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RELEASE AND PURIFICATION OF *MICROCOCCUS LYSODEIKTICUS* ATPase FROM MEMBRANES EXTRACTED WITH *n*-BUTANOL

MILTON R. J. SALTON and MARIAN T. SCHOR

Department of Microbiology, New York University, School of Medicine, New York, N.Y. 10016 (U.S.A.)

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SUMMARY

The ATPase associated with the membranes of *Micrococcus ysodeikticus* has been released into the aqueous phase (i.e. solubilized) by extracting the membranes with *n*-butanol in a two-phase system modified from the procedure of Maddy, A.H. (1964) Biochim. Biophys. Acta 88, 448-449. A procedure for the release and purification of the ATPase from the membranes extracted with *n*-butanol is described as an alternate method to that previously used for the shock-wash ATPase. Upon extracting the membrane suspensions with *n*-butanol the soluble ATPase released into the buffer phase no longer exhibits stimulation by trypsin in contrast to the shock-wash type of ATPase. As shown by Salton, M. R. J. and Schor, M. T. (1972) Biochem. Biophys. Res. Commun. 49, 350-357, the shock-wash ATPase possesses associated protein(s) as determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis whereas these are absent from the purified ATPase released by the *n*-butanol method. The specific activities of the purified ATPase released by the two methods were generally similar, the *n*-butanol type being consistently somewhat higher.

INTRODUCTION

Bacterial membrane ATPases appear to be the least tightly integrated of the major membrane proteins and the relative ease with which they can be released from the membrane was first reported by Abrams [1] in his study of *Streptococcus faecalis* ATPase. The ATPase of *Micrococcus lysodeikticus* was also selectively released from the membranes when they were subjected to a low-ionic strength wash (referred to as a shock wash) [2–4] and similar results have been obtained with other bacteria [5–8]. Following the early observations of Morton [9] on the release of particle-bound enzymes by extracting the lipids with *n*-butanol, Maddy [10, 11] demonstrated the solubilization of membrane proteins by extraction in this two-phase system [9]. For many years we have used in this laboratory a modification of Maddy's [10] procedure for the release of *M. lysodeikticus* membrane proteins including ATPase and a fast-moving component characterized as a relatively hydrophobic protein [12]. In the light of our recent discovery that the purified ATPase released by the shock-wash

method is more complex than that obtained by *n*-butanol extraction [13] we wish to describe the results obtained with this latter procedure as an alternate method for bacterial ATPase purification.

MATERIALS AND METHODS

Preparation of membranes

Membranes from *M. lysodeikticus* (NCTC 2665) were isolated from cells grown on the peptone–water–yeast extract medium as previously described [14, 15]. The membranes were subjected to the standard washing procedure in 0.03 M Tris–HCl buffer, pH 7.5, used in this laboratory and described in earlier papers [4, 15]. Batches of membranes which had received the standard five washes in the 0.03 M Tris buffer were used throughout and were either subjected to the shock wash in 0.003 M Tris–HCl buffer, pH 7.5, as described previously for the selective release of ATPase [3, 4] or subjected to the *n*-butanol extraction outlined below.

Extraction of membranes with n-butanol for ATPase release and purification

Washed membranes suspended in 0.03 M Tris–HCl buffer, pH 7.5, were given an EDTA wash (5 mM EDTA in 0.03 M Tris–HCl buffer, pH 7.5) as described by Nachbar and Salton [16] to release much of the NADH dehydrogenase components from the membranes prior to *n*-butanol extraction. The EDTA wash supernatant was discarded and the membranes were resuspended in 0.03 M Tris buffer to give approx. 5 mg membrane protein/ml suspension and extracted at 0 °C by mixing with ice-cold *n*-butanol (certified ACS grade from Fisher Scientific Company, Fair Lawn, N. J.). To 4 vol. of suspension, 3 vol. of ice-cold *n*-butanol were added with stirring and the preparations were allowed to stand for 15 min in an ice bath. The organic-solvent phase was then separated by centrifugation at 27 000 × *g* for 15 min at about −5 °C. The clear upper *n*-butanol layer containing carotenoids and lipids was removed and discarded. The extraction process was repeated with fresh ice-cold butanol until the upper organic-solvent layer was devoid of carotenoid pigment and some six to seven successive extractions and separations by centrifugation were generally required to achieve this. During the course of the extractions there was a progressive decrease in the interfacial "emulsion" layer but at the end of the extraction and centrifugation sequence there was an insoluble pellet, an aqueous phase, an insoluble interfacial layer and upper *n*-butanol phase. After removal of most of the solvent layer, the aqueous phase was carefully withdrawn with a cold syringe and placed in a dialysis sac and immediately dialysed against several changes of ice-cold 0.005 M Tris–HCl buffer, pH 7.5, to remove the butanol dissolved in the aqueous phase. Dialysis was continued at 0–4 °C against changes of buffer until the preparations were free from the odour of butanol. The dialysed aqueous-phase preparations containing released ATPase and solubilized proteins were then centrifuged at 27 000 × *g* for 30 min at 0 °C and the supernatant fractions were concentrated in an Amico Ultrafiltration apparatus fitted with a PM 10 filter and the solutions were then subjected to high-speed centrifugation at 133 000 × *g* for 1 h at 0 °C. The pellets contained little ATPase activity and were discarded and the supernatant solutions were concentrated further by ultrafiltration so that a protein concentration in excess of about 5 mg/ml was achieved. The solutions were dialysed against 0.03 M Tris–HCl buffer,

