SUBUNIT STRUCTURE AND PROPERTIES OF TWO FORMS OF ADENOSINE TRIPHOSPHATASE RELEASED FROM MICROCOCCUS LYSODEIKTICUS MEMBRANES

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SUMMARY: The Ca^{2+}-activated ATPase of M. lysodeikticus membrane has been released in two forms: (i) a "shock wash" form which is trypsin stimulated, capable of rebinding to ATPase-depleted membranes in the presence of Mg^{2+} and possesses additional associated protein(s) giving rise to 1-3 minor protein bands in addition to the two major subunits detected by SDS-polyacrylamide gel electrophoresis; (ii) a form released into the aqueous phase on extracting the membranes with n-butanol (referred to as "n-butanol type"), which is not stimulated by trypsin, does not rebind to depleted membranes and contains only two major protein subunits. The two protein subunits of both types have molecular weights of 62,000 and 60,000 daltons and reduction followed by alkylation or performic oxidation does not appear to significantly alter the behavior of these subunits. It is suggested that the additional protein(s) of the shock wash ATPase have attachment and regulatory functions.

INTRODUCTION: Investigations in our laboratory have been concerned with establishing the structure-function relationships in the multifunctional bacterial membrane system of Micrococcus lysodeikticus (1, 2). The membrane ATPase has been released by a low-ionic strength shock wash (3) similar to that reported for the Strep. faecalis ATPase by Abrams (4) and the particulate nature, purification and reaction with specific antibody described in previous publications (5-7). The striking similarity of the M. lysodeikticus ATPase in its appearance in negatively-stained preparations viewed in the electron microscope (5) to that of mitochondrial ATPase (8) has been pointed out and its stimulation by trypsin and "latency" in the membrane-

bound form reinforce these general similarities (6, 9). In this paper, we wish to report a comparison of some of the properties of the ATPase released by the "shock wash" procedure (3) with that released into the aqueous phase on extracting the membranes with n-butanol.

MATERIALS AND METHODS: Membranes of M. lysodeikticus were prepared as previously described (10) and washed with 0.03 M Tris-HCl buffer, pH 7.5, before being subjected to the treatments for releasing the ATPase. A modification of the previous shock-wash treatment (3) has involved the prior exposure of the membranes to a wash in 0.03 M Tris-HCl containing 0.005 M EDTA, holding the membranes overnight at 0-4°C and removing the EDTA wash containing NADH-dehydrogenase activity (11) before the low ionic strength wash in 0.003 M Tris-HCl, pH 7.5, which releases the ATPase. The n-butanol method of releasing ATPase from the membranes has involved the preparation of standard, washed membranes and releasing the NADH-dehydrogenase with EDTA as above and then extracting the membranes suspended in 0.03 M Tris-HCl, pH 7.5, with n-butanol (4:3, by volume) at 0°C, separating the organic solvent phase by centrifugation at 0°C and recovering the ATPase in the aqueous phase, after 3-4 successive extractions, removal of the n-butanol phase and dialysis to remove dissolved butanol. All preparations were concentrated at 0°C by ultrafiltration in the Amicon apparatus fitted with a type XM 50 membrane. Both types of ATPase were purified on Sephadex G-200 as previously described (6). All enzyme assays were performed in the presence and absence of trypsin as previously reported (6).

The method of Weber and Osborn (12) was used for the determination of the molecular weights and detection of subunit structures of the ATPases

in the sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis system.

The ability of the purified ATPases to rebind to the depleted membranes was tested by adding the preparations to "stripped" membranes in 0.05 M Tris-HCl, pH 7.5, containing 5-30 mM Mg^{2+}, allowing them to interact for 45 min at 0°C and then removing unbound ATPase by centrifuging for 30 min at 30,000 X g at 0°C. The supernatant fluids and membrane pellets were dialysed against 0.01 M Tris-HCl, pH 7.5, to remove excess Mg^{2+} and assayed for ATPase activity in the presence and absence of trypsin as indicated above.

