ATP SYNTHESIS BY AN ARTIFICIAL PROTON GRADIENT IN RIGHT-SIDE-OUT MEMBRANE VESICLES OF ESCHERICHIA COLI

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SUMMARY: Membrane vesicles formed from spheroplasts of E. coli lysed in the presence of ADP and P_i produced ATP when an artificial proton gradient (acid outside) was formed across the membrane. ATP synthesis required Mg^{2+} and ADP, was inhibited by dicyclohexylcarbodiimide and carbonyl cyanide p-trifluoromethoxyphenylhydrazone, and stimulated by valinomycin in the presence of KCl. Synthesis was absent in a mutant lacking the Mg^{2+}-ATPase. The optimum external pH was 2.5 when the internal pH was 8.2. Oxidative phosphorylation driven by D-lactate or succinate was also observed.

According to the formulation of Mitchell (1), the Mg^{2+}-ATPase of energy-transducing membranes is a primary active transport system for protons, so that hydrolysis of ATP can generate an electrochemical gradient of protons (acid on the side of the membrane trans to the Mg^{2+}-ATPase), while energy from a previously-established proton gradient can be coupled to the reverse reaction, phosphorylation of ADP. The total driving force for the reaction is a result of both the chemical and electrical gradients of protons, termed the "protonmotive force". While the physiological origin of the proton gradient would be oxidation of substances by the electron transport chain, the classic experiments of Jagendorf and Uribe (2) established that a proton gradient of the proper polarity artificially established across an energy-transducing membrane, in this case, the chloroplast membrane, could drive the phosphorylation of ADP to form ATP.

Since that time ATP synthesis driven by an artificially-imposed proton gradient or membrane potential or both has been shown to occur in mitochondria (3,4), inverted submitochondrial vesicles (5) and whole bacteria (6,7). Analogous experiments have not been reported for right-side-out bacterial vesicles. A major difficulty with such experiments is due to the sidedness of the membrane. The membrane of most bacteria is impermeable to adenine nucleotides, so that exogenously added ADP is not accessible to the Mg^{2+}-ATPase, which is located primarily on the inner surface of the bacterial inner membrane (8).

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Likewise oxidative phosphorylation cannot be measured in right-side-out vesicles (9-11), while inverted vesicles do carry out that process (12,13). Incubation of right-side-out vesicles with substrates of the Mg^{2+}-ATPase for long periods at low temperature have been found to allow for a small amount of ATP synthesis (14).

In this paper we report a procedure for loading right-side-out membrane vesicles of E. coli with ADP. These vesicles are capable of oxidative phosphorylation and ATP synthesis driven by a pH jump (rapid shift in external pH from 8.2 to 2.5).

MATERIALS AND METHODS: E. coli strains 7 and NR70, a Mg^{2+}-ATPase negative derivative (15), were grown in basal salts medium (16) supplemented with 68 mM glycerol and were harvested in exponential phase. Membrane vesicles were prepared by a modification of the procedure of Kaback (17) in order to load with ADP. In this modified method, spheroplasts were lysed in a small volume (20 ml/g wet spheroplasts) of 50 mM potassium phosphate, pH 6.6, containing 5 mM ADP, by repeated passage of the spheroplasts through a syringe equipped with a 22 guage needle. EDTA, $MgSO_4$ and DNase were added sequentially, unbroken cells and debris were removed by centrifugation, and the vesicles were collected as a pellet, all according to the method of Kaback (17). The vesicles were washed once with 50 mM potassium phosphate, pH 6.6, containing 5 mM ADP, and once with 10 mM potassium phosphate, pH 8.2, containing 5 mM $MgCl_2$ and 0.28 M sucrose. The vesicles were resuspended in the latter solution at a concentration of about 20 mg/ml of membrane protein and used within several hours of preparation. As noted in the text, constituents of the buffer solutions were omitted in certain experiments.

Oxidative phosphorylation was assayed at 23° in a buffer consisting of 10 mM potassium phosphate, pH 6.5, containing 5 mM $MgCl_2$, 0.28 M sucrose and 1 mg/ml of membrane protein. The reaction was initiated by the addition either of 20 mM D-lactate or 20 mM succinate. Portions (0.1 ml) were withdrawn at the indicated times and immediately diluted with 0.1 ml of cold 12% perchloric acid. ATP content was measured using a firefly luciferin-luciferase assay as described by Berger and Heppel (18).

