PURIFICATION OF THE YEAST PLASMA MEMBRANE ATPase SOLUBILIZED WITH A NOVEL ZWITTERIONIC DETERGENT

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1. Introduction

ATPases with similar kinetic properties have been identified in the plasma membranes of *Neurospora crassa* [1,2], *Schizosaccharomyces pombe* [3] and *Saccharomyces cerevisiae* [4]. In these fungal cells the active transport of nutrients is coupled to the proton gradient and therefore it has been suggested that this ATPase operates as a proton pump [5–7]. In order to obtain direct evidence for this important physiological role it would be necessary to purify the enzyme, incorporate it into liposomes and look for ATP-driven proton transport in these structures [8].

The purification of membrane enzymes requires in most cases their solubilization with detergents and, as stated in [9], the optimal detergent for a particular membrane protein has to be found empirically. The plasma membrane ATPase of *Schizosaccharomyces pombe* has been solubilized with lysolecithin [10] but this agent, as well as many other conventional detergents were inoperative in our hands for the solubilization of the enzyme from *Saccharomyces cerevisiae*.

Synthetic zwitterionic detergents have been largely neglected in the study of membrane proteins [9]. However, in our hands, the yeast plasma membrane ATPase could be solubilized satisfactorily only with the novel detergent 3-(tetradecyldimethyl ammonium)-1-propanesulfonate (zwittergent TM314 [11]). This detergent may also be useful for the solubilization in active form of other membrane enzymes refractory to conventional detergents. The results presented here constitute the first report to our knowledge on the utilization of a synthetic zwitterionic detergent for the purification of membrane enzymes.

2. Materials and methods

The detergent 3-(tetradecyldimethylammonium)-1-propane-sulfonate was a generous gift from Dr A. Gonene (Calbiochem) and it is commercially available under the name zwittergent TM314. Soy bean phospholipids (phosphatidylcholine type II-S) and egg lysolecithin (type I) were obtained from Sigma.

Plasma membranes were obtained from commercial baker's yeast as in [4]. A medium containing 10% glycerol, 10 mM Tris–HCl (pH 7.5), 2 mM 2-mercaptoethanol and 1 mM EDTA was used throughout; in the gradients, only the glycerol concentration was changed. Treatment of the membranes with detergents was made at 2 mg protein/ml and solubilization was assesed by centrifugation for 45 min. at 35 000 rev./min in a Beckman 60 Ti rotor.

ATPase activity was measured as in [4], except at pH 6.5 and, when indicated, 500 μg sonicated soy bean phospholipids were included. Protein was determined by the microassay in [12] with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was done as in [13] except that acrylamide and SDS were 7.5% and 0.1%, respectively. Samples were prepared with 0.1–0.5 mg protein/ml, 2.5% SDS and 2% 2-mercaptoethanol and incubated for 10 min at 30°C.

3. Results and discussion

3.1. *Solubilization of yeast plasma membrane ATPase*

The ATPase activity associated with yeast plasma membranes could not be solubilized (<5%) by the

following detergents: cholate (≤50 mg/ml), deoxycholate (≤15 mg/ml). Triton X-100 (≤30 mg/ml) digitonin (5 mg/ml), egg lysolecitin (≤10 mg/ml) and SDS (≤1.5 mg/ml, higher concentrations inactivate). Combinations of detergents, raising to 20°C, sonication, lowering 0.2 mg protein/ml and the addition of salts (≤1 M ammonium sulfate or KCl) did not improve ATPase solubilization by these detergents. A similar resistance to solubilization has been described for the plasma membrane ATPase of *Neurospora crassa* [1].

A novel zwitterionic detergent with great solubilizing power: the sulfobetaine derivative 3-(tetradecyldimethylammonio)-1-propanesulfonate described [11], is referred in the following as zwittergent. Although it was stated [11] that proteins were not denatured by the detergent we found that at >1.5 mg/ml the yeast plasma membrane ATPase was inactivated. If glycerol was omitted from the medium the concentration of zwittergent tolerated by the enzyme was even lower. Nevertheless, this synthetic detergent at 1.5 mg/ml solubilized 20–30% of the ATPase and it has been in our hands the only detergent capable of solubilizing the yeast plasma membrane ATPase in an active form. The superiority of the zwittergent may be related to the sulfobetaine polar head [11] because lysolecithin, which has a similar hydrophobic tail and is also zwitterionic, was ineffective.

