ADENOSINETRIPHOSPHATASE OF MICROCOCCUS LYSODEIKTICUS: SELECTIVE RELEASE AND RELATIONSHIP TO MEMBRANE STRUCTURE

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Received July 5, 1968

INTRODUCTION: Further studies of a Ca^{++}-dependent adenosinetriphosphatase (EC 3.6.1.3) associated with Micrococcus lysodeikticus membranes (Munoz et al., 1968) revealed that the enzyme, when attached to the membranes was only able to catalyze adenosinetriphosphate hydrolysis if activated by trypsin (Muñoz and Salton, to be published). This property enabled the detection of residual activity associated with the membranes and led to a reinvestigation of conditions and factors involved in a complete release of adenosinetriphosphatase from the protein-membrane complex; the results are reported in this study. "Solubilization" of the ATPase is compared to that of polynucleotide phosphorylase, an enzyme associated with the membranes of Streptococcus faecalis (Abrams and McNamara, 1962).

METHODS: M. lysodeikticus (NCTC 2665) was grown and harvested as previously described (Salton and Freer, 1965). Protoplasts were prepared and burst as reported by Muñoz et al. (1968), with the modification of adding 0.005 M $MgCl_2$ to the bursting mixture. The lysate was centrifuged (30 min.

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** Public Health Service Training Fellow, P.H.S. Grant GM 00466

at 30, 000 xg), cytoplasm and membranes were separated and the pellets were washed with various buffers, adjusted to pH 7.5. Washings were performed so that the first four washes were finished on the same day, the pellets stored at 4°C and the washing resumed the next day. After each wash the membranes were sedimented at 30, 000 xg for 30 min. Protein was determined by the method of Lowry et al., 1951. Adenosine triphosphatase (ATPase) activity was measured by the liberation of inorganic phosphorus (Pi) from ATP. The test system contained: 4 µmoles ATP, 4 µmoles $CaCl_2$, tris-HCl buffer, pH 7.5, 45 µmoles and 100 µmoles of each supernatant in a final volume of 500 µl. Incubation was carried out at 37°C for 10 min. and the reagents for Pi determination (Vambutas and Racker, 1965) were added directly to the reaction mixtures. Appropriate blanks were run each time to determine background levels of Pi in the supernatant washes. One unit of ATPase activity is defined as the amount of enzyme able to liberate 1 µmole of Pi/10 min. under the assay conditions. Polynucleotide phosphorylase (PNPase) activity was also measured by the Pi liberation in a test system that contained: 4 µmoles ADP, 2 µmoles $MgCl_2$, 25 µmoles tris-HCl buffer, pH 9.0, and 100 µl of each supernatant in a final volume of 500 µl. Incubation was carried out at 37°C for 1 hour and the Pi liberated was measured as described above. One unit of enzyme activity is defined as the amount of enzyme liberating 1 µmole Pi/1 hr. Confirmation of the existence of this activity was also obtained from the kinetics of Pi liberation, the hypochromicity that develops by nucleotide polymerization and from the sensitivity of the final product to ribonuclease. Electrophoresis of the supernatant fractions in polyacrylamide gels was performed as described by Salton (1967 a, b). Zones of the two enzymatic activities were visualized by the staining technique described by Weinbaum and Markman (1966).

RESULTS: The partition of protein, ATPase units and PNPase units in a series of experiments is illustrated in TABLE I. Of interest was the detection of ATPase in the cytoplasm where it accounted for 10% of the maximum total number of ATPase units. Although we could not rule out the possibility that its presence resulted from mechanical dissociation, its fairly constant value suggested that it might exist as an active enzyme in the cytoplasmic fraction of the stationary phase cells.

