RESTORATION OF ACTIVE CALCIUM TRANSPORT IN VESICLES OF AN MG^{2+}-ATPASE MUTANT OF ESCHERICHIA COLI BY WILD-TYPE MG^{2+}-ATPASE[1]

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SUMMARY: Strain NR70, a mutant of E. coli lacking the Mg^{2+}-adenosine triphosphatase (E.C. 3.6.1.3.) was previously shown to be defective in amino acid and sugar transport in whole cells and right-side-out membrane vesicles. It is shown here that the mutant is also deficient in the uptake of calcium into inverted membrane vesicles. Treatment of inverted vesicles from the wild-type strain with ethylenediamine tetraacetate removes the Mg^{2+}-adenosine triphosphatase and results in an inability to transport calcium. Addition of a crude fraction containing the wild-type Mg^{2+}-adenosine triphosphatase restores active uptake of calcium both to vesicles from the mutant and depleted vesicles from the wild-type.

INTRODUCTION: The role of the Mg^{2+}-ATPase[2] in energy transduction has traditionally been considered to be as an acceptor of energy from the electron transport chain during synthesis of ATP, and, in the reverse reaction, as the generator of utilizable energy from the hydrolysis of ATP. According to Mitchell's hypothesis (1), the driving force for many biological reactions is derived from the electrochemical gradient of protons established by the electron transport chain during respiration. Thus, the electron transport chain can be considered a permease for protons. The Mg^{2+}-ATPase should also be a proton permease when coupled to a specific membrane protein (or proteins) which acts as a proton ionophore. During ATP hydrolysis, that system acts as a H^+-translocating ATPase (2), just as other ATPases are translocators of Na^+, K^+, and Ca^{2+} (3, 4).

If the H^+-translocating protein has only a passive role in the transport of protons, then it must be regulated by the Mg^{2+}-ATPase in order to maintain the gradient of protons. Genetic loss of the Mg^{2+}-ATPase leads to inability to regulate the proton channel and to maintain the protonmotive force (5, 6), resulting in an uncoupling of energy-linked functions such as

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[2]Abbreviations: Mg^{2+}-ATPase, magnesium dependent adenosine triphosphatase; EDTA, ethylenediamine tetraacetate; DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

active transport (5-7). Active transport in E. coli can be restored chemically by treatment with DCCD, an inhibitor of the membrane bound Mg^{2+}-ATPase (5-7). DCCD has been shown to inhibit the E. coli Mg^{2+}-ATPase secondarily by binding to a membrane site (8). Since DCCD simultaneously decreases the H^+ permeability of the membrane (5, 6, 9), the DCCD-binding site may prove to be the proton-translocating protein. Further mutations in the gene for the Mg^{2+}-ATPase in Mg^{2+}-ATPase mutants can restore the ability to maintain the gradient of protons without concomitant restoration of the enzymatic activity of the Mg^{2+}-ATPase (6), suggesting a role for the protein independent of its catalytic role, namely regulation of the protonmotive force.

We have previously shown that membranes derived from Mg^{2+}-ATPase mutants lack energy-dependent quenching of quinacrine fluorescence, as do wild-type membranes from which that enzyme has been stripped (6). Addition of a crude active Mg^{2+}-ATPase restores both ATP-driven and respiratory-driven fluorescence quenching (6). Energy-dependent transhydrogenase activity has also been shown to be restored by binding of purified Mg^{2+}-ATPase (10, 11). In this communication we demonstrate that a crude active Mg^{2+}-ATPase can be bound to inverted membranes of an Mg^{2+}-ATPase mutant or to wild-type membranes from which that enzyme has been stripped. This reconstitution results in restoration of active calcium transport in inverted membranes.

MATERIALS AND METHODS: E. coli strains 7 and NR70, a Mg^{2+}-ATPase negative derivative (7), were grown in a basal salts medium (12) supplemented with 68 mM glycerol. Cells were harvested in exponential phase for the preparation of transport vesicles and in stationary phase for the preparation of crude soluble Mg^{2+}-ATPase.

