ATP SYNTHESIS DRIVEN BY A pH GRADIENT IMPOSED ACROSS THE CELL MEMBRANES OF LIPOIC ACID AND UNSATURATED FATTY ACID AUXOTROPHS OF *ESCHERICHIA COLI*

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1. Introduction

Griffiths et al. have suggested that lipoic and oleic acids are involved in oxidative phosphorylation [1–7]. In the proposed cycle of reactions ('oleoyl cycle') the energy available from substrate oxidation through the respiratory chain is coupled to the formation of oleoyl-*S*-lipoate from reduced lipoate and oleate. Oleoyl-*S*-lipoate, through the intermediate formation of an oleoyl-*S*-acyl-carrier protein, is converted to oleoyl phosphate with incorporation of inorganic phosphate from the medium. Oleate is regenerated from oleoyl phosphate by transphosphorylation of ADP. The terminal reactions of this cycle are catalyzed by the ATPase synthetase complex [1,8].

In [9] we showed that intact cells and membrane vesicles from a lipoic acid auxotroph of *Escherichia coli* could carry out several energy-dependent functions (active transport of proline, aerobic and ATP-driven proton translocation and transhydrogenation of $NADP^+$ by NADH, oxidative phosphorylation) in the absence of lipoic acid. Similar results have been reported in [10]. In these experiments oxidative phosphorylation was measured in everted membrane vesicles. This is not the ideal system for such measurements since P:O ratios are generally much lower than would be predicted from theoretical considerations. Recent studies have shown that imposition of a pH gradient across the membrane of intact cells of *E. coli* leads to the formation of ATP in a process involving the ATP synthetase complex of oxidative phosphorylation [11,12]. We have used this method here to show that ATP formation driven by an artificially-imposed proton gradient will occur in the absence of lipoic acid. Furthermore, we have found that oleic acid also is not required for this process in cells of an unsaturated fatty acid auxotroph of *E. coli.*

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone

2. Methods

E. coli W1485 *lip*2 [13], a lipoic acid auxotroph, and *E. coli* K1060 [14], an unsaturated fatty acid auxotroph, were used. These strains were kindly supplied by Drs J. R. Guest (University of Sheffield) and Dr P. Overath (Max-Planck-Institut für Biologie, Tübingen). *E. coli* W1485 *lip*2 was grown aerobically at 37°C on a minimal medium containing 11 mM glucose, 4 mM sodium acetate and 4 mM sodium succinate, as in [9]. *E. coli* K1060 was grown on M63 medium supplemented with 0.5% glycerol, 0.25% Triton X-100, 0.1% casein amino acids, and 0.01% elaidic acid as in [15]. The cells were harvested at the end of the exponential phase of growth and washed twice with the respective minimal medium supplemented with (K1060) or without (W1485 lip2) 0.25% Triton X-100. Cells of *E. coli* K1060 were washed twice more with minimal medium lacking Triton X-100.

2.1. *Measurement of ATP formed in response to an artificially imposed pH gradient*

The cells were depleted of endogeneous reserves by the method in [16]. Cells, suspended at 2 mg protein/ml in the respective minimal medium containing 40 mM sodium azide and 20 mM α-methyl glucoside, were incubated with aeration for 2 h at 37°C. The cells were sedimented by centrifugation and washed twice with 0.2 M sodium phosphate buffer (pH 8.0) and resuspended in the same buffer to give an A_{650} of 100–140. The cells were pre-equilibrated for 30 min at 22°C and then diluted 25-fold with the same buffer. After incubation for 10 min at 22°C (W1485 *lip*2) or 37°C (K1060), ATP synthesis was initiated by lowering the external pH from 8.0 to 3.1 by the addition of 2 N HCl [11,12]. At timed intervals 1.0 ml samples were pipetted into 1.4 M perchloric acid. Intracellular ATP was extracted and measured as in [17].

2.2. *Measurement of membrane potential*

Washed cells of *E. coli* W1485 *lip*2 were resuspended in 50 mM potassium phosphate buffer (pH 6.8) disrupted in a French press, and everted vesicles prepared as in [9]. The membrane potential was measured from the equilibration of thiocyanate as detailed in [18].

3. Results and discussion

E. coli W1485 *lip*2 grown in the presence of acetate and succinate yields lipoic acid-free cells [9,13]. The absence of lipoic acid in each batch of cells was verified by measuring the inability of the cells to oxidize α-ketoglutarate in the absence of added lipoic acid. In some instances, lipoic acid was shown to be absent by direct analysis [19]. The unsaturated fatty acid auxotroph, *E. coli* K1060, cannot synthesize or degrade unsaturated fatty acids. Although we did not examine the unsaturated fatty acid content of the cells used in our experiments, other workers have clearly shown that growth on elaidic acid-containing medium will result in this acid being the only unsaturated fatty acid present in the cell [14,20,21]. In our experiments the cells were adapted to growth in elaidic acid by several serial transfers into elaidic acid-containing medium. It is unlikely that a significant amount of another unsaturated fatty acid could be carried over from the stock culture of this organism. For our experiments, *E. coli* K1060 was grown on elaidic acid (*trans*-Δ^9-$C_{18:1}$) since this acid could not replace oleic acid (*cis*-Δ^9-$C_{18:1}$) in the reactions of the oleoyl cycle [3,4].

