DEPENDENCE ON Mg^{2+} FOR DIFFERENT STATES OF THE MEMBRANE-BOUND ADENOSINE TRIPHOSPHATASE OF *MICROCOCCUS LYSODEIKTICUS**

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1. Introduction

Previous work has shown the existence of an ATPase† associated with *Micrococcus lysodeikticus* cytoplasmic membranes [1–3]. The selective solubilization of the enzyme from isolated 'standard' membranes [1, 2], its purification and partial characterization [3] have been reported. In these previous reports, the 'allotopic' properties of the protein, i.e. the Mg^{2+}-trypsin dependence in its membrane-bound state and the Ca^{2+} activation of the 'soluble'-trypsin independent ATPase, were emphasized [3]. The responsibility of one protein for these different Ca^{2+}, Mg^{2+} activities has been previously substantiated [3].

These studies suggested an indirect role for divalent cations in maintaining the *M. lysodeikticus* ATPase–membrane complex [2, 4]. Salton (unpublished results) showed that *M. lysodeikticus* protoplasts required divalent cations in the high osmolality medium to stabilize them. Moreover, Ishikawa [5, 6] repeatedly reported the essential role of Mg^{2+} in oxidative phosphorylation in this organism.

The unexplained function of divalent cations in *M. lysodeikticus* membrane integrity and the absence of a protease-induced activation for membrane-bound ATPase [3], prompted us to investigate the possible relationships between Mg^{2+} and the ATPase–membrane complex. This communication describes the estimation of the apparent intracellular Mg^{2+} concentration in *M. lysodeikticus*, the detection of a trypsin-independent state of the membrane-bound ATPase and its dependence on Mg^{2+} during membrane isolation. The possible role of ATPase in cation transport is discussed.

2. Materials and methods

Micrococcus lysodeikticus (NCTC 2665) was used throughout these studies. Cell membranes were prepared as described previously either by direct lysis or by bursting preformed protoplasts [1–3]. Membranes of type A were obtained and washed five times in 50 mM tris-HCl buffer, pH 7.5 containing 50 mM $MgCl_2$. Type B membranes were obtained and washed (5X) in 50 mM tris-HCl buffer pH 7.5, 10 mM $MgCl_2$. Type C membranes were obtained from cells lysed in 50 mM tris, pH 7.5–50 mM $MgCl_2$ but the membranes were washed (5X) in the same buffer without added cations. Type D membranes were prepared and washed (5X) in 100 mM tris-HCl buffer at the same pH. For further work, each membrane preparation was suspended in the initial volume of the respective washing buffer with protein concentrations ranging from 3 mg/ml (type A) to 0.8 mg/ml (membranes D).

ATPase activity was assayed as described previous-

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† *Abbreviations:*
ATPase : Adenosine triphosphatase (EC 3.6.1.3)
TIM-ATPase : trypsin-independent membrane-bound ATPase
TDM-ATPase: trypsin-dependent membrane-bound ATPase

Table 1
Magnesium estimation in membrane and supernatant fractions of different types of *Micrococcus lysodeikticus* membrane preparations.

Membrane preparation	Membrane μeq Mg^{2+} per	Supernatant ml suspension*
A	16.14	43
B	6.52	10.5
C	5.15	0.28
D	3.33	0.19

The characteristics of each membrane preparation are given in the text.
* Mean values of duplicate experiments.

ly [2]. Incubations were carried out at 37° for 30 min. One unit of enzyme activity is defined as the amount of enzyme able to liberate 1 μmole of Pi/30 min at 37°.

Magnesium was determined according to Orange and Rhein [7]. In soluble samples Mg^{2+} estimation was performed either directly or after protein precipitation, no major difference being detected between both procedures. For Mg^{2+} determination in membrane pellets, samples were first mineralized by heating in H_2SO_4–$HClO_4$–H_2O (4:8:1:10 by vol.) Appropiate blanks and standards were run each time. Ca^{2+} was shown not to interfere with Mg^{2+} measurements by performing the determinations after oxalate precipitation or in the presence of known amounts of Ca^{2+}. Extracellular water was determined by the difference between the wet weights of the two cell pellets obtained from 5 ml of cell suspension (100–150 mg wet weight/ml) by centrifugation at 5,000 *g* for 15 min and by filtration over a 0.8 μm Millipore filter; it was 13± 2% of the total wet weight of the centrifuged pellet. The dry weight of this pellet was 19% of its wet weight and intracellular water, by difference, was 68% of the wet weight. These figures are within the range found in other bacteria [8]. Protein was determined by the method of Lowry et al. [9].

3. Results

3.1. *Intracellular Mg^{2+} concentration*

The intracellular amount of Mg^{2+} was measured in the supernatant fluid after lysis of a known volume of cell suspension. Actual figures were: 54–60 μg of Mg^{2+} per 0.111 ml of intracellular water content which gave an apparent concentration for magnesium of 20–23 mM.

3.2. *Mg^{2+} bound by cell membranes*

The measurements of Mg^{2+} bound to the different types of membrane preparations was carried out after centrifugation of 5 ml of each suspension. The determination was performed as described under Material and methods for both supernatants and pellets. Results are represented in table 1 and expressed as μeq per ml of initial suspension to facilitate comparison between the different membrane preparations. It must be noted that type C membranes still show high figures of bound magnesium despite washing in absence of divalent cation. Cation binding capacity of *M. lysodeikticus* membranes has been previously studied by Cutinelli et al. [10]. A more detailed description of magnesium binding to our membrane preparations will be reported elsewhere.