Inhibition studies were performed with phloretin (13, 14) purchased from K and K Laboratories, Inc. and 1-anilino-naphthalene-8-sulphonate (ANS). The former compound was dissolved in 50% ethanol and diluted as required in 0.1 M Tris-HCl, pH 7.5, for addition to the enzyme assay mixtures. ANS was dissolved and diluted in 0.1 M Tris-HCl buffer.

RESULTS AND DISCUSSION: SDS-polyacrylamide gel electrophoresis system of Weber and Osborn (12) has not only proved to be valuable in determining the molecular weights of protein subunits but it has also been of great value in monitoring the homogeneity of enzyme preparations. The results obtained with the purified "shock wash" and the "n-butanol" forms of M. lysodeikticus ATPases are illustrated in Fig. 1. Although the shock wash ATPase complex appears to be homogeneous by several criteria (e.g. a single band obtained in the normal polyacrylamide gel electrophoresis system; one peak on ultracentrifugal analysis; a single line of precipitation on immunoelectrophoresis against specific antibody -- refs. 6, 7) examination of purified fractions by SDS-polyacrylamide gel electrophoresis has revealed the pres-

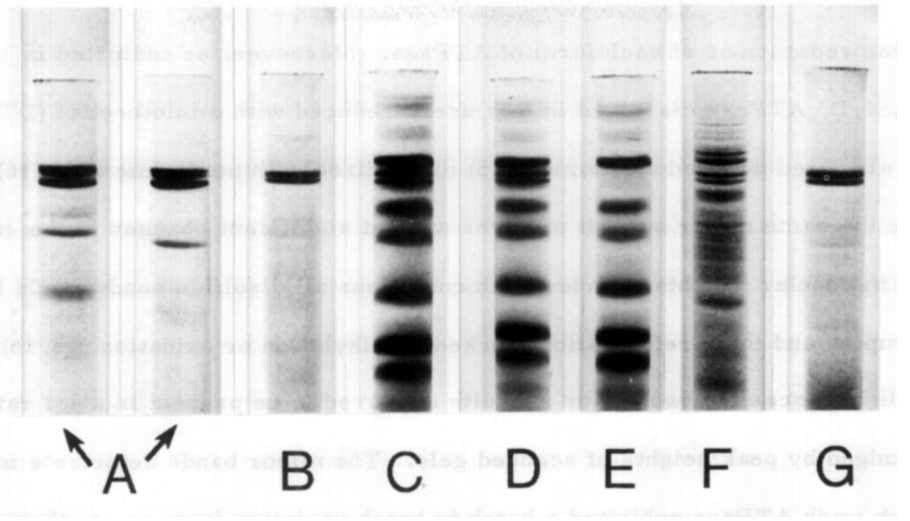

Fig. 1. SDS-polyacrylamide gels prepared and stained with Coomassie blue by the Weber and Osborn (12) method. A. Shock wash ATPase preparations showing two major subunits and additional minor protein bands. The principal minor band has a molecular weight close to that of pepsin (i. e. about 35, 000). B. n-butanol type ATPase showing two subunits identical to those found in shock wash ATPase. C. Shock wash ATPase + protein standards; bands from top to bottom of gel are bovine serum albumin, ATPase subunits, ovalbumin, pepsin, trypsin, myoglobin and hemoglobin; the principal minor band overlaps with pepsin. D. ATPase reduced with DTT and alkylated with iodoacetamide (ref. 15) together with standard proteins as in gel C. Identity of bands as for gel C. Similar results were obtained with both types of ATPase and with performic acid oxidized preparations. E. Protein standards alone, as in gels C. and D. F. Subunit pattern for whole membranes of M. lysodeikticus. G. n-butanol type ATPase showing two major subunits and corresponding bands in whole membranes (gel F.).

