STUDIES OF ENERGY-LINKED REACTIONS: A LIPOIC ACID REQUIREMENT FOR OXIDATIVE PHOSPHORYLATION IN *ESCHERICHIA COLI*

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1. Introduction

A role for lipoic acid (or a lipoic acid conjugate) in oxidative phosphorylation catalysed by yeast and heart mitochondria has been demonstrated and net synthesis of ATP by ATP–synthase preparations has been shown to require dihydrolipoate and an unsaturated fatty acid as cofactors [1,2]. It is proposed that lipoic acid residues provide a link between the respiratory chain and the ATP–synthase complex and that the terminal reactions of oxidative phosphorylation involve a cycle of transacylation and transphosphorylation reactions, analogous to those involved in substrate level phosphorylation [1].

The availability of lipoic acid auxotrophs of *Escherichia coli* [3] provides an experimental system for evaluating the role of lipoic acid in the oxidative phosphorylation system present in the plasma membrane of *E.coli*. This paper describes studies of oxidative phosphorylation in membrane vesicles from a lipoic acid requiring mutant of *E.coli* and demonstrates a requirement for lipoic acid in oxidative phosphorylation with NADH, succinate and D-lactate as substrates.

2. Materials and methods

DL-Lipoic acid, DL-lipoamide and hydroxyquinoline-*N*-oxide were obtained from Sigma Chemical Company. DL-8-Methyllipoic acid was a gift from Dr J. R. Guest, University of Sheffield and checked for purity by 90 mHz NMR. Other components have been described previously [1,2].

The lipoic acid auxotroph W1485 lip 2 was obtained from Dr J. R. Guest, and its general properties have been described previously [3], as have the conditions for growth on minimal medium with or without lipoate additions and a mixture of glucose (0.2%), acetate (4 mM) and succinate (4 mM) as substrates [4]. Cells were grown aerobically in a shaking incubator at 37°C for 18 h and isolated by centrifugation at 5000 × *g* for 15 min, washed and resuspended in 250 mM sucrose, 50 mM Tris–Cl, pH 8.0. Spheroplasts were prepared by lysozyme treatment of washed cells as described by Witholt et al. [5] and stabilised with 20 mM $MgCl_2$, washed by centrifugation at 10 000 × *g* and broken by osmotic shock by resuspension in 20 mM potassium phosphate buffer, pH 8.0, containing DNAase (20 μg/ml). Unbroken cells and large fragments were removed by centrifugation at 10 000 × *g* for 15 min. Membrane vesicles were isolated by centrifugation of the 10 000 × *g* supernatant at 100 000 × *g* for 20 min, resuspended and further purified by centrifugation through a discontinuous sucrose density gradient as described by Schnaitman [6]. The polarity of these vesicles has not been established but the capacity for rapid oxidation of NADH and phosphorylation of ADP indicates that a high proportion of the vesicles are 'inside out,' but it is likely that the preparation is a mixed population [7].

ATP synthesis was assayed in a glucose–hexokinase trap system by the disappearance of inorganic phosphate or glucose-6-phosphate formation [1,2]. The

Abbreviations: DCCD dicyclohexylcarbodiimide, FCCP carbonyl cyanide p-trifluoromethoxyphenylhydrazone, HOQNO 2-heptyl-4-hydroxyquinoline-*N*-oxide, 8-MLA DL-8-methyl lipoic acid

reaction medium (1.0 ml) contained 250 mM sucrose, 20 mM glucose, 1 mM $MgCl_2$, 1 mM ADP, 4 mM phosphate, 20 mM Tris–Cl buffer, pH 7.3, hexokinase 30 units, membrane vesicles 1 mg protein. Vesicles were preincubated for 5 min with inhibitors and cofactors as indicated in the legends to tables, prior to initiation of the assay by addition of substrate.

Incubation was for 20 min at 30°C by shaking in air. As the purpose of the experiments is to measure restoration of phosphorylation capacity, P/O ratios were not estimated directly. However, the rate of oxygen uptake was estimated for 3–5 min in parallel assays using an oxygen electrode, but utilising 2.0 ml of complete incubation medium. Assuming that the rate of oxygen uptake is constant throughout the reaction period, then an estimate of the apparent P/O ratios can be made. Apparent P/O ratios with succinate (0.75–0.85), NADH (0.5–0.6) and D-lactate (0.45–0.65) were obtained.

Cytochrome spectra of vesicles from lipoate deficient and lipoate supplemented cells were determined after freezing in liquid nitrogen in a low temperature attachment to a Unicam SP 1800 spectrophotometer (courtesy of Dr D. Lloyd, University of Cardiff). No significant differences in the cytochrome concentration or cytochrome species present were observed.

