphokinase reaction (table 2). The sensitivity of the reaction to DCCD and the F_1-ATPase inhibitor, efrapeptin, characterises the reaction as a representative partial reaction of the oxidative phosphorylation sequence. The reaction is not inhibited by oligomycin, triethyltin and most uncoupling agents. However, the reaction is sensitive to 2,4-dinitrophenol and the reaction catalysed by soluble ATPase is also sensitive to the uncoupler '1799' (table 1). The marked stimulation of oleoyl phosphatase activity by 2,4-dinitrophenol and '1799' (table 4) provides an explanation for inhibition of the oleoyl phosphokinase reaction and the associated oleoyl phosphate-dependent P_i–ATP exchange reaction. These effects of dinitrophenol and of '1799' at levels equivalent to those which uncouple oxidative phosphorylation suggest that stimulation of the oleoyl phosphatase reaction could be a major uncoupling site of action of dinitrophenol, i.e., catalysis of the hydrolysis of an acyl phosphate intermediate as originally proposed by Lardy and Wellman [20]. Regulation of the level of oleoyl phosphate and/or oleoyl-enzyme or phospho-enzyme derivatives may be an important control point in oxidative phosphorylation and can account for varying levels of mitochondrial ATPase, e.g., the stimulation of mitochondrial ATPase by dinitrophenol. However, the well established inhibitory effect of dinitrophenol and other uncouplers, on mitochondrial energy-linked reactions driven by the respiratory chain which do not involve the ATP synthase complex, indicates that there is at least one other major site of action of dinitrophenol and other uncoupling agents which also must be taken into consideration.

The demonstration of the oleoyl phosphokinase reaction in the soluble ATPase [10] obtained by chloroform extraction of a variety of bioenergetic membranes provides a simple experimental system of minimal complexity for study of the terminal reactions of oxidative phosphorylation and photophosphorylation which is also a reference point for comparison with less complex preparations such as F_1-ATPase and more complex preparations such as the ATP synthase (OS–ATPase) complex [17,21].

Examination of the differences in polypeptide composition of the soluble ATPase [10] as compared with the purified F_1-ATPase should give information as to whether the oleoyl phosphokinase reaction is due to loss of an enzymically active polypeptide component or structural modification and disorganisation during the preparation of F_1-ATPase. The demonstration of catalysis of an oleoyl phosphate-dependent P_i–ATP exchange activity by the soluble ATPase indicates that it contains a group capable of oleoylation by oleoyl phosphate and the possible participation of a pantotheine containing polypeptide with an acyl carrier protein function which has been shown to be present in yeast ATP synthase [2,22].

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