pH 7.5, and solid $(NH_4)_2SO_4$ (0.35 g/ml) was added with constant stirring at 0 °C to give approx. 60% level of saturation. The suspension was adjusted to pH 7.0–7.5 by the addition of small volumes of 1 M NaOH. After the addition of the $(NH_4)_2SO_4$ the suspension was held for 1 h in an ice bath and then the precipitate deposited by centrifugation at 0 °C. The supernatant fraction was carefully removed and the precipitate was dissolved in 2–3 ml 0.03 M Tris–HCl buffer and any insoluble material was removed by centrifugation for 30 min at 27 000 × *g*. The supernatant fractions were dialysed against 0.03 M Tris–HCl buffer before application to a Sephadex G-200 column, using gel filtration and elution conditions previously described [4]. The tubes containing the peak of ATPase activity and minimal contamination with other proteins as determined by polyacrylamide gel electrophoresis under standard conditions, were pooled, concentrated by ultrafiltration and placed on a second Sephadex G-200 column for further purification. Fractions from the second cycle of gel filtration on Sephadex G-200 which were judged to be homogeneous by polyacrylamide gel electrophoresis and those of similar specific activities were pooled as the final purified product.

Shock-wash ATPase

For comparative purposes, ATPase fractions were prepared by the shock-wash procedure essentially as previously described from this laboratory [3, 4]. Modifications to this procedure included prior EDTA washing of the membranes to release the NADH dehydrogenase [16] and removing this EDTA-wash supernatant before subjecting the membranes to the shock-wash step for ATPase release. The supernatant shock-wash fractions were then centrifuged at 133 000 × *g* for 1 h after concentration by ultrafiltration in the Amico apparatus using a PM 10 filter. In some instances the shock-wash ATPase concentrates were subjected to $(NH_4)_2SO_4$ precipitation and followed by gel filtration on Sephadex G-200 as for the *n*-butanol type of ATPase.

ATPase assays

Activities were determined by estimating the liberation of P_i as previously described [3, 4] using an incubation temperature of 37 °C for 10 min to develop the color. The specific activities of the ATPase (EC 3.6.1.3) are given in this paper as 1 μmole P_i liberated/min per mg protein and not for a 10-min period as used in previous papers [3, 4]. A unit of enzyme is thus given as the amount of ATPase liberating 1 μmole P_i/min.

Assays of enzyme activities were routinely performed in the presence and absence of trypsin (Calbiochem Grade A, crystalline, salt-free enzyme from pancreas) in view of the latency found with this ATPase and the conditions used in the trypsin–ATPase assay were identical to those previously described [3, 4].

Protein determination

Protein contents were determined by the method of Lowry et al. [17] using bovine serum albumin (Pentex, Kankakee, Ill., crystalline Grade A) as a standard with appropriate Tris-buffer blanks.

Polyacrylamide gel electrophoresis

All fractions monitored for ATPase were examined by electrophoresis in the

standard polyacrylamide gels as described in our previous papers [3, 4] and stained with Coomassie Blue and/or stained for enzymatic activity in the gels by the method of Weinbaum and Markman [18].

Where information was required on the variety of polypeptide chains in the fractions the sodium dodecylsulfate–polyacrylamide gel electrophoresis technique of Weber and Osborn [19] was used and the gels stained with Coomassie Blue as described by these investigators.