3.3. *States of the membrane-bound ATPase*

Tables 2 and 3 illustrate the properties of two states of the membrane-bound ATPase. As shown in table 2, TIM-ATPase is dependent on the Mg^{2+} concentration of the fluids used for membrane isolation and washing, being more clearly present in membranes of types A and B. The results with these two types of membrane preparations also indicate that TIM-ATPase preferentially needs Mg^{2+}-ATP as substrate but the ratio ATP/Mg^{2+} in the reaction mixture does not seem to be very critical. From table 2, a broad optimum ranging from 0.7 to 3 is observed. It is noteworthy that membrane-bound ATPase of types C and D recovers its trypsin independent activity when Mg^{2+} is added to the reaction mixture; better results are obtained with type C membranes because of their bound Mg^{2+} (see table 1). Ca^{2+} can replace Mg^{2+} as substrate but is less efficient.

Table 3 presents the characteristics of TDM-ATPase for the same types of membranes. Unlike TIM-ATPase, the trypsin-dependent state of the enzyme is obviously manifested in membranes isolated in low Mg^{2+} and it also requires Mg^{2+}-ATP as substrate with a stricter ATP/Mg^{2+} ratio for optimal activity. It is very interesting to note the progressive loss of Ca^{2+} efficiency in replacing Mg^{2+} as activator (see D membranes).All these results confirm our previous ones [3].

Table 2
TIM-ATPase in *Micrococcus lysodeikticus* isolated membranes.

Background $[Mg^{2+}]$ in enzyme prep. (mM)	Additions	ATP / Me^{2+}	ATPase activity	
			μmoles Pi/mg prot.	U/100 ml susp.
Membrane preparation A				
11.2	–	0.7	1.6	440
11.2	$MgCl_2$*	0.52	1.0	280
11.2	$CaCl_2$**	0.41	0.6	140
Membrane preparation B				
2.8	–	2.85	1.8	500
2.8	$MgCl_2$	1.17	2.0	540
2.8	$CaCl_2$	0.74	1.6	450
Membrane preparation C				
1.8	–	7.4	0.10	20
1.8	$MgCl_2$	1.57	1.02	200
1.8	$CaCl_2$	0.88	0.87	170
Membrane preparation D				
0.7	–	11.43	0.0	0
0.7	$MgCl_2$	1.70	0.60	90
0.7	$CaCl_2$	0.91	0.20	30

The reaction mixture contained, in a volume of 0.5 ml, 40 mM tris-HCl buffer pH 7.5 8 mM ATP (Pabst Laboratories) and 0.1 ml of membrane preparation equivalent to about 300–80 μg of protein. Results are the average values of triplicate experiments. Conditions for incubation are given in the text.

* 4 mM $MgCl_2$
** 8 mM $CaCl_2$

Table 3
TDM-ATPase in *Micrococcus lysodeikticus* isolated membranes.

Background $[Mg^{2+}]$ in enzyme prep. (mM)	Additions	ATP / Me^{2+}	ATPase activity	
			μmoles Pi/mg prot.	U/100 ml susp.
Membrane preparation A				
11.2	Trypsin*	0.7	2.1	600
11.2	Trypsin + $MgCl_2$**	0.52	1.6	500
11.2	Trypsin + $CaCl_2$***	0.41	1.4	430
Membrane preparation B				
2.8	Trypsin	2.85	5.6	1,500
2.8	Trypsin + $MgCl_2$	1.17	4.7	1,100
2.8	Trypsin + $CaCl_2$	0.74	3.1	700
Membrane preparation C				
1.8	Trypsin	7.4	1.12	220
1.8	Trypsin + $MgCl_2$	1.57	6.15	1,200
1.8	Trypsin + $CaCl_2$	0.88	2.66	520
Membrane preparation D				
0.7	Trypsin	11.43	0.40	60
0.7	Trypsin + $MgCl_2$	1.70	6.10	920
0.7	Trypsin + $CaCl_2$	0.91	1.45	220

The experimental conditions were the same as those given in the legend to table 2.

* 0.5 mg/ml trypsin
** 4 mM $MgCl_2$
*** 8 mM $CaCl_2$

4. Discussion

The detection of a trypsin-independent ATPase activity in cell membranes from *M. lysodeikticus* is highly relevant to the understanding of the physiological role of the membrane-bound enzyme. Our results on its magnesium dependence during membrane isolation and on the membrane-bound cation are validated by the high intracellular magnesium concentration found in this organism which indicates the existence of a pump for this divalent cation. (Evidence for a Mg^{2+} pump in *Bacillus cereus* has recently been obtained by Hurwitz and Schmid [11]). On this experimental basis, it is tempting to postulate that the TDM-ATPase plays a role in Mg^{2+} transport, regulating the hydrolysis of ATP, which will drive cation movement as a function of Mg^{2+} and ATP concentrations. On the other hand, the physiological interpretation TDM-ATPase is more difficult. Up to now no ATPase activation by proteases has been found [3]. But this may be a special mechanism functioning only during certain phases of cell growth.

The simpler explanation of two enzymes accounting for two activities must be ruled out by previous experimental evidence [2, 3, 12]. A more plausible interpretation is that Mg^{2+} induces conformational changes in the membrane complex resulting in different properties of the membrane-bound enzyme. It must be emphasized that the Mg^{2+} effect could act on neighbouring molecules and not directly on ATPase. The partial reversibility observed with membranes from C and D treatments makes this a feasible interpretation; this possibility is being investigated.

Finally we should like to add that studies of topological properties of membrane-associated enzymes can be a very useful approach to the understanding of structural–functional relationships in those structures.

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