PROPERTIES OF THE MEMBRANE–ADENOSINE TRIPHOSPHATASE COMPLEX OF *MICROCOCCUS LYSODEIKTICUS*: REVERSIBILITY OF THE Mg^{2+}-DEPENDENT STATES OF THE ATPase

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1. Introduction

In a previous report [1] we have described the detection of different states of the membrane-bound ATPase of *Micrococcus lysodeikticus* depending upon the magnesium concentration during membrane isolation. These states were characterized by distinct dependence on trypsin and divalent cation requirements to hydrolyze ATP. The physiological implications of the magnesium role were inferred from the high cation concentration found in the intracellular fluid. The exact mechanism by which the membrane complex controlled the ATPase activity was not clear at that time although, as a possible explanation, we suggested the existence of conformational changes in the membrane complex [1].

In order to gain some insight into the above mentioned phenomenon, we have attempted the restoration of the magnesium-dependent states of *M. lysodeikticus* ATPase which had been deprived of that cation. The present report describes the partial reversibility of the Mg^{2+}-induced states of the membrane bound ATPase from *M. lysodeikticus*. These results provide biochemical support to the hypothesis of conformational changes in this bacterial membrane complex. They can be interpreted on the light of the model proposed by Changeux and Thiery [2, 3] to explain regulatory processes which involve biological membranes.

2. Materials and methods

M. lysodeikticus (NCTC 2665) was used for these studies. Membranes were isolated as described previously [4–6]. Four types of membranes were obtained by using different buffer systems for isolation and washing [1]. The characteristics of each type of membrane preparation (A, B, C and D) have been detailed before [1]. They can be summarized as follows: type A membranes were obtained and washed with 50 mM $MgCl_2$, type B preparation was prepared and washed in the presence of 10 mM $MgCl_2$, whereas type C membranes were obtained with 50 mM $MgCl_2$ but washed without magnesium. Membranes D were obtained and washed with magnesium-free buffer. In some instances the membranes were further washed with 50 mM Tris-HCl buffer, pH 7.5 or with the same buffer supplemented with 5 mM EDTA as it will be marked out in the text. To restore the Mg^{2+}-dependent states, membranes were suspended at a protein concentration of 0.8–1 mg per ml in either 50 mM Tris-HCl, pH 7.5 containing 50 mM $MgCl_2$ or 50 mM Tris buffer alone. In the last case they were then dialyzed against several changes of Tris-Mg^{2+} buffer in a volume 100 times that of the membrane suspension. Dialysis was carried out indistinctly at room temp or in the cold without detectable change. ATPase activity was measured as previously indicated [5]. 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 P_i/30 min at 37°. During the obtention and washing of the different membrane preparations, their protein content is subject to variations. Therefore, ATPase activity is expressed in units per 100 ml of membrane suspension, since this volume is kept constant, to unify the results presented herein.

3. Results

3.1. *Modification and restoration of the Mg^{2+}-dependent states in type A membranes*

When membranes of type A were washed with 50 mM Tris-HCl buffer, a rapid change in the properties of ATPase occurred as is illustrated in table 1. As the data show, the most important effect was a net increase in all type of assays of the trypsin-dependent ATPase. However, the trypsin-independent ATPase did not show a substantial change when it was measured in absence of cation, but a marked augmentation was observed when assayed in presence of either extra-Mg^{2+} of Ca^{2+}. The later results strongly suggest a loss in the sensitivity of the membrane-bound ATPase to the low ATP/divalent cation ratio and to Ca^{2+} as compared with the original type A membranes [1]. The same effect is also observed for the trypsin-dependent activity. When the membranes, after this Mg^{2+}-free wash, were resuspended again in magnesium-containing Tris buffer, an almost complete restoration of the ATPase to the Mg^{2+}-induced states of type A preparation was obtained (compare first and third columns in table 1).