RESULTS

Release and purification of ATPase from membranes subjected to n-butanol extraction procedure

The ATPase activity in the initial membranes, in the EDTA-wash supernatant and membrane residue and that released following the *n*-butanol extraction procedure as described in Materials and Methods are summarized in a typical experiment presented in Table I. The purification steps subsequent to the release of the enzyme into aqueous phase are also illustrated in Table I. All assays of the ATPase were performed in the presence and absence of trypsin as previously described [3, 4] in order to determine the presence of "latent" or "masked" activity.

TABLE I

RELEASE AND PURIFICATION OF *M. LYSODEIKTICUS* ATPase BY *n*-BUTANOL EXTRACTION PROCEDURE

All assays were performed in the presence and absence of trypsin as described in Materials and Methods.

Purification step	Total protein (mg)	Units ATPase	Units ATPase (trypsin assay)	Specific activity	Specific activity (trypsin assay)
Initial membrane suspension	692	155	233	0.23	0.33
EDTA wash	119	9	13	0.08	0.11
Membrane residue	571	71	211	0.12	0.37
n-Butanol aqueous phase	202	328	303	1.62	1.50
n-Butanol aqueous phase after ultrafiltration, high-speed centrifugation	65	409	349	6.3	5.4
After $(NH_4)_2SO_4$ precipitation	15	80	74	5.3	5.0
Sephadex column I*	12.1	108	106	8.9	8.7
Sephadex column II*	6.7	67	54	10.0	8.1

* Values given are for pooled fractions recovered from the columns judged to be principally ATPase (Column I) or homogeneous ATPase (Column II) on basis of polyacrylamide gel electrophoresis.

It can be seen from the results presented in Table I that the initial membrane preparation and the membrane residue after the EDTA-wash step both exhibited some stimulation by trypsin. However, after the *n*-butanol extraction step no trypsin stimulation of activity was observed; on the contrary, the ATPase showed varying degrees of sensitivity to trypsin (Table I). In accord with our previous experience [16], the EDTA wash did not generally result in the release of substantial ATPase activity. One other feature worthy of mention is that the $(NH_4)_2SO_4$ precipitation step resulted in losses of enzyme units without significant gains in the specific activity of the ATPase. For reasons as yet unknown to us, the $(NH_4)_2SO_4$ precipitation step has given rather variable results and at this stage it cannot be recommended as a useful procedure in this purification. The overall purification in this particular experiment is approx. 50-fold based on the initial specific activity in the membrane and that of the final homogeneous preparation.

The release and purification of the ATPase has been monitored throughout by polyacrylamide gel electrophoresis. A typical series of gels stained with Coomassie Blue illustrating the aqueous-phase fraction containing solubilized membrane proteins following the *n*-butanol extraction procedure and the series of fractions from the first and second separations on Sephadex G-200 are presented in Fig. 1. The major protein band detected in the aqueous phase following the *n*-butanol extraction method and revealed by staining with Coomassie Blue (Fig. 1, Gel A) corresponds to the ATPase as revealed by the enzymatic stain of Weinbaum and Markman [18]. Other proteins solubilized by this procedure include the fast-moving component studied by Fukui et al. [20] and bands corresponding to the NADH dehydrogenase [16] although much of this was released from the membrane by the EDTA-wash step in the purification sequence. The separation of the ATPase from these other solubilized proteins is achieved by gel filtration on the Sephadex G-200 column and the polyacrylamide gel electrophoresis of the fractions from the column is illustrated in Fig. 1, Gels B–H and shows the elution of the NADH dehydrogenase and fast-moving component after the main peak of ATPase activity. Only fractions containing the ATPase

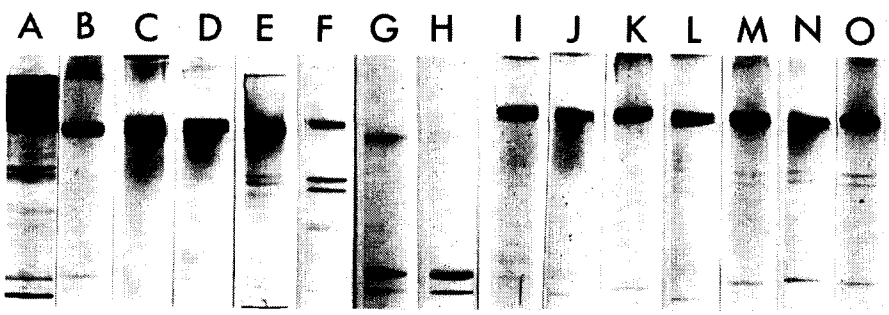

Fig. 1. Polyacrylamide gel electrophoresis of the *M. lysodeikticus* membrane proteins released into the aqueous phase following *n*-butanol extraction is shown in Gel A and the peak of ATPase activity and late fractions from gel filtration on Sephadex G-200 are illustrated in Gels B–H. The major band in the initial *n*-butanol-solubilized fractions and Gels B–E is the ATPase and the principal band in Gels G and H corresponds to the fast-moving component investigated by Fukui et al. [20]. The polyacrylamide gel electrophoresis of the fractions from the second cycle of separation on Sephadex G-200 is shown in Gels I–O.

