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STUDIES OF ENERGY-LINKED REACTIONS : STIMULATION OF THE MITOCHONDRIAL Pi-ATP EXCHANGE REACTION BY OLEOYL LIPOATE, OLEOYL CoA AND OLEOYL PHOSPHATE

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SUMMARY: Oleoyl lipoate, oleoyl CoA, oleoyl phosphate and dihydrolipoate have been shown to stimulate Pi-ATP exchange activity in sub-mitochondrial particles. A different sensitivity to uncouplers and inhibitors of oxidative phosphorylation is observed, indicating that the various oleoyl derivatives interact at different levels of a multistep reaction sequence, a postulated 'oleoyl cycle.'

INTRODUCTION : Pi-ATP exchange activity is characteristic of energy conservation reactions in mitochondria, chloroplasts, chromatophores and bacterial membranes. Inhibition of this activity by uncoupling agents, energy transfer inhibitors and F_1-ATPase inhibitors all indicate that Pi-ATP exchange activity represents a series of reactions involved in the mechanism of oxidative phosphorylation [1]. By analogy with the mechanism of substrate level phosphorylation, these reactions are assumed to involve phosphorolysis of an acyl intermediate as expressed in the following series of reactions [1,2]:

$$X \sim I + Y \rightleftharpoons X \sim Y + I \quad (1)$$

$$X \sim Y + P_i \rightleftharpoons X \sim P + Y \quad (2)$$

$$X \sim P + ADP \rightleftharpoons ATP + X \quad (3)$$

The overall rate of the Pi-ATP exchange reaction is thus determined by the individual rate constants and the steady state concentrations of the postulated intermediates. Addition of an intermediate or a component which generates or is in equilibrium with an intermediate thus leads to an enhancement of Pi-ATP exchange activity. Oleoyl lipoate, oleoyl Co-A (as an oleoyl acyl carrier protein) and oleoyl phosphate are postulated intermediates in an 'oleoyl cycle' [3], catalysed by ATP synthase preparations from heart and yeast mitochondria and other bioenergetic membranes including chloroplasts. The capacity of the postulated intermediates to stimulate Pi-ATP exchange activity has been investigated and this paper demonstrates enhancement of mitochondrial Pi-ATP exchange activity by oleoyl lipoate, oleoyl CoA and oleoyl phosphate, and provides further evidence for the involvement of equivalent enzyme bound components in the mechanism of the Pi-ATP exchange reaction and the mechanism of oxidative

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phosphorylation. In addition, dihydrolipoate or a derivative formed from it in solution is also shown to stimulate Pi-ATP exchange activity, presumably by generation of enzyme bound oleoyl lipoate and other oleoyl derivatives. The differential sensitivity of Pi-ATP exchange activity stimulated by dihydrolipoate, oleoyl lipoate, oleoyl CoA and oleoyl phosphate to inhibitors and uncouplers of oxidative phosphorylation is also demonstrated.

MATERIALS AND METHODS : The source of inhibitors, reagents , and the method of preparation of ox heart mitochondria and sub-mitochondrial particles (SMP) are as described previously [4]. ^{33}Pi was obtained as carrier free orthophosphoric acid from New England Nuclear GmbH, Dreieichenain, West Germany. Oleoyl lipoate and oleoyl phosphate were synthesised as previously described [4,5] and dissolved in dimethyl formamide. Oleoyl CoA, dithiothreitol and lipoamide were obtained from Sigma Chemical Company. Reduced lipoamide was prepared by borohydride reduction [6].

Dihydrolipoic acid was obtained from Sigma Chemical Company or was prepared by borohydride reduction of lipoic acid [6]. A 0.2M solution of dihydrolipoate was prepared by suspension of dihydrolipoic acid in half volume of 0.25M sucrose, 10mM Tris-Cl, 1mM EDTA, pH 8. The dihydrolipoic acid was brought into solution by careful addition of small amounts of 1M Tris base with constant mixing, using a vortex mixer. The solution is then made up to final volume with sucrose-Tris-EDTA solution. This solution (final pH 7.5 -8.0) contains 90-95% of the theoretical thiol content as estimated by use of the Ellman reagent [7].