ATP synthesis driven by a proton gradient was measured at 23° by a 20-fold dilution of the vesicles into the same buffer solution adjusted to pH 2.5 with HCl. Portions (0.1 ml) were withdrawn at 5 sec intervals following the pH shift, diluted into perchloric acid, and assayed for ATP content as given above. Each assay was performed in parallel with assays in which the dilution buffer had the same pH as that in which the vesicles were suspended. The values found in such assays were consistently quite large relative to the increases found in the pH jump assays (0.13 to 0.19 nmole/mg protein without a pH jump, compared to 0.4 to 0.6 nmole/mg 5 sec after a pH jump) but did not change with time. The average value for assays without a pH jump were subtracted from those with a pH jump. The results in Table I and Fig 3 were obtained 5 sec after the pH jump and are expressed as nmole ATP synthesized/min/mg of membrane protein. In experiments designed to determine the necessity for Mg^{2+}, the order of addition of EDTA and $MgSO_4$ was reversed during the incubation at 37° following lysis of the spheroplasts. The concentration of $MgSO_4$ was reduced from 15 mM to 5 mM in that step. $MgCl_2$ was omitted from the later steps. To examine the effect of valinomycin, FCCP[2] or DCCD, those reagents were added to both the vesicles and

[2]Abbreviations: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; DCCD, dicyclohexylcarbodiimide.

the acid solution at the concentrations indicated below 10 min prior to the pH shift.

Protein concentrations were measured by a modification of the method of Lowry et al (19).

Firefly lantern extract (FLE-50), ADP, ATP, and valinomycin were all purchased from Sigma Chemical Co. FCCP was the generous gift of Dr. P. G. Heytler of the E. I. Dupont de Nemours Co. All other compounds were reagent grade and purchased from commercial sources. ADP, ATP, and succinate were used as the sodium salts. D-lactate was used as the lithium salt.

RESULTS: Since right-side-out vesicles are reported not to carry out oxidative phosphorylation (9-11), it was necessary first to find conditions permitting such vesicles to phosphorylate ADP. As shown in Fig. 1, oxidation of D-lactate

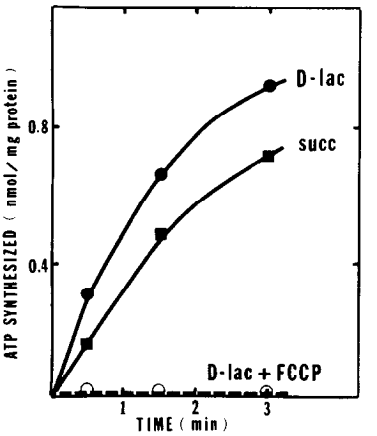

Fig 1 - Oxidative phosphorylation in ADP-loaded membrane vesicles. Preparation of vesicles and oxidative phosphorylation were measured as described in Methods. (●—●), + 20 mM D-lactate; (■—■), + 20 mM succinate; (○--○), + 20 mM D-lactate and 5 μM FCCP.

or succinate could supply energy for the phosphorylation of ADP in vesicles prepared from spheroplasts lysed in the presence of ADP. While the P/O values are low (less than 0.1), the uncoupler FCCP completely prevented ATP synthesis. Oxidative phosphorylation in right-side-out vesicles is currently under investigation in our laboratory, and further details will be presented in a later communication.

Since ADP-loaded vesicles were capable of oxidative phosphorylation, it was of interest to determine if an artificial proton gradient could drive the phosphorylation of ADP. When the vesicles were prepared in a buffer of pH 8.2 and diluted into a solution of pH 2.5, rapid formation of ATP was observed (Fig 2). Maximal synthesis occurred in about 10-15 sec, followed by a decline in ATP

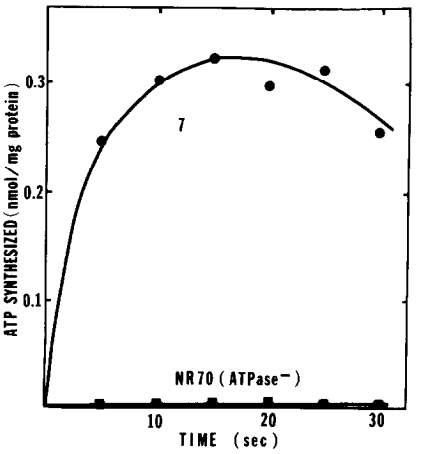

Fig 2 - ATP synthesis driven by an artificial proton gradient. Vesicles were prepared as described in Methods in buffer at pH 8.2. The reaction was initiated by dilution of vesicles into pH 2.5 solution. (●—●), strain 7; (■—■), strain NR70.

TABLE I: Effect of various factors on phosphorylation of ADP driven by an artificial pH gradient. Vesicles were prepared and assayed for ATP synthesis as described under Methods. The inner pH was 8.2 and the outer, 2.5.