Table 1
Oxidative phosphorylation in membrane vesicles from lipoic-deficient and lipoic-supplemented *E.coli* (W1485 lip 2)

Additions		ATP synthesis (nmol/min)		Oxygen uptake (ng atom/min)
		Δ P_i	ΔG6P	
A	Lipoic-deficient vesicles			
	None	0	0	< 10
	Lipoic acid	0	0	< 10
	NADH	0	0	200[a]
	D-lactate	0	0	170[a]
	Succinate	0	0	180[a]
B	Lipoic-supplemented vesicles			
	None	0	0	30
	Lipoic Acid	0	0	30
	NADH	101	102[b]	200[a]
	D-lactate	98	100[b]	210[a]
	Succinate	94	80[b]	115[a]

[a] Respiration and any attendant phosphorylation is inhibited (>95%) by 5 μg/ml HOQNO for all substrates and by 5 μmol malonate for succinate as substrate only. The low 'endogenous' rate of respiration in the presence of glucose and hexokinase is non-phosphorylating and is not inhibited by malonate, but is partially inhibited by 5 μg HOQNO.

[b] FCCP (5 μg) and DCCD (5 μg) inhibit phosphorylation (100%) with all substrates without inhibition of respiration

In the presence of 1 μmol lipoic acid, phosphorylation rates are 10% higher

ATP Synthesis was assayed in a glucose–hexokinase trap system [1,2] as described in Materials and methods. Substrate and cofactor additions were NADH (10 μmol), D-lactate (10 μmol), succinate (10 μmol) and lipoic acid (1 μmol). The assay mixture was incubated aerobically with shaking for 20 min at 30°C. Membrane vesicles, 1 mg protein. Glucose 20 μmol, hexokinase 30 units. Oxygen uptake was measured for 3–5 min in parallel assays, using an oxygen electrode as described in Materials and methods.

3. Results

The lipoic acid auxotroph W1485 lip 2 is capable of fermentative growth on glucose and the lipoic acid requirement may be partially met by the addition of both acetate and succinate [3]. However, strain W1485 lip 2 is unable to grow on oxidisable substrates such as acetate and succinate in the absence of added lipoic acid and the growth yield on glucose is greater in the presence of lipoic acid, indicating a role for lipoic acid in oxidative metabolism. Similar results have been fully described by Herbert and Guest [3] and they have been ascribed to a requirement for lipoic acid in the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes.

The effect of lipoic acid depletion and addition on oxidative phosphorylation is shown in tables 1 and 2. The results described in table 1 show that membrane vesicles from lipoate-deficient cells have a competent respiratory chain capable of HOQNO sensitive oxidation of NADH, succinate and D-lactate. The oxidation rates are comparable to those of lipoate supplemented cells, except for succinate oxidation, which is approximately 50% higher in lipoate deficient vesicles.

The lipoate-deficient vesicles do not catalyse oxidative phosphorylation but are able to do so on addition of lipoic acid to the incubation mixture (table 2). Phosphorylation restored by lipoic acid is sensitive to dicyclohexyl carbodiimide, respiratory chain inhibitors and to uncoupling agents with all substrates. The amounts of lipoic acid added in these experiments (1 μmol) is 100–200-times higher than the lipoic acid (lipoic acid conjugate) content of *E.coli* membrane vesicles (M. Partis and D. E. Griffiths, unpublished results) and well above the concentrations con-

Table 2
Restoration of oxidative phosphorylation in membrane vesicles from lipoic-deficient *E.coli* (W1485 lip 2)

Additions	ATP synthesis (nmol/min)		Oxygen uptake (ng atom/min)
	ΔP_i	ΔG6P	
None	0	0	< 10
Lipoic acid	0	0	< 10
NADH + lipoic acid	135	124	205
D-lactate + lipoic acid	127	115	180
Succinate + lipoic acid	153	144	170
NADH + lipoic acid + 5 μg HOQNO[a]	0	0	30
D-lactate + lipoic acid + 5 μg FCCP[a]	0	0	210
Succinate + lipoic acid + 5 μmol malonate[a]	0	0	20
Succinate + lipoic acid + 5 μg DCCD[a]	0	0	170

[a] Experiments which are not listed in the table show that phosphorylation and respiration with all substrates were inhibited by 5 μg HOQNO. Phosphorylation and respiration were inhibited by malonate (5 μmol) with succinate as substrate only. FCCP (5 μg) and DCCD (5 μg) abolished phosphorylation with all substrates but did not inhibit respiration. FCCP gives only slight stimulation (20–30%) of respiration as the reaction mixture contains hexokinase.

ATP Synthesis and oxygen uptake were assayed in a glucose–hexokinase trap system [1,2] as described in Materials and methods. Additions of substrates were as described in the legend to table 1. Lipoic acid (1 μmol) was added 5 min before the addition of substrate. *E.coli* membrane vesicles from lipoic-deficient cells, 1 mg protein.