Some variation in the preparation of active solutions of dihydrolipoate has been noted. About 25% of the dihydrolipoate solutions prepared in this manner are inactive in stimulating Pi-ATP exchange activity and are also inactive in the dihydrolipoate-dependent ATP synthesis reaction [4]. It has been noted that solutions of dihydrolipoate which are inactive in the dihydrolipoate dependent ATP synthesis reaction [4] are generally potent inhibitors of the Pi-ATP exchange reaction and of oxidative phosphorylation. It has also been observed that many inactive (inhibitory) solutions of dihydrolipoate become active after storage at 5°. The reasons for these variations in activity have not yet been elucidated. However, the sensitivity of lipoate and dihydrolipoate to light, pH, metal catalysed oxidation and the capacity to form polymers is well documented [8 , 9]. It should be noted that a mixture of lipoic acid and dithiothreitol is not active in stimulation of Pi-ATP exchange or in the dihydrolipoate-dependent ATP synthesis assay.

Pi-ATP exchange activity was estimated by the method of Pullman [10] as described by Hatefi et al [11] with the following modifications: Bovine serumalbumen was omitted from the incubation mixture as no effect of oleoyl derivatives and dihydrolipoate was observed in its presence. Samples containing dihydrolipoic acid were oxidised with bromine water as described by Fisher et al [12] before addition of molybdate and extraction with isobutanol/benzene.

RESULTS : Fig. 1 shows the marked stimulation of mitochondrial inner membrane Pi-ATP exchange activity by oleoyl lipoate, oleoyl CoA, oleoyl phosphate and dihydrolipoate. Rates of over 2000 nmol/mg/min have been observed with high levels of dihydrolipoate, oleoyl lipoate and oleoyl phosphate. No stimulation of Pi-ATP exchange activity is observed with saturated fatty acyl derivatives such as palmitoyl-lipoate, palmitoyl CoA and palmitoyl phosphate (Table 2). These acyl derivatives do

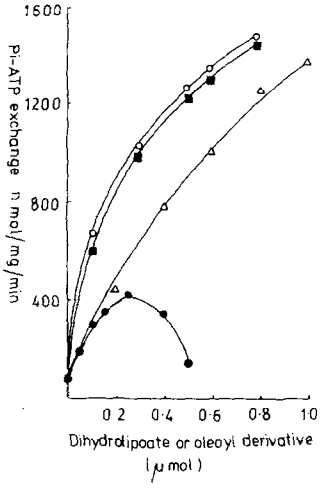

Figure 1. Stimulation of Pi-ATP exchange activity by oleoyl derivatives and by dihydrolipoate

Pi-ATP exchange activity was assayed at 30° as described by Hatefi et al [11] except for the omission of bovine serum albumen from the incubation medium. The reaction mixture contained 25mM Tris-SO, pH 7.5, 15 mM $MgCl_2$, 30 mM sucrose, 20mM potassium phosphate containing 10^7 cpm ^{33}Pi and 0.5 mg of submitochondrial particles (suspended in 250 mM sucrose, 10mM Tris-Cl, pH 7.5, 1mM EDTA at 10 mg protein/ml). The reaction mixture was preincubated with added oleoyl derivatives or dihydrolipoate with or without inhibitors for 5 min at 30° and the reaction initiated by addition of 50 μl of 0.24M ATP (pH 7.5) to a final concentration of 12 mM. Final volume 1.0 ml. Oleoyl lipoate and oleoyl phosphate were added as solutions in dimethylformamide.

After 5 min. incubation at 30° the reaction was stopped by addition of 200 μl of 30% perchloric acid and the precipitated protein removed by centrifugation. A 500 μl sample of the supernatant was assayed for incorporation of ^{33}Pi into ATP as described previously [10, 11]. The supernatant was also sampled for assay of inorganic phosphate content. Under the conditions of the assay, less than 10% of the ATP added was hydrolysed. No correction for ATP hydrolysis was made in calculation of Pi-ATP exchange activity.

△——△, dihydrolipoate; ■——■ Oleoyl-S-lipoate ;
●——●, Oleoyl CoA O——O Oleoyl phosphate.

not inhibit the basal Pi-ATP exchange activity at levels of 100 nmol/ml but inhibit the stimulation by equimolar amounts of oleoyl derivatives. Elaidoyl lipoate and elaidoyl phosphate do not stimulate P_i-ATP exchange and elaidoyl phosphate is a potent inhibitor in contrast to palmitoyl derivatives (data not shown). These results are consistent with previous findings of a specific requirement for cis unsaturated fatty acids in the dihydrolipoate dependent ATP synthesis reaction [4, 13].