Figure 1 shows that imposition of an artificial pH gradient across the membranes of lipoate-deficient cells of *E. coli* W1485 *lip*2 and oleate-deficient cells of *E. coli* K1060 results in the formation of ATP. As has been shown using other strains [12], the formation of ATP under these conditions is dependent on the presence of an intact ATP synthetase complex since mutants in the F_1 component of the complex were unable to form ATP. ATP synthesis was abolished by DCCD, an inhibitor of the ATP synthetase complex [19], and by the uncoupler FCCP (fig.1). Thus, the ATP generated in our experiments involved the oxidative phosphorylation system and was not produced by substrate level phosphorylation. The amount of ATP formed (2–8 nmol/mg protein) is consistent with the results in [11,12].

The membrane potential generated in everted membrane vesicles by substrate oxidation through the respiratory chain can be determined using an ion-selective electrode by measuring the equilibration of

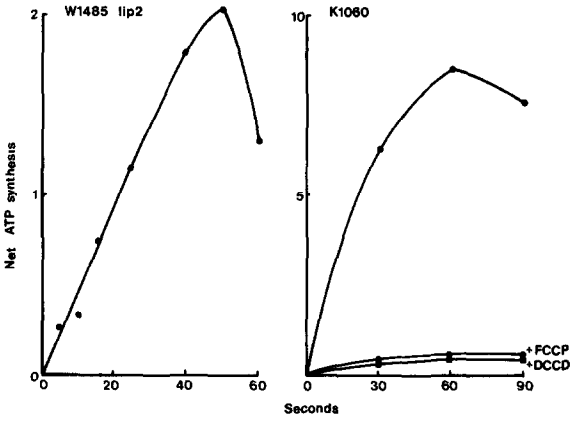

Fig.1. ATP formation in intact cells of *E. coli* W1485 *lip*2 and K1060 in response to the imposition of a pH gradient. ATP synthesis, expressed as nmol/mg cell protein, is corrected for endogenous levels of ATP (W1485 *lip*2, 1.22 nmol ATP/mg protein; K1060, 0.25 nmol ATP/mg protein). DCCD and FCCP were present at 1 mM and 20 μM, respectively.

Table 1
Formation of a membrane potential in lipoic acid-deficient everted vesicles of *E. coli* W1485 *lip*2

Substrate	Oxidase activity		Membrane potential (mV)	
	– lip	+ lip	– lip	+ lip
NADH	568	568	107 ± 7 (4)	106 ± 7 (2)
Succinate	56	57	95 ± 5 (3)	93 ± 3 (2)
D-Lactate	27	26	69 ± 3 (3)	67 ± 1 (2)

Everted membrane vesicles were prepared in 100 mM potassium phosphate buffer (pH 6.8) containing 5 mM magnesium acetate. They were suspended at 33.8 mg protein/ml in 100 mM sodium phosphate (pH 7.5) containing 5 mM magnesium acetate. The membrane potential was measured in this buffer using 1.0 ml membrane suspension in final vol. 4.6 ml. NADH, succinate and D-lactate were 2 mM, 5 mM and 5 mM, respectively. Oxidase activity is expressed in ng atoms oxygen used/min/mg protein. The membrane potential is expressed as the mean of several values. The number of separate determinations is shown in parenthesis. Lipoic acid (lip) was present at 0.2 mM in the assay medium where indicated

thiocyanate across the membrane in response to the potential difference [18,20]. Oxidation of NADH and succinate lead to the generation of membrane potentials (interior of vesicle positive) of 95–110 mV in everted vesicles from lipoic acid-deficient cells (table 1). These values are in good agreement with membrane potentials of 97 mV and 125 mV determined for the oxidation of succinate and NADH by everted vesicles from wild-type cells grown on a glucose medium [18]. However, the membrane potential of 69 mV generated by oxidation of D-lactate is lower than the value of 110 mV found with wild-type cells [18]. This is probably due to the 7-fold lower lactate oxidase activity of the membrane vesicles used in the present experiments.

According to the chemiosmotic hypothesis [21], the energized state which is used to drive energy-dependent reactions associated with the plasma membrane of bacteria or the mitochondrial membrane, is an electrochemical gradient of protons consisting of a transmembrane pH difference and a membrane potential. Since the pH difference and/or membrane potential can provide energy for ATP formation [12], one of the 'high energy' compounds of the oleoyl cycle, oleoyl-*S*-lipoate, oleoyl acyl-carrier protein, or oleoyl phosphate, would have to be in equilibrium with the electrochemical gradient of protons. Our results show that the formation of ATP could be driven by the pH difference in either lipoic or oleic acid-deficient cells. That is, in cells in which these high energy compounds would not be present. Furthermore, a membrane potential could be generated by substrate oxidation through the respiratory chain in the absence of lipoic acid. Thus, it seems unlikely that lipoic or oleic acids are on the pathway which generates an energized state of the membrane by substrate oxidation or uses it in the formation of ATP.

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