The preparation of transport vesicles and the assay of calcium transport were as described previously (13) with two modifications. First, the cells were washed with and lysed in 0.1 M potassium phosphate buffer, pH 6.6, containing 10 mM EDTA, instead of 10 mM Tris buffer, pH 7.2, containing 0.15 M KCl, 1 mM β-mercaptoethanol, and 10% (v/v) glycerol (TKMG buffer). The membranes were washed with and resuspended in TKMG buffer as in the previously described method (13). Second, the pH of the transport assay buffer was altered from pH 8.5 to pH 8.0. The concentration of $^{45}Ca^{2+}$ was 1 mM in all assays. Stripped membranes and crude soluble Mg^{2+}-ATPase were prepared as previously described (6). Transport vesicles which had been treated with stripping buffer were washed once with and resuspended in TKMG buffer. Reconstitution of membranes was performed by resuspending stripped vesicles in the crude soluble fraction, followed by the addition of $MgCl_2$ to 10 mM. The vesicles were then washed with and resuspended in TKMG buffer. In

typical reconstitution experiments between 0.7 and 1 mg of membrane protein was resuspended in 1 ml containing 10 to 15 units of ATPase activity. A unit is defined as the liberation of 1 μmole of P_i/min. The crude soluble fraction was found to have no ability to accumulate calcium nor were control membranes from strain 7 stimulated by that fraction.

ATPase activity was measured in a reaction mixture (0.3 ml) containing 50 mM Tris·HCl, pH 8, 2.5 mM $MgCl_2$, 5 mM ATP, and protein. The assay was initiated by placing samples in a water bath at 37° and terminated after 5 min by the addition of 0.7 ml of cold 0.5 M trichloroacetic acid. Inorganic phosphate was determined by the method of Fiske and SubbaRow (14). Protein concentrations were estimated by a modification of the method of Lowry et al (15).

$^{45}CaCl_2$ (1.3-1.4 Ci/mmol) was purchased from New England Nuclear Corp. All other compounds were analytical grade. Solutions of DCCD and FCCP were prepared in ethanol.

RESULTS: Whole cells and right-side-out membrane vesicles of strain NR70 show reduced respiratory-driven active transport for proline, lactose and other compounds compared to strain 7 (7). These reductions could be phenotypically corrected by treatment with DCCD (5, 7). Calcium transport activity is also impaired in inverted vesicles of strain NR70 and vesicles of strain 7 which have been stripped of the Mg^{2+}-ATPase, and the activity is restored by treatment with DCCD (Fig. 1A).

When crude Mg^{2+}-ATPase is added to stripped membranes of either strain the membranes are able to rebind the enzyme (Table I). While it is not necessary to strip membranes of strain NR70, better reconstitution occurs if the membranes have first undergone the EDTA treatment. It should be pointed out that the values for Mg^{2+}-ATPase activities of strains 7 and NR70 which were previously reported (7) were obtained under different conditions. By both the assays reported here and elsewhere (16), NR70 has 1% or less of the ATP hydrolyzing activity of strain 7.

Reconstitution of stripped membranes from either strain restores the ability of those membranes to utilize either ATP or respiratory substrates such as NADH to drive calcium uptake (Fig. 2). DCCD inhibits ATP-driven calcium uptake in either control or reconstituted membranes, as shown in Fig. 1B. Respiratory-driven calcium uptake is stimulated by DCCD in stripped membranes of either strain or control membranes of strain NR70 (Fig. 1A and Table I). Reconstituted membranes of strain 7 are similar to control membranes of strain 7 in that DCCD is slightly inhibitory, while reconstituted membranes of NR70 are stimulated slightly by DCCD (Table I). Thus, the capacity for

TABLE I: Reconstitution of Mg^{2+}-ATPase and restoration of respiratory-driven calcium uptake in inverted vesicles.