Experiment	Addition or Deletion	ATP Synthesized nmol/min/mg protein	% Control
1	control	3.31	100
	-ADP	0.05	2
	$-Mg^{2+}$	0.00	0
	+5 mM $MgCl_2$*	1.99	60
2	control	3.06	100
	+2 μg/ml valinomycin	4.61	150
3	control	4.56	100
	+5 μM FCCP	0.00	0
	+50 μM DCCD	2.56	56
	+100 μM DCCD	1.99	44

*$MgCl_2$ was added to the assay mixture and to Mg^{2+}-depleted vesicles 10 min prior to the pH jump.

levels. After a few min the ATP level fell to the basal level (data not shown). Strain NR70, which lacks the Mg^{2+}-ATPase, was unable to synthesize ATP (Fig 2).

The process required both ADP and Mg^{2+}. Vesicles not loaded with ADP showed extremely little ATP synthesis (Table I), and the basal level decreased by about 30-40%. Likewise, vesicles in which Mg^{2+} was removed by a reversal of the EDTA and $MgSO_4$ steps and omission of Mg^{2+} from all other buffers were unable to catalyze ATP synthesis (Table I). Addition of Mg^{2+} to both the assay mixture

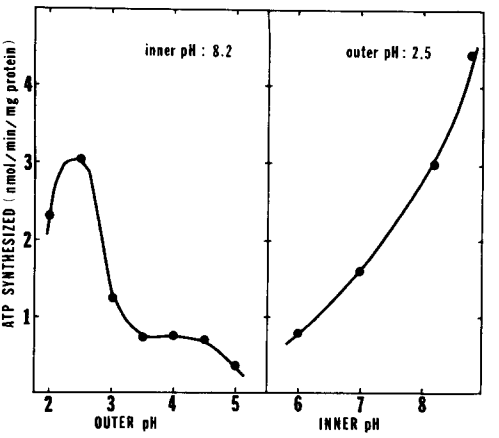

Fig 3 - Dependency of ATP synthesis on inner and outer pH. Vesicles were prepared and assayed for ATP synthesis as described in Methods, except that the pH of the solutions were varied as shown. Left: vesicles were prepared in a solution of pH 8.2, and the reaction was initiated by dilution in solutions of the indicated pH values. Right: vesicles were prepared in solutions of the indicated pH values and diluted into a solution of pH 2.5.

and vesicles10 min prior to the pH jump restored most of the activity (Table I). As shown also in Table I, valinomycin stimulated ATP synthesis 50%, while FCCP completely abolished synthesis. DCCD was also inhibitory, but only to the extent of about 50%.

The magnitude of the pH shift was examined in the experiments shown in Fig 3. When the inner pH was 8.2, maximal synthesis occurred with a pH of 2.5 in the external solution, but decreased markedly when the pH of the external solution was lower or higher. When the pH of the solution in which vesicles were prepared was varied with the outer solution being pH 2.5, the amount of synthesis of ATP increased as the pH increased. However, a precipitate believed to be a magnesium phosphate salt formed within several hours after preparation of the vesicles if the pH were greater than 8.5.

DISCUSSION: We have found that right-side-out E. coli vesicles can be loaded with ADP by lysis of spheroplasts in the presence of ADP. Futai (20) has used a similar procedure for loading vesicles with NAD^+ and alcohol dehydrogenase. These vesicles are capable of a low level of oxidative phosphorylation but, more importantly, synthesize ATP with a base to acid shift. Since a shift of base to acid is necessary and since ADP loaded into inverted vesicles would not reach the Mg^{2+}-ATPase, we can eliminate the possibility of the observed synthesis being catalyzed by inverted vesicles. That the synthesis is catalyzed by the Mg^{2+}-ATPase is probable, since vesicles from a mutant lacking that enzyme is unable to phosphorylate ADP, and since synthesis is sensitive to DCCD, an inhibitor of the Mg^{2+}-ATPase.

The effects of valinomycin and FCCP are consistent with the proton gradient as the primary driving force for ATP synthesis. According to the chemiosmotic hypothesis, the Mg^{2+}-ATPase acts as an electrogenic proton pump (1). The uptake of protons during ATP synthesis in this artificial system would, therefore, be expected to create a membrane potential, positive inside, which would slow further proton uptake. Valinomycin should allow for an efflux of potassium in response to the potential, permitting a more rapid influx of protons and a stimulation of ATP synthesis, as was found. FCCP, on the other hand, produced an electrogenic inward movement of protons, leading to the formation of a membrane potential, positive inside. The combination of a partial depletion of the proton gradient coupled with the formation of a membrane potential, positive inside, should prevent movement of protons through the Mg^{2+}-ATPase and inhibit ATP synthesis. This is in accord with our observations (Table I).

It has been reported a number of times that right-side-out bacterial vesicles lack oxidative phosphorylation ability (9-11) and yet are a major tool for the study of active transport. There have even been studies on the conversion of "transport vesicles" to "phosphorylating vesicles" (21). The implication has been that vesicles can have active transport or phosphorylation, but not both. Our data demonstrate that such distinctions are unreal.

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