3.2. *Partial restoration of Mg^{2+}-dependent states of the ATPase bound to membranes of types C and D**

It is possible to obtain a gradation in the states of the *M. lysodeikticus* membrane-bound ATPase by controlled and selective treatments of the membranes of type A (unpublished observations). In any way, it was of particular relevance to the objective of this work to attempt the restoration of the Mg^{2+}-dependent states of the ATPase in membranes of types C and D, i.e. membranes which had lost those ATPase states by washing out of magnesium [1]. The results for the trypsin-independent and -dependent activities are illustrated in tables 2 and 3, respectively. It is noteworthy from table 2, the good restoration of trypsin-independent activity assayed in the absence of cations. Actual figures amount to 70–105% of that ATPase activity detected in membranes of type A. However, a marked increase of the ATPase assayed in the presence of extra cations is observed. As a matter of fact, 140–215% of ATPase units measured in the presence of 4 mM Mg^{2+} are recovered; higher values (300–485%) are obtained for the ATPase determined in presence of 8 mM Ca^{2+}. These results are, in general, very similar regardless of which type of membrane C or D is used and of which kind of subsequent washing procedure is applied. Nevertheless, a progressive increment in the number of Ca^{2+}-ATPase units corresponds to the progressive wash of the membrane preparations. To rule out that the increase in Ca^{2+}-ATPase units was due to a solubilization of the enzyme which would then show higher activation with calcium [6], aliquots of the membrane suspensions were spun down (27,000 g for 30 min) and the supernatants were assayed for ATPase. Less than 10% of total ATPase units were found in the soluble fractions. These results indicate that membranes of type C and D are able to restore trypsin-independent ATPase activity in its membrane-bound state. However, they also suggest that a profound change is observed in the membrane complex with respect of Ca^{2+} sensitivity in particular, and $[Me^{2+}]$ requirements in general.

Table 3 illustrates the results obtained in the restoration of trypsin-dependent ATPase activity. They agree to a great extent with those reported in table 2 and may be interpreted on a similar basis.

* Restoration experiments with membranes of type B gave intermediate results between those of type A membranes and preparations of types C and D.

4. Discussion

Changeux and Thiery [3] have proposed the analogy of excitable membranes with regulatory enzymes. They based this analogy on the existence of the following characteristics in both macromolecular structures: i) presence of regulatory sites; ii) capacity of the structure to undergo reversible conformational changes; iii) the organization of the macromolecular units into *ordered* structures.

Although we are aware of the preliminary character of our research we believe that the properties of the ATPase-membrane system of *M. lysodeikticus* can be explained in terms of the Changeux and Thiery proposal. In previous reports we have discussed the "allotopic" properties of the enzyme [6] and shown the existence of different states of the membrane-bound enzyme mediated by Mg^{2+} as effector [1]. Circumstantial evidence has been presented [4, 7] for the *ordered* association of the ATPase with *M. lysodeikticus* membrane. The results described in this paper have shown the

Table 1
Trypsin-independent and -dependent ATPase activities in type A membranes of *M. lysodeikticus*

Assay additions	ATPase activity (units/100 ml suspension)		
	Membrane preparation		
	Unmodified	Washed Tris buffer	Restored Tris-Mg^{2+}
———	440	545	370
4 mM $MgCl_2$	280	610	230
8 mM $CaCl_2$	140	385	180
Trypsin (0.5 mg/ml)	600	1485	450
Trypsin + 4 mM $MgCl_2$	500	1560	380
Trypsin + 8 mM $CaCl_2$	430	885	320

The reaction mixture contained, in a 0.5 ml vol, 40 mM Tris-HCl buffer pH 7.5, 8 mM ATP (Pabst Laboratories) and 0.1 ml of membrane preparation. Conditions for incubation and details on membrane preparations are given in the text.

Table 2
Trypsin-independent ATPase activity in magnesium-deprived membranes of *M. lysodeikticus* after dialysis against Tris-Mg^{2+} buffer.

Membrane preparation	Assay additions	ATPase activity	
		(Units/100 ml suspension)	Activity of type A membrane* (%)
Unmodified type C	–	350	79.5
Unmodified type C	4 mM $MgCl_2$	390	139
Unmodified type C	8 mM $CaCl_2$	420	300
Type C washed with Tris	–	420	95.5
Type C washed with Tris	4 mM $MgCl_2$	460	164.2
Type C washed with Tris	8 mM $CaCl_2$	550	392.6
Type C washed with Tris-EDTA	–	460	104.5
Type C washed with Tris-EDTA	4 mM $MgCl_2$	600	214.2
Type C washed with Tris-EDTA	8 mM $CaCl_2$	680	485
Unmodified type D	–	400	90.9
Unmodified type D	4 mM $MgCl_2$	420	150
Unmodified type D	8 mM $CaCl_2$	500	357.1
Type D washed with Tris	–	385	87.5
Type D washed with Tris	4 mM $MgCl_2$	460	164.2
Type D washed with Tris	8 mM $CaCl_2$	630	450
Type D washed with Tris-EDTA	–	310	70.4
Type D washed with Tris-EDTA	4 mM $MgCl_2$	460	164.2
Type D washed with Tris-EDTA	8 mM $CaCl_2$	680	485