The solubilization effected by the zwittergent could be increased to 50–80% of the ATPase by the following procedure. First the membranes were treated with 5 mg cholate/ml to remove easily solubilized proteins. The residual pellet was resuspended at 2 mg/ml and treated with 1.5 mg zwittergent/ml. After 10 min incubation at 20°C the sample was sonicated for 3 min in a bath sonicator and centrifuged.

3.2. *Purification of the solubilized enzyme*

Solubilized enzyme (4 ml) was applied to 20 ml linear glycerol gradient (20–50%, v/v) containing 0.10 mg/ml of zwittergent and centrifuged in the cold at 37 000 rev./min during 14 h in a Beckman 60 Ti rotor. As shown in fig.1A, the ATPase was associated with a small peak of protein of high mobility and most of the protein moved much more slowly. From 20–30% of the ATPase was recovered as a pellet, this probably corresponds to large aggregates of incompletely solubilized enzyme.

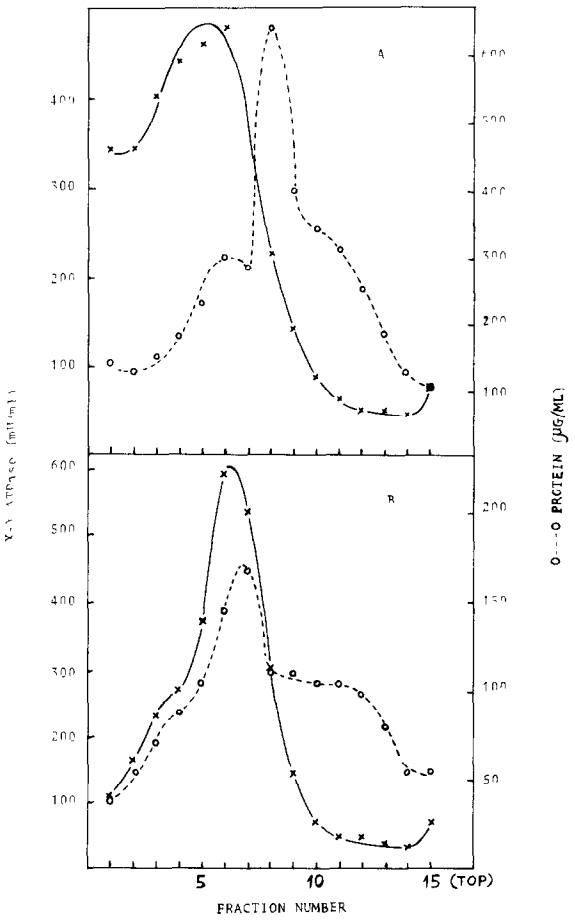

Fig.1. Distribution of solubilized ATPase and protein after glycerol gradient centrifugation. The conditions were described in the text. ATPase activity was measured in the presence of 500 μg soy bean phospholipids (A) 4 ml zwittergent extract were applied to the gradient. (B) Fractions 2–6 from the above gradient were pooled and applied to a second gradient as in the text.

Fractions 2–6 from the gradient were combined and diluted 2-fold to reduce the concentration of glycerol. At this stage it was important to include in the dilution medium the protease inhibitors [14] *p*-aminobenzamidine (10 mM) and phenylmethylsulfonylfluoride (1 mM) because a latent protease contaminant became activated which degraded the ATPase (F. M., R. S., unpublished). The sample was concentrated to 1/3rd original vol. by pressure filtration in an Amicon cell (model 52) with a Diaflo PM 30 membrane. Concentrated material (4 ml) was supplemented with 80 μg zwittergent/ml and applied to a second glycerol gradient which differed from

the first only in that zwettergent was 40 μg/ml. Higher concentrations in this second gradient inactivated the ATPase. The activity exhibited the same mobility as in the first gradient and the residual contaminant protein again lagged behind (fig.1B). The results of a typical purification are summarized in table 1. The ATPase was purified 6-fold with a recovery of 20%. In the course of the purification the enzyme was depleted of essential phospholipids and it only exhibited full activity in the presence of exogenous phospholipids. The purified enzyme was stable at 0°C for several days and at −20°C for several weeks. This compares favourably with the ATPase purified from *Schizosaccharomyces* [10], which was highly unstable and was obtained in much lower yield.