In experiment A, using the standard membrane washing procedure described by Salton (1967 a, b), 25% of the total cellular protein remained associated with the membrane and no detectable loss of cytochromes or carotenoids occurred. Under these conditions there was virtually no ATPase release from the membranes and the quantity of "residual" ATPase (trypsin activated) was large. In contrast, the polynucleotide phosphorylase activity was completely solubilized. In experiments B and C, a complete release of ATPase enzyme was obtained by exposure to low ionic strength buffer (washing Nos. 5 and 6), a treatment analogous to an "osmotic shock". Prior washing with 0.03 M tris or tris + 0.001 M EDTA buffers did not cause very much ATPase release but did solubilize polynucleotide phosphorylase activity, which was more rapidly released when EDTA was present. Moreover, in experiment C, the residual membranes contained only 16% of the starting pellet protein, compared to 60% for the residual membranes in experiment A. This substantial protein solubilization was accompanied by the release of carotenoids, as judged by the absorption spectra of the washes and would seem to indicate a progressive dissolution of the membranes. Other authors (Lukoyanova et al., 1961) had reported destruction of M. lysodeikticus membranes by EDTA treatment. However, the pellet from our experiment

TABLE I The effect of different treatments of M. lysodeikticus membrane upon the partition of protein, adenosine triphosphate and polynucleotide phosphorylase

		WASHING BUFFER	PROTEIN (mg)	ATPase UNITS	PNPase UNITS
Cytoplasm		—	560	130	560
Pellet (Membrane)		—	440	+++*	+++
Experiment					
	1st wash	0.1M tris	73	10	140
	2nd "	"	43	6	90
	3rd "	"	28	10	60
A	4th "	"	10	15	80
	5th "	"	10	20	190
	6th "	"	10	10	80
				Total	Total
	Membranes	—	266	+++* 201	— 1,210
	1st wash	0.03M tris	80	32	140
	2nd "	"	45	20	90
	3rd "	"	22	22	60
B	4th "	"	22	45	80
	5th "	0.003M tris	80	750	240
	6th "	"	26	104	20
				Total	Total
	Membranes	—	165	±* 1,103	— 1,190
	1st wash	0.03M tris-0.001M EDTA	140	69	252
	2nd "	"	70	97	225
	3rd "	"	75	100	180
C	4th "	"	40	100	48
	5th "	0.003M tris	40	600	—
	6th "	"	5	85	—
				Total	Total
	Membranes	—	70	—* 1,181	— 1,265
	1st wash	0.03M tris-0.05M $MgCl_2$	80	—**	150
	2nd "	"	18	—**	80
	3rd "	"	18	—**	40
D	4th "	"	5	—**	—
	5th "	"	3	—**	40
	Membranes	—	316	+++*	+
	Wash a	0.003M tris	56	60	140
	Wash b	0.03M-0.001M EDTA	30	20	200
				Total	Total
	Wash c	0.003M tris	20	300 510	— 1,210

* Indicates (+→ +++) relative amount present or (-) absence of trypsin activated ATPase; owing to complexity of kinetics on trypsin

** activation exact units not given.

Assayed after dialysis against tris-HCl buffer 0.03M pH 7.5. Mg^{++} inhibits Ca^{++}- ATPase activity (Muñoz et al., 1968).

C showed in the electron microscope, a membrane-structure of modified form, characterized by the disappearance of structured particles and the appearance of the residual membranes as smoother sheets. It is tempting to suggest that a basal structure still remained and that the associated components had been removed. This important EDTA effect seemed less related to ATPase solubilization than to the release of other proteins, e.g. polynucleotide phosphorylase and DPNH-dehydrogenase activities (M. S. Nachbar, unpublished). Experiment D demonstrated the stabilizing effect on the membranes of Mg^{++} (Ca^{++} gave similar results) with 32 % of the initial protein still membrane associated after five washes, with the retention of some PNPase and all ATPase activities. Treatment of these stable membranes with the "osmotic shock" (wash a) resulted in a poorer solubilization of adenosinetri-