Strain	Conditions	Mg^{2+}-ATPase Activity	Carbon Source	Addition	$^{45}Ca^{2+}$ Uptake	% Strain 7 Control
7	Control	4.2	D-lactate	none	88.0	100
				DCCD	69.7	79
				FCCP	8.4	10
			none	none	7.3	8
	Stripped	0.2	D-lactate	none	7.4	8
				DCCD	57.1	65
				FCCP+DCCD	4.6	5
	Reconstituted	3.5	D-lactate	none	81.5	93
				DCCD	72.4	82
				FCCP	5.8	7
NR70	Control	0.05	D-lactate	none	25.9	29
				DCCD	60.3	69
				FCCP	6.8	8
			none	none	0	0
	Stripped	0.03	D-lactate	none	2.9	3
				DCCD	48.8	55
	Reconstituted	3.1	D-lactate	none	43.4	49
				DCCD	60.5	69
				FCCP	11.4	13

Mg^{2+}-ATPase activity is expressed as μmoles P_i/min/mg protein. Calcium uptake activity is expressed as nmoles/mg/30 min. DCCD and FCCP were used at 30 μM and 5 μM, respectively, and D-lactate at 20 mM.

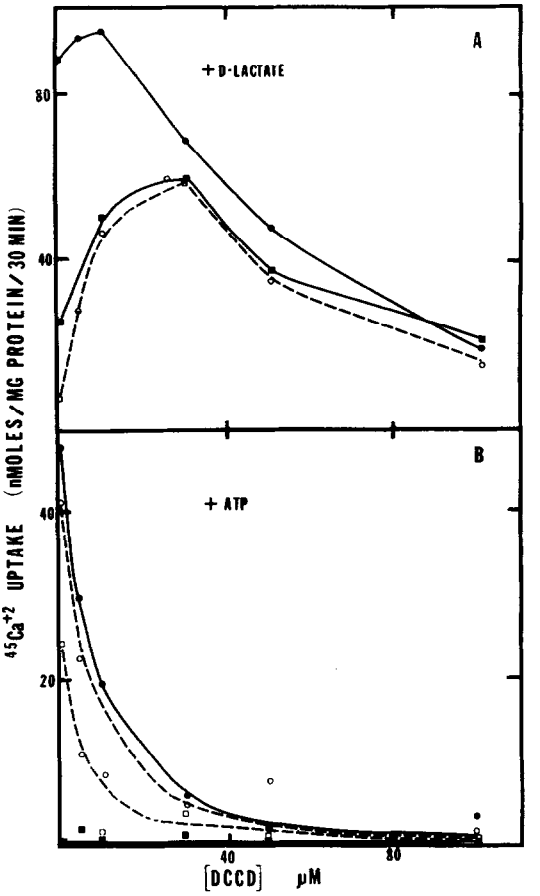

Fig. 1. Effect of DCCD on calcium transport in inverted vesicles. A- Respiratory-driven uptake with 20 mM D-lactate as energy source. —●—, strain 7 control membranes; —○—, strain 7 stripped membranes; —■—, strain NR70 control membranes. B- ATP-driven uptake with 5 mM ATP and 5 mM $MgCl_2$ as energy source. —●—, strain 7 control membranes; —○— strain 7 stripped membranes reconstituted with crude active Mg^{2+}-ATPase; —■—, strain NR70 control membranes; —□—, strain NR70 stripped membranes reconstituted with crude active Mg^{2+}-ATPase.

stimulation by DCCD is a gross measure of the extent of reconstitution. FCCP, a proton conductor, inhibits calcium transport in control vesicles, reconstituted vesicles and DCCD-treated stripped membranes (Table I), consistent with the conclusion that there is a relation between calcium uptake and a protonmotive force (13).