were pooled for the second cycle of gel filtration, thereby removing most of the NADH dehydrogenase and fast-moving component. Polyacrylamide gel electrophoresis of the ATPase fractions from the second Sephadex column is shown in Fig. 1, Gels I–O. Pooled fractions from this latter column were judged to be homogeneous by several criteria including immunoelectrophoresis [20, 21], centrifugation in the Spinco Model E analytical ultracentrifuge and by the uniformity of the appearance of the particles as seen by negative staining in the electron microscope [21, 22]. Moreover, preparations examined by the sodium dodecylsulfate–polyacrylamide gel electrophoresis method of Weber and Osborn [19] showed the presence of two polypeptide subunits and no associated proteins, in confirmation of our previous results reported for this form of ATPase [13].

Shock-wash ATPase

For comparative purposes we have purified the shock-wash ATPase released from membranes which have also been subjected to a prior EDTA-wash step to release NADH dehydrogenase components [16] as in the *n*-butanol procedure. The inclusion of this step and high-speed centrifugation after concentration of the shock-wash by ultrafiltration represent modifications of the method previously reported from this laboratory [4]. The results of a typical experiment illustrating the purification of this form of ATPase are presented in Table II and by assaying in the presence and absence of trypsin the latency of this form of ATPase is demonstrated. Unlike the *n*-butanol-released ATPase which was generally sensitive to trypsin, the shock-wash ATPase activity was stimulated upon assaying in the presence of trypsin. The $(NH_4)_2SO_4$ precipitation step illustrated in the purification of *n*-butanol-released ATPase (Table I) has also been tried repeatedly with the shock-wash ATPase and has given similar variable results with marked losses of units of enzyme and not infrequently, reductions in specific activities.

TABLE II

RELEASE AND PURIFICATION OF *M. LYSODEIKTICUS* SHOCK-WASH ATPase

All assays were performed in the presence and absence of trypsin as described in Materials and Methods.

Purification step	Total protein (mg)	Units ATPase	Units ATPase (trypsin assay)	Specific activity	Specific activity (trypsin assay)
Initial membrane suspension	855	89	150	0.10	0.18
EDTA wash	145	29	29	0.20	0.20
Membrane residue	660	136	360	0.21	0.54
0.003 M Tris shock wash	324	192	465	0.60	1.43
High-speed spin and ultrafiltration	23	120	240	5.20	10.4
Sephadex column I	16	91	164	5.7	10.3
Sephadex column II	10.7	107	140	10.0	13.1

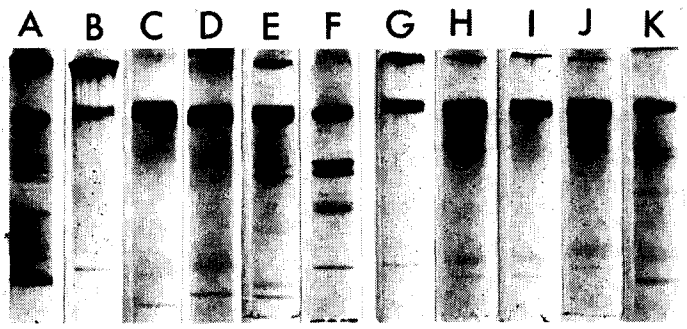

Fig. 2. Polyacrylamide gel electrophoresis of the initial shock-wash ATPase fraction from *M. lysodeikticus* membranes is shown in Gel A and the first and second cycles of separation on the Sephadex G-200 column as described in Materials and Methods are shown, respectively, in gels B–F and Gels G–K.

As with the *n*-butanol-released ATPase, the purification of the shock-wash ATPase has been followed by electrophoresis in polyacrylamide gels. Typical results are illustrated in Fig. 2 which shows the initial shock-wash fraction obtained from EDTA-washed membranes (Gel A) and the peak fractions from first and second consecutive separations by gel filtration on Sephadex G-200 columns are shown in Gels B–F and G–K, respectively.