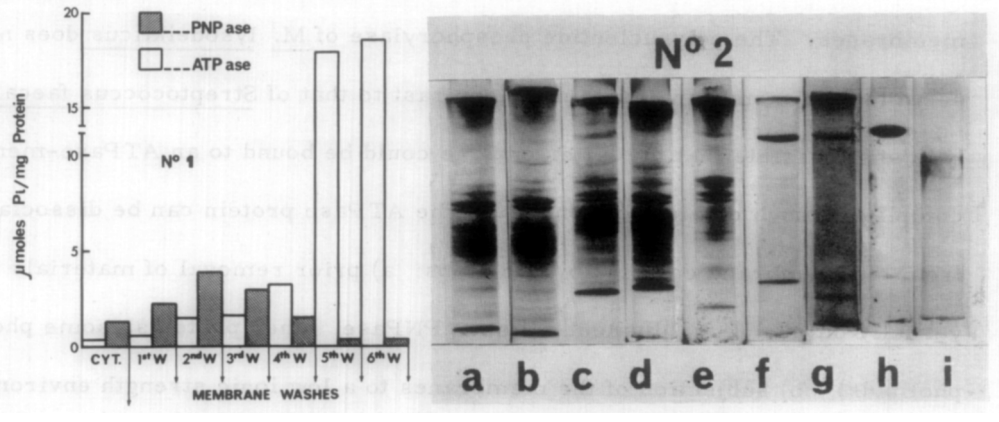

Figure 1. Experiment C showing ATPase and PNPase activities released from membranes. No. 1: Specific activities in the different supernatant fractions under the assay conditions described in Methods. No. 2: Disc electrophoresis showing aniline black staining of proteins in a) cytoplasm; (b-g) six consecutive washes of the membranes, and enzymatic staining for ATPase (h) and PNPase (i). Migration to the anode (bottom of gels).

phosphatase enzyme.[1] The good yield of ATPase activity in wash c with 0.003 M tris buffer, after previous treatment with EDTA (wash b) confirmed that ATPase solubilization depended not only upon treatment of the membranes with low ionic strength buffer but also upon the stability and complexity of membranes as modified by prior treatment.

In all the experiments described in TABLE I, a Mg^{++}-dependent inorganic pyrophosphatase activity was found largely in the cytoplasmic fraction. By disc gel electrophoresis it was possible to identify the bands corresponding to ATPase and PNPase activities. The gels from one experiment of Type C, together with the specific activities for both enzymes in the different washes, are shown in Figure 1. The ATPase band corresponds to the slow moving component described previously by Salton (1967 a, b) as a protein component of *M. lysodeikticus* membranes.

DISCUSSION: From the results reported here the following points emerge: 1) ATPase protein appears as a well defined component of *M. lysodeikticus* membranes. The polynucleotide phosphorylase of *M. lysodeikticus* does not seem to be a membrane protein, in contrast to that of *Streptococcus faecalis*; it is still possible that the latter enzyme could be bound to an ATPase-membrane complex through divalent cations. 2) The ATPase protein can be dissociated from the membrane complex by two steps: a) prior removal of materials not yet fully defined (e.g. divalent cations, PNPase, other proteins, some phospholipids); b) subjection of the membranes to a low ionic strength environment. This second step is more specific and is more effective when the first step has been performed under optimal conditions. It seems clear that hydrophobic

1. ATPase solubilization by the low ionic strength wash was less efficient when applied directly to membranes not previously washed.

and/or ionic linkages are the most important bonds in the attachment of the ATPase "particles" previously described (Muñoz et al., 1968), to a so-called basal membrane. The selective release of ATPase has proved useful as a method for the partial purification of this enzyme.

The "solubilization" of ATPase enzyme from M. lysodeikticus membranes shows a marked similarity to the release of this enzyme from S. faecalis membranes (Abrams, 1965, Abrams and Baron 1967, 1968) but, as the present report indicates, the release is not a simple "all or none" phenomenon, being dependent on well defined conditions of preparation. Despite these similarities of the M. lysodeikticus and S. faecalis ATPases, a generalization of the type of association of membrane ATPases in bacterial systems would seem inappropriate. Thus, an ATPase activity present in isolated membranes of Staphylococcus aureus (Gross and Coles, 1968) was not released from the membranes by washing with dilute buffers.

From these studies of bacterial membranes it has become apparent that carefully controlled methods of membrane treatments, covering a wide range of conditions, are necessary in order to determine the sites of localization of "functional" proteins on or in the membrane structures.

ACKNOWLEDGEMENTS: This work was supported by a National Science Foundation Grant (GB 4603). We wish to thank Dr. D. J. Ellar for his helpful discussions and for the electron microscopic work for this study.

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