DISCUSSION Based on the loss of energy-coupling exhibited by certain Mg^{2+}-ATPase mutants of E. coli, we have proposed that the Mg^{2+}-ATPase has a dual role in energy transduction: a catalytic role in the synthesis and utilization of ATP and a regulatory role in the maintenance of the protonmotive force (6). A similar proposal has been used to explain the phenomenon of

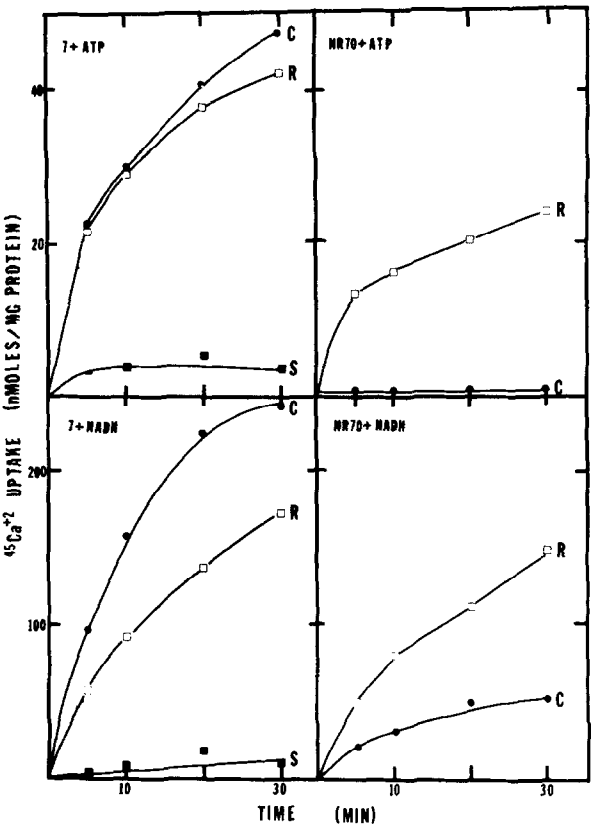

Fig. 2. Restoration of active calcium transport in inverted vesicles. TOP - ATP-driven uptake with 5 mM ATP and 5 mM $MgCl_2$ as energy source. BOTTOM - Respiratory-driven uptake with 5 mM NADH as energy source. ●, control membranes; ■, stripped membranes; □, stripped membranes reconstituted with crude active Mg^{2+}-ATPase from strain 7.

acceptor control of respiration in mitochondria (1). Our results showing the simultaneous restoration of active transport ability and the ability to maintain a transmembrane pH gradient by treatment with DCCD supported this concept (5, 7). These results have been confirmed by Altendorf et al (9) with strain DL-54, another Mg^{2+}-ATPase mutant. Even stronger evidence is the data from partial revertants of an Mg^{2+}-ATPase mutant (5). Here a secondary lesion in the Mg^{2+}-ATPase has restored the ability to maintain the pH gradient without a corresponding restoration of enzymatic activity, showing the separate nature of the two functions of that protein.

But the strongest evidence would be the restoration of energy-coupling by biochemical reconstitution with purified wild-type Mg^{2+}-ATPase. This has not been possible for active transport systems measured in whole cells or right-side-out vesicles because the Mg^{2+}-ATPase-binding site is on the inner surface of the cytoplasmic membrane. On the other hand, the Mg^{2+}-ATPase

could be bound to stripped inverted vesicles by simply incubating in the presence of Mg^{2+}-ATPase (10). However, systems which transport molecules from the medium to cytosol cannot be assayed in inverted vesicles (13, 17). The discovery that calcium transport occurs in such vesicles (13) has allowed the measurement of transport in Mg^{2+}-ATPase-depleted vesicles after reconstruction with active Mg^{2+}-ATPase. The restoration of ATP-driven calcium transport lends credence to the idea that the transport of small molecules can be supported by ATP hydrolysis via the Mg^{2+}-ATPase. But, more important, the restoration of NADH- and D-lactate-driven calcium transport by a crude soluble Mg^{2+}-ATPase strongly supports the hypothesis that it is the absence of the Mg^{2+}-ATPase in strain NR70 or stripped membranes of strain 7 which leads to the loss of energy-coupling. A more conclusive demonstration would be the effect of purified Mg^{2+}-ATPase on these processes. This has been accomplished for the energy-linked transhydrogenase (10, 11). Such an approach to the restoration of calcium transport is presently in progress in this laboratory.

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