The incubation mixtures were as indicated under table 1. Details of membrane preparations and their treatments are given in the text.

* Expressed as recovery with respect to the primitive membranes of type A [1].

Table 3
Trypsin-dependent ATPase activity in magnesium-deprived membranes of *M. lysodeikticus* after dialysis agaisnt Tris-Mg^{2+} buffer.

Membrane preparation	Assay additions	ATPase activity	
		(Units/100 ml suspension)	Activity of type A membrane* (%)
Unmodified type C	–	765	125.8
Unmodified type C	4 mM $MgCl_2$	780	130
Unmodified type C	8 mM $CaCl_2$	772	179.5
Type C washed with Tris	–	1,060	176.6
Type C washed with Tris	4 mM $CaCl_2$	935	187
Type C washed with Tris	8 mM $CaCl_2$	935	217.4
Type C washed with Tris-EDTA	–	1,240	200.6
Type C washed with Tris-EDTA	4 mM $MgCl_2$	1,170	234
Type C washed with Tris-EDTA	8 mM $CaCl_2$	1,170	272
Unmodified type D	–	715	119.1
Unmodified type D	4 mM $MgCl_2$		
Unmodified type D	8 mM $CaCl_2$		
Type D washed with Tris	–	900	150
Type D washed with Tris	4 mM $MgCl_2$	800	160
Type D washed with Tris	8 mM $CaCl_2$	900	209.3
Type D washed with Tris-EDTA	–	965	160.8
Type D washed with Tris-EDTA	4 mM $MgCl_2$	1,035	207
Type D washed with Tris-EDTA	8 mM $CaCl_2$	965	224.4

Incubation mixtures were as indicated in table 1 but supplemented with trypsin (0.5 mg/ml). For further details see the text.

* Expressed as recovery with respect to the primitive membranes of type A [1].

feasibility of getting a restoration of the Mg^{2+}-induced states of *M. lysodeikticus* membrane ATPase, even in those membranes which had been thoroughly washed out of magnesium. This reversibility gives, therefore, biochemical support to the likelihood of conformational changes as responsible for the states of the ATPase-membrane complex. However, the reversibility is not absolute since progressive changes in the sensitivity of the membrane ATPase to Ca^{2+} were found during membrane manipulation. This is an indication of the extreme complexity of the relationships between cations, membrane and ATPase. Further report on the nature and number of cation ligands will be reported elsewhere. At the present time, we think that the changement in Ca^{2+} response might be due to an irreversible modification, during membrane manipulation, of the regulatory sites and/or of the specific interactions responsible for Ca^{2+} effect or to the loss of a component(s) which regulate(s) the action of Ca^{2+} ions onto the membrane-bound ATPase.

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References

[1] M. Lastras and E. Muñoz, FEBS Letters 14 (1971) 69.
[2] J.P. Changeux and J. Thiery, J. Theoret. Biol. 17 (1967) 315.
[3] J.P. Changeux and J. Thiery, in: Regulatory Functions of Biological Membranes (Elsevier, Amsterdam, London, New York, 1968) p. 116.
[4] E. Muñoz, J.H. Freer, D.J. Ellar and M.R.J. Salton, Biochim. Biophys. Acta 150 (1968) 531.
[5] E. Muñoz, M.S. Nachbar, M.T. Schor and M.R.J. Salton, Biochem. Biophys. Res. Commun. 32 (1968) 539.
[6] E. Muñoz, M.R.J. Salton, M.H.Ng. and M.T. Schor, European J. Biochem. 7 (1969) 490.
[7] D.J. Ellar, E. Muñoz and M.R.J. Salton, Biochim. Biophys. Acta 225 (1971) 140.