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ence of two major subunits and several (1-3) additional minor protein bands derived from associated protein(s) of the complex. Thus, the shock wash ATPase is chemically more heterogeneous than the form released by extracting membrane suspensions with n-butanol. Both forms possess two major subunits with molecular weights of 62, 000 and 60, 000 daltons as determined by relative mobilities in the SDS-polyacrylamide gel electrophoresis system (12) with internal protein standards as indicated in Fig. 1, C. These values represent means of a minimum of 5 determinations on each batch of three or

more preparations of each form of ATPase. Moreover, as indicated in Fig. 1, D, ATPase dissolved in 8 M urea, reduced with dithiothreitol (DTT) and alkylated with iodoacetamide (15) or oxidized with performic acid (16) gave the same major subunit patterns without significant changes in the subunit molecular weights. Under such conditions all disulfide bonds would be disrupted and their reformation blocked by alkylation or oxidation (15, 16). In all instances the two major subunits appeared to be present in a 1:1 ratio as judged by peak heights of scanned gels. The minor bands detectable in the shock wash ATPase exhibited a batch to batch variation from one to three extra components. The existence of two major subunits and a variable number of additional minor "subunits" is thus strikingly similar to the results obtained with beef heart and yeast mitochondrial ATPase complexes (see Tzagoloff, ref. 17) and the Strep. faecalis ATPase (18). The reasons for the variation in the additional proteins found in the shock wash form of ATPase are not known, but it is conceivable that a spontaneous dissociation and loss of some proteins of the complex may occur during purification. They are, however, consistently of the same molecular weight species and do not appear to be a gross mixture of the other membrane protein subunits seen in the pattern of whole membranes (Fig. 1, F) and would thus argue against a random contamination of the complex.

Some of the properties of the two forms of ATPase released from the M. lysodeikticus membrane are summarized in Table 1. Two outstanding properties are clearly correlated with the presence of the additional proteins in the shock wash ATPase complex. The n-butanol released ATPase is chemically more homogeneous but lacks the ability to rebind to depleted membranes, is not stimulated by trypsin and indeed exhibits some sensitivity to trypsin even in the presence of the substrate ATP. The shock wash ATPase shows

TABLE 1

Properties of Two Forms of Adenosine Triphosphatase Released from M. lysodeikticus Membranes

	"Shock wash" ATPase	"n-butanol type" ATPase
Protein subunits		
Major	2	2
Molecular weights	62,000 60,000	62,000 60,000
Minor	1-3	None
Trypsin stimulation	150-200% of control	100-80% of control
Rebinding to depleted membranes	++*	–
% Inhibition		
Phloretin 0.4×10^{-3} M	46.0	52.5
0.3×10^{-3} M	38.5	35.0
ANS 0.4×10^{-3} M	60.0	53.5
0.3×10^{-3} M	49.0	44.0

* ATPase rebound to membranes varied from about 50-70% of added enzyme; the unbound activity in the supernatant was not stimulated by trypsin.

marked stimulation with trypsin and will reassociate with the depleted membranes when Mg^{2+} is present. It is worthy of note that the ATPase activity in the supernatant fraction (about 30% of added enzyme units) following rebinding of shock wash ATPase to the membranes does not exhibit stimulation by trypsin. Such an observation is not unexpected and would be in accord with the above suggestion that there is some "spontaneous" dissociation of the additional protein(s) involved in reassociation. Moreover, double-bands of shock wash ATPases have been frequently observed in the standard poly-

acrylamide gel electrophoresis system (7, 19) and this can now be explained by the slightly faster mobility of the ATPase form (e. g. n-butanol type) free of the additional protein(s). There seems little doubt that one of the additional proteins is identical to the mitochondrial junction proteins (17) and nectin (20). In addition to its role in attaching the enzyme to the membrane we would like to suggest that the additional protein(s) of the M. lysodeikticus ATPase complex also have a regulatory function, controlling hydrolytic and oxidative phosphorylation activities in the membrane associated state. Such a regulatory protein may also account for the changes in cation responses observed by Muñoz and coworkers (21, 22).

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