3.3. *Subunit composition of the purified enzyme*

SDS–polyacrylamide gel electrophoresis revealed the enrichment of 105 000 mol. wt band in the course of the purification (fig.2). Planimetric measurements of the gel scans indicated that this band represents ~15%, 55% and 85% of the total protein in the original membrane and in the purified enzyme after the first and second glycerol gradient, respectively. This enrichment is in good agreement with the increase of specific activity of the preparations (table 1). A striking feature of this polypeptide is that it becomes aggregated and does not enter the gels if the samples for electrophoresis are boiled.

About 15% of the protein in the purified preparation was distributed between three small bands of 50 000, 44 000 and 15 000 mol. wt. They probably represent firmly bound contaminants and not components of the enzyme because in the passage from the first to the second glycerol gradient the specific activity was increased while these bands decreased proportionally (fig.2B,C).

Table 1
Purification of yeast plasma membrane ATPase

Fraction	Protein (mg)	ATPase (μmol/min × mg)
Plasma membranes	60	0.70
Cholate-treated membranes	40	1.50
Zwittergent extract	30	1.70[a]
First glycerol gradient	6.4	3.00[a]
Second glycerol gradient	2.1	4.00[a]

[a] Assayed in the presence of 500 μg sonicated soy bean phospholipids

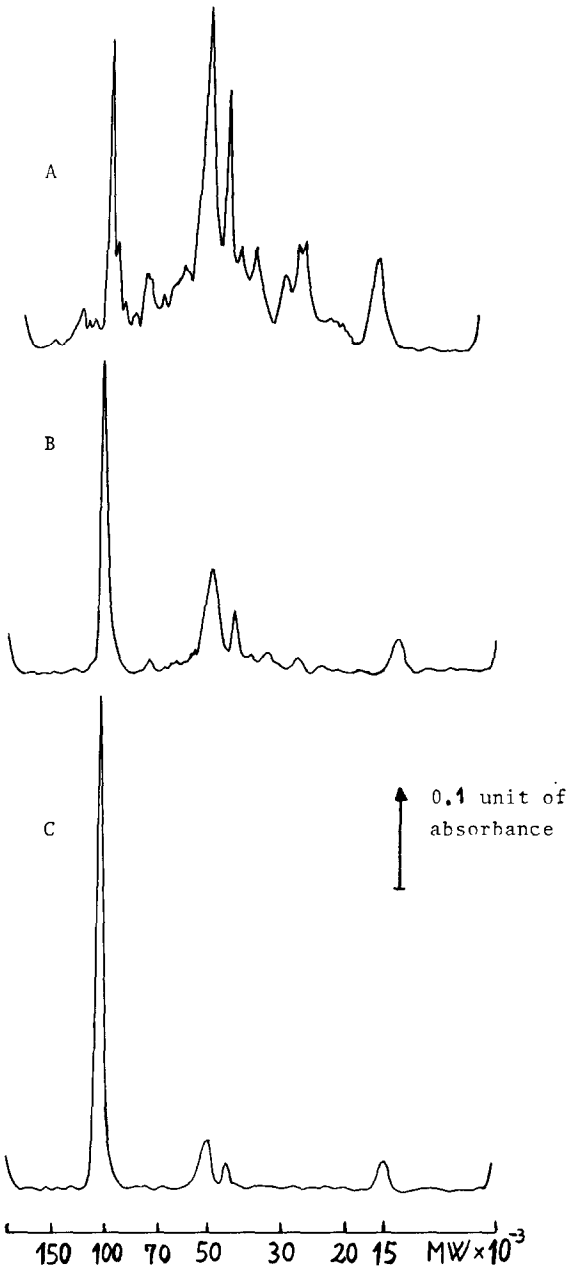

Fig.2. SDS–polyacrylamide gel electrophoresis of plