OXIDATIVE PHOSPHORYLATION AND PROTON TRANSLOCATION IN A LIPOATE-DEFICIENT MUTANT OF *ESCHERICHIA COLI*

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1. Introduction

On the basis of experiments performed in a large variety of energy-transducing systems and on isolated ATPase complex from mitochondria, a fundamental role in the mechanism of membrane-associated ATP synthesis is played by lipoic acid has been suggested, which should provide the link coupling the electron-transport chains and the ATP-synthetase complexes [1–3]. According to [1,4] the terminal reactions of phosphorylation in membranous systems involve a cycle of transacylation and transphosphorylation reactions analogous to those involved in substrate level phosphorylation. The main evidence in support of this proposal is the observation that membrane vesicles from the lipoic acid-auxotroph mutant *Escherichia coli* W1485 lip2 are not able to carry out electron flow-dependent ATP synthesis and that incubation for a short time of these preparations with lipoic acid restores this capability fully [3].

Using vesicles of the same organism respiration- and ATP-driven proton translocation was demonstrated [6], as monitored by quenching of atebrine fluorescence. The extent and the response to inhibitors of the quenching was the same as observed in vesicles prepared from *E. coli* W1485 lip2 grown in the presence of lipoic acid. However, no data on oxidative phosphorylation are reported in this work.

Here we confirm the findings [6] that proton translocation occurs in particles from the *lip*$^-$ mutant and we present evidence that the same membranes can synthetize ATP associated to NADH oxidation. Similar P/2e$^-$ ratios and identical sensitivity to inhibitors are found in membranes prepared from the same organism grown without or with lipoic acid. In addition we have tested the effect of DBCT on ATPase activity of membranes from the same organism. This compound has been reported to bind covalently to lipoic acid in submitochondrial particles [1,7].

While this work was in progress data pointing to the same conclusions was published [8].

2. Materials and methods

DL-lipoic acid was obtained from Sigma (St Louis), DCCD from Merck (Darmstadt), DBCT was a gift from D. E. Griffiths, University of Warwick. *Escherichia coli* W1485 lip2 was from Dr H. Guest's collection and was given to us by M. D. Partis, University of Warwick. Details on its isolation and general properties have been extensively described [9–11].

For large scale growth, single colonies of the mutant were isolated by replica-plating technique and grown essentially as in [10]. The cells used in each experiment were tested for the possible presence of revertants by polarographic assays of pyruvate and succinate-dependent respiration [10] and by specific growth tests. For membrane preparations the cells were harvested, washed twice with 50 mM Tris–Cl

Abbreviations: DBCT, dibutylchloromethylthynchloride; DCCD, dicyclohexylcarbodiimide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis-(α-aminoethylether)*N,N'*-tetraacetic acid; FCCP, carbonyl-cyanide-*p*-trifluoro-methoxyphenylhydrazone; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide

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buffer (pH 8.0) containing 0.25 M sucrose and 10 mM $MgCl_2$ and resuspended in 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.3) containing 0.25 M sucrose, 10 mM $MgCl_2$, 0.25 mM EGTA. Inside-out vesicles were prepared by passage through a French pressure cell operating at 8000 p.s.i. Membranes were isolated by differential centrifugation and resuspended in Hepes buffer and used within a few hours. ATP synthesis was measured as in [12] with [^{32}]phosphate in a hexokinase trap system. The reaction was carried out at 30°C in a 1.7 ml oxygraph vessel with a Clark-type electrode (Yellow Spring) for simultaneous recording of oxygen consumption. Proton translocation was monitored by atebrin fluorescence as in [13]. ATPase activity was measured as in [14].

3. Results and discussion

The lipoic acid-deficient cells of *E. coli* W1485 lip2 used in our experiments were isolated by replica plating and grown in the complete absence of lipoic acid. They were routinely checked for revertants by plating on succinate media with or without addition of lipoic acid and by their inability to oxidize pyruvate in absence of added lipoic acid. We also tested the 2-oxoglutarate dehydrogenase activity.

As shown in table 1 in vesicles prepared from these cells by the above method NADH-dependent oxidative phosphorylation occurs. The P/O values observed in different preparations vary between 0.2 and 0.6 and were in the same range of those found in vesicles prepared from cells grown in the presence of lipoic acid or in wild-type cells (strain K12). Oxidative phosphorylation was sensitive to classical inhibitors of different kinds, such as FCCP, valinomycin plus nigericin, aurovertin, DBCT, HOQNO. Incubation with lipoic acid as in [3] had no stimulatory effect. Phosphorylating particles of *E. coli* W1485 lip2 grown in presence or absence of lipoic acid always showed proton translocation as measured by quenching of atebrin fluorescence (fig.1). The quenching driven by respiration (NADH as substrate) or by ATP hydrolysis were inhibited by FCCP. If the vesicles were prepared as in [3] (lysozyme–EDTA particles) much lower values of phosphorylating activity were detected; however lipoic acid had very little, if any, restorative effect.

DBCT has been reported to inhibit ATPase in submitochondrial particles or in oligomycin-sensitive ATPase; it has been suggested that this inhibition is due to a specific binding to lipoic acid [1,7]. It was therefore of interest to test the effect of this inhibitor on ATPase activity in membranes from the lipoic acid-deficient mutant. Figure 2 shows that DBCT inhibits ATPase activity in membranes from *E. coli* W1485 lip2, even if at a lower degree than in membranes

Table 1
Oxidative phosphorylation and the effect of various inhibitors in membrane preparations from *E. coli* W1485 lip2, grown in the absence or in the presence of lipoic acid

Additions to the assay	– Lipoic acid			+ Lipoic acid		
	ATP synthesis	Oxygen uptake	P/O	ATP synthesis	Oxygen uptake	P/O
–	49.1	133.0	0.37	42.2	122.0	0.35
FCCP (5 μM)	0	173.5	0	0	164.7	0
Valinomycin (1 μg/ml) + nigericin (1 μg/ml) + KCl 50 mM	0.4	161.9	0			
Aurovertin (10 μM)	7.0	141.7	0.05	6.1	119.8	0.05
DBCT (10 nmol/mg prot.)	3.5	104.1	0.03			
HOQNO (5 μg/ml)	3.0	17.4	0.17			
Lipoic acid (0.5 mM)	24.6	130.1	0.19			

NADH-dependent ATP synthesis and oxygen uptake were expressed, respectively, as $nmol.min^{-1}.mg^{-1}$ protein and ng $atoms.min^{-1}.mg^{-1}$ protein

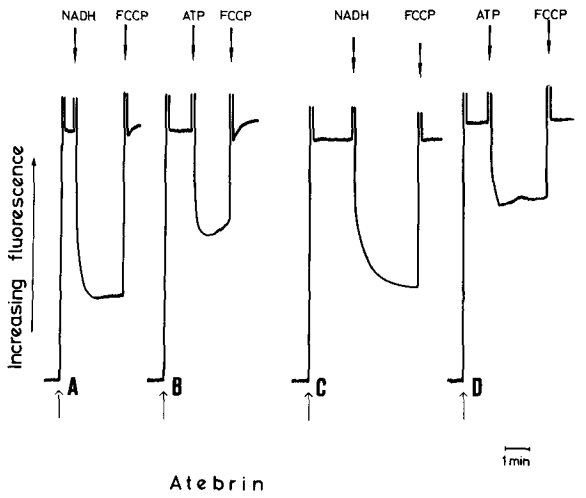

Fig.1. Quenching of atebrin fluorescence in vesicles of *E. coli* W1485 lip2 grown in absence or presence of lipoic acid. Additions: 0.7 mM NADH, 2 mM ATP and 1 μM FCCP. (A,B) Vesicles from unsupplemented cultures. (C,D) Vesicles from lipoate-supplemented cultures.

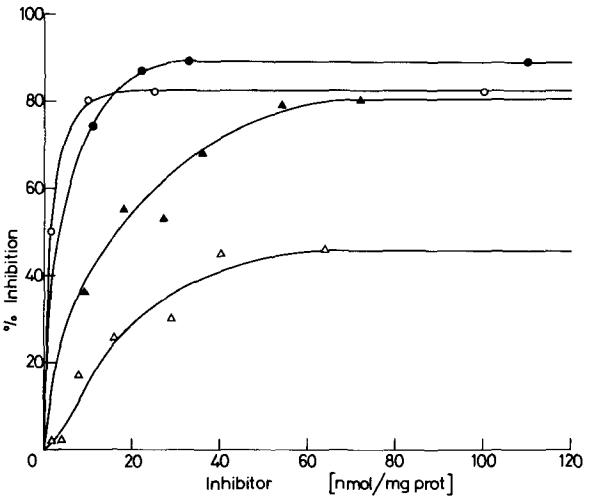

Fig.2. Inhibition of ATPase activity by DBCT and DCCD in vesicles of *E. coli* W1485 lip2 grown in absence or presence of lipoic acid. Symbols: vesicles from unsupplemented cultures: DBCT (△ – △), DCCD (○ – ○); from supplemented cultures: DBCT (▲ – ▲), DCCD (● – ●).

from lipoic acid-supplemented cells. The inhibitory effect by DCCD was similar in both types of vesicles.

The data presented confirm the results in [6] on the occurrence of proton translocation and are in agreement with the conclusions in [8]. The complete absence of D-lipoic acid in the membrane preparations used in the present experiments was confirmed by bioassays, performed by us and kindly duplicated in Dr Griffiths' laboratory on extracts obtained either by solvent extraction or alkaline hydrolysis of the membranes [10]. These analytical results were in agreement with the characteristics of growth of the cells which showed an absolute requirement for exogenous lipoate, when grown on a minimal medium containing succinate as the only carbon source.

In conclusion our experimental results strongly indicate that oxidative phosphorylation can take place 'in vitro' in membrane of *E. coli* free of any detectable amount of free or bound lipoate. These findings are in contrast with [1–6]. In addition the observation of a DBCT-sensitive ATPase activity in these membranes casts some doubts on the proposal that DBCT inhibition is only due to a specific and covalent interaction with membrane-associated lipoic acid.

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