TABLE 1

Stimulation of the P_i-ATP exchange activity of ox heart SMP by dihydrolipoate

Assay conditions were as described in the legend to Fig.1. The enzyme was preincubated with dihydrolipoate with or without inhibitors for 5 min at 30° and the reaction was initiated by addition of 50 µl of 0.24M ATP (pH 7.5). Reaction period, 5 min. Temperature, 30° . Additions of ionophores, uncouplers and inhibitors were 2 µg each.

Additions	P_i-ATP exchange activity (nmol/mg/min)
1. None (basal rate) *	79
2. Dihydrolipoate (200 nmol)	486
3. Lipoate (200 nmol)	78
4. Dithiothreitol (200 nmol)	98
5. Lipoate (200 nmol) + Dithiothreitol (400 nmol)	64
6. Dihydrolipoate (200 nmol)	83
7. Lipoamide (200 nmol) + Dithiothreitol (400 nmol)	75

Experiment B

1.	Dihydrolipoate (200 nmol)	475
2.	Dihydrolipoate (200 nmol) + FCCP	0
3.	" + S-13	0
4.	" + '1799'	0
5.	" + DNP	5
6.	" + TTFB	469
7.	" + Oligomycin	0
8.	" + DCCD	0
9.	" + Efrapeptin	0
10.	" + Valinomycin + Nigericin	0

* The basal P_i-ATP exchange activity was inhibited (> 95%) by 2µg each of the following : carbonylcyanide-m-fluoro phenyl hydrazone (FCCP); 5-chloro-3-t-butyl-2'-chloro-4'-nitrosalicylanilide (S-13), bis(hexafluoroacetonyl)acetone(1799); valinomycin plus nigericin; gramicidin; oligomycin; venturicidin; triethyltin sulphate; dicyclohexylcarbodiimide (DCCD) and also by tetrachlorotrifluoromethyl benzimidazole (TTFB).

Table 1 illustrates the specific requirement for dihydrolipoate in stimulation of the P_i-ATP exchange reaction. Dihydrolipoamide is inactive as has also been shown in the case of reversal of dibutylchloromethyltin inhibition of oxidative phosphorylation

TABLE 2

Inhibitor and uncoupler sensitivity of P_i-ATP exchange activity stimulated by oleoyl-S-lipoate, oleoyl CoA and oleoyl phosphate

P_i-ATP exchange activity was assayed as described in the legends to Fig.1 and Table 1. Additions were : 100 nmol Oleoyl-S-lipoate, 100 nmol Oleoyl CoA and 100 nmol Oleoyl phosphate in the experiments listed in columns A, B and C respectively. All other additions of ionophores, uncouplers and inhibitors were 2μg each. The basal exchange rate was 80 ± 5 nmol/min/mg of protein.

	P_i-ATP exchange activity (nmol/min/mg of protein)		
	A	B	C
	Oleoyl-S-lipoate	Oleoyl CoA	Oleoyl phosphate
1. None	229	390	314
2. + Valinomycin + Nigericin	0	0	315
3. + Gramicidin	0	0	324
4. + S - 13	0	387	305
5. + FCCP	0	390	324
6. + 1799	0	370	319
7. + TTFB	219	374	311
8. + DNP	0	0	0
9. + Oligomycin	0	0	322
10. + Venturicidin	0	0	n.t.
11. + Triethyltin	0	0	n.t.
12. + DCCD	0	0	0
13. + Efrapeptin	0	0	0
14. + Palmitoyl derivatives (100 nmol)	79	80	n.t.
15. + Elaidoyl derivatives (100 nmol)	75	n.t.	0

[14], dihydrolipoate-dependent ATP synthesis and restoration of ATP synthesis in a lipoate deficient E.coli mutant [4, 15]. Dihydrolipoate-stimulated Pi-ATP exchange is sensitive to all inhibitors and uncouplers of oxidative phosphorylation except tetrachlorotrifluoromethylbenzimidazole (TTFB), as found also in studies of the dihydrolipoate-dependent ATP synthesis reaction (Griffiths, D.E. & R.L.Hyams, unpublished work). However, dihydrolipoate dependent ATP synthesis is not

sensitive to ionophores, in contrast to the marked sensitivity to ionophores of the dihydrolipoate stimulated Pi-ATP exchange reaction (Table 1).