Table 3
Cofactor role of lipoic acid and specificity of restoration of oxidative phosphorylation in membrane vesicles from lipoic-deficient *E.coli* (W1485 lip 2)

Additions	ATP synthesis (nmol/min)		Oxygen uptake (ng atom/min)
	ΔP_i	ΔG6P	
None	0	0	< 10
Lipoic acid (1 μmol)	0	0	< 10
Succinate	0	0	172
Succinate + lipoic acid (1 μmol)	154	144	170
Succinate + lipoic acid (100 nmol)	135	129	170
Succinate + lipoic acid (10 nmol)	99	93	n.t.
Succinate + lipoic acid (5 nmol)	52	50	n.t.
Succinate + lipoic acid (5 nmol) + 8-MLA (5 nmol)	29	30	165
Succinate + lipoic acid (5 nmol) + 8-MLA (10 nmol)	7	5	170
Succinate + lipoamide (1 μmol)	0	0	165

ATP synthesis and oxygen uptake was assayed as described in Materials and methods and the legend to table 1. Cofactors and inhibitors were added to the reaction medium 5 min before initiation of the reaction with succinate (10 μmol). Incubation was for 20 min at 30°C. *E.coli* membrane vesicles from lipoic-deficient cells, 1 mg protein. 8-MLA was added as the reduced compound. n.t.=not tested.

sistent with a membrane-bound cofactor function for lipoic acid. Table 3 shows that with succinate as substrate, 33% of maximal phosphorylation is restored by 5 nmol DL-lipoic acid/mg protein and 65% of maximal phosphorylation is restored by 10 nmol DL-lipoic acid/mg protein. These values are consistent with a membrane bound cofactor function for lipoic acid, particularly when it is considered that the incubation conditions for maximal incorporation of lipoic acid, have not been delineated and that a racemic mixture of lipoates is used.

Table 3 also illustrates the specificity of oxidative phosphorylation restoration by lipoic acid. Lipoamide is unable to replace lipoic acid, indicating a structural requirement for the carboxyl group. 8-Methyl lipoate acid, an analogue of lipoic acid, is a known inhibitor of aerobic growth and lipoic acid requiring reactions [8,9] and dihydro-8-methyllipoic acid has been shown to inhibit oxidative phosphorylation and ATP-driven reactions in heart mitochondria [10]. Table 3 also shows that dihydro-DL-8-methyl lipoic acid added in equimolar amounts with DL-lipoic acid leads to marked inhibition of restoration of oxidative phosphorylation by lipoic acid.

4. Discussion

The restoration of oxidative phosphorylation by lipoic acid in membrane vesicles utilising NADH, succinate or D-lactate as respiratory chain substrates indicates a specific function for lipoic acid in oxidative phosphorylation, unrelated to its cofactor function in pyruvic dehydrogenase and α-ketoglutarate dehydrogenase.

It is not known if added free lipoic acid acts as the cofactor or whether it is incorporated during the preincubation period into a lipoic acid conjugate, which is the true cofactor. The inability of lipoamide to replace lipoic acid would suggest that a free carboxyl group is required for incorporation of lipoic acid into a conjugate form. Further studies utilising lipoic acid esters and lipoyl-adenylate, together with identification of the conjugate form of lipoic acid

present in *E.coli* membrane vesicles, are required. The marked inhibition of restoration of ATP synthesis by dihydro-8-methyl lipoic acid is consistent with its action as a known inhibitor of energy-transfer reactions in mitochondrial oxidative phosphorylation [10] its ability to inhibit growth of *E.coli* [8] and other lipoic acid requiring reactions [9].

The presence of apparently normal respiratory chain oxidation pathways for NADH, succinate and D-lactate in lipoate-deficient membrane vesicles also indicates that lipoate is not required for non-phosphorylating electron-transport. These findings are consistent with other studies that lipoic acid is a cofactor which serves a 'coupling' or energy-transfer function between the electron-transport chain and the ATP–synthase complex [1,10] and is involved in the terminal reactions of mitochondrial oxidative phosphorylation [1,2] and chloroplast photophosphorylation (Griffiths, D. E. and Hyams, R. L., unpublished observations) and phosphorylation in chromatophores of *Rhodopseudomonas capsulata* (Melandri, B. and Bertoli, E., unpublished observations).

The general properties of lipoate-requiring mutants (low growth on glucose, lack of growth on oxidisable substrates) are similar to those of oxidative phosphorylation mutants in *E.coli* described by many authors [11] and the inability of lipoate auxotrophs to grow on acetate and succinate has been commented on previously [9]. As lipoate auxotrophs contain a functional respiratory chain, they provide a suitable experimental system for evaluation of the role of lipoic acid and lipoic acid derivatives, not only in oxidative phosphorylation, but also in respiration-driven proton translocation, ion-transport and energy-linked reactions.

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