Comparison of the release and purification of ATPase by the n-butanol and shock-wash procedures

From a considerable number of individual experiments on the release and purification of the ATPase by the two procedures described above we have gained some idea of the extent of variability of the initial and final specific activities of this

TABLE III

A COMPARISON OF THE SPECIFIC ACTIVITIES OF ATPase IN INITIAL MEMBRANES AND MEMBRANE FRACTIONS OF *M. LYSODEIKTICUS* ASSAYED IN THE PRESENCE AND ABSENCE OF TRYPSIN

Mean values and the range of values for a minimum of four individual experiments are presented for each preparation.

Preparation	Assay conditions	
	Control (Specific activity)	Trypsin present (Specific activity)
Initial membranes	0.30 (0.10– 0.60)	0.39 (0.18– 0.72)
Membrane residue after EDTA wash	0.30 (0.10– 0.80)	0.50 (0.31– 0.83)
0.003 M Tris shock wash	0.68 (0.25– 1.30)	1.30 (0.45– 1.50)
n-Butanol aqueous phase from membranes	1.68 (1.00– 2.42)	1.60 (0.96– 2.52)
Purified shock-wash ATPase	9.1 (8.0 –10.0)	11.3 (9.4 –13.1)
Purified butanol-released ATPase	13.2 (9.7 –19.8)	9.2 (7.8 –11.6)

membrane enzyme. By performing all assays in the presence and absence of trypsin we have obtained an indication of the latency of the ATPase at various stages of the release and purification procedures. The data for a minimum of four individual experiments is presented in Table III indicating the ranges of specific activities encountered throughout these studies. Quite a wide range in the initial ATPase activities in the membranes is apparent and this of course will have a marked effect on the overall purification factors. Until the factors governing latency or the masked state of membrane enzymes are more clearly understood and until the methods become available for determining true initial activities, purification factors can only be regarded as being very approximate.

In general, the final specific activities of the ATPase released by the *n*-butanol extraction procedure and the shock-wash method are rather similar although those of the former were consistently higher probably because of more complete dissociation of associated proteins from the *n*-butanol type of ATPase as shown by Salton and Schor [13].

DISCUSSION

The studies of Maddy [10, 11] were the first to establish the solubilization of membrane proteins (those of ox erythrocytes) by extraction of the lipids into *n*-butanol in what is essentially a two-phase system. The release of proteins from a variety of membranes into aqueous media by *n*-butanol and other aliphatic alcohols has been the subject of a number of studies [23–26]. Investigations in our laboratory have shown release of several membrane-bound enzymes (ATPase and NADH dehydrogenase) into solution by extracting membrane suspension with *n*-butanol and other alcohols [26]. We have developed the *n*-butanol procedure as an alternate method to the shock-wash release method and this paper compares the results of the purification of the ATPase by the two methods. The *n*-butanol procedure will undoubtedly be valuable in future structural studies of the ATPase protein and its subunits, since the enzyme prepared by this method contains two subunits and none of the associated polypeptide chains found in the shock-wash ATPase examined by sodium dodecylsulfate–polyacrylamide gel electrophoresis [13]. Moreover, a comparison of the properties of the two types of ATPase should enable us to elucidate the role of associated proteins in attachment and regulation of the enzymatic activity of the free and membrane-bound forms.

Although both the *n*-butanol-released ATPase and the shock-wash ATPase behave homogeneously as judged by column chromatography, polyacrylamide gel electrophoresis in standard conditions preserving enzymatic activity, immunoelectrophoresis, ultracentrifugation and uniformity of particle appearance in the electron microscope [4, 13, 20, 21], dissociation of the shock-wash ATPase and sodium dodecylsulfate–polyacrylamide gel electrophoresis revealed the presence of polypeptides additional to the two major subunits [13]. At the present time the amounts of such additional associated protein(s) and their functions are largely unknown although it has been suggested by Salton and Schor [13] that they are involved in rebinding to depleted membranes and regulation of the active site as indicated by the stimulating effects of trypsin. However, despite these differences in the properties of the ATPase obtained by the two procedures, their mean average molecular weights of 345 000

for the *n*-butanol type compared to 352 000 for the shock wash (unpublished results) and their final specific activities are quite similar. The *n*-butanol procedure described in this paper yields an ATPase preparation which is chemically more homogeneous than the shock-wash ATPase [13] and should enable us to characterize its two polypeptide chains as well as investigating the reconstitution of a particle capable of binding to depleted membranes through association with the nectin [27] class of protein and any other regulatory polypeptides.

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