A differential sensitivity to ionophores, uncouplers and inhibitors of oxidative phosphorylation is shown in Table 2. Oleoyl lipoate stimulated Pi-ATP exchange, like dihydrolipoate stimulated P_i-ATP exchange activity, is sensitive to all ionophores, inhibitors and uncouplers except for the uncoupler TTFB. In contrast, oleoyl CoA-stimulated P_i-ATP exchange is insensitive to the uncouplers FCCP, S-13, 1799, as well as TTFB, but is sensitive to DNP, ionophores and inhibitors of oxidative phosphorylation. Oleoyl phosphate stimulated Pi-ATP exchange activity is insensitive to ionophores, the uncouplers FCCP, S-13, 1799 and TTFB, and to oligomycin , but the reaction is sensitive to DCCD, efrapeptin and also to dinitrophenol. The action of ionophores , inhibitors and uncouplers is thus the same as that reported for the oleoyl phosphokinase reaction catalysed by submitochondrial particles [5] and the effect of dinitrophenol is explained by its capacity to stimulate a rapid oleoyl phosphatase reaction [5], thus leading to inhibition of P_i-ATP exchange.

DISCUSSION : The stimulation of the P_i-ATP reaction by oleoyl lipoate, oleoyl CoA and oleoyl phosphate indicates the involvement of equivalent membrane-bound acyl derivatives in the mechanism of the mitochondrial P_i-ATP exchange reaction and supports the conclusion that a series of acylation, transacylation, phosphorolysis and transphosphorylation reactions are involved in the terminal reactions of oxidative phosphorylation [4, 13]. The specificity for acyl derivatives of cis unsaturated fatty acids is similar to that demonstrated for the fatty acid requirement for dihydrolipoate dependent ATP synthesis by ATP synthetase preparations [4, 14].

The P_i-ATP exchange activity stimulated by the various oleoyl derivatives show a differential sensitivity to uncoupling agents and inhibitors of oxidative phosphorylation, indicating that these reagents react at different sites in a multistep reaction sequence. The differential sensitivity to uncouplers and inhibitors also indicates that the various oleoyl derivatives integrate into the reaction sequence at different levels, e.g. oleoyl lipoate stimulated P_i-ATP exchange would involve reactions 1, 2 and 3, while the oleoyl CoA stimulated reaction would involve reactions 2 and 3 only and the oleoyl phosphate stimulated exchange activity would be representative of the terminal reaction (s) of the sequence. The sensitivity to uncouplers and inhibitors parallels that observed for dihydrolipoate-dependent ATP synthesis [4, 14 , Griffiths, D.E. and Hyams, R.L., unpublished results], oleoyl lipoate dependent ATP synthesis [4]

and oleoyl phosphate dependent ATP synthesis (oleoyl phosphokinase)[5], and indicates a site of action of uncouplers such as FCCP, S-13 and 1799 at the level of oleoyl-lipoate linked reactions and a site of action of oligomycin, venturicidin and triethyltin at the phosphorolysis step in the reaction sequence. The site of action of DCCD is distinct from that of oligomycin as previously indicated from studies of the oleoyl phosphokinase reaction [5] and the sensitivity to DNP is readily explained by its capacity to stimulate a rapid oleoyl phosphatase activity [5].

These studies and similar studies of the uncoupler and inhibitor sensitivity of purified ATP synthase preparations [4, Griffiths, D.E. & Hyams , R.L., unpublished work] indicate the presence of interaction sites for uncouplers in the ATP synthase complex and provide supporting evidence for the studies of Hanstein and Hatefi [16,17] on uncoupler binding sites in ATP synthase and biochemical genetic studies in this laboratory on uncoupler resistant mutants [18]. However, these uncoupler interaction sites in the ATP synthase complex may not be the primary site of action of uncouplers as oxidative phosphorylation is sensitive to all uncoupling agents including TTFB and an uncoupler sensitive succinate driven transhydrogenase is still present in particles where the total free lipoic acid pool has been titrated by dibutylchloromethyltin chloride [Griffiths, D.E. & Hyams, R.L., unpublished work]. These findings indicate that there is a primary site of action of uncoupling agents associated with the primary energisation events at the level of the respiratory chain which are not associated with the acyl transfer, phosphorolysis and transphosphorylation events catalysed by the ATP synthase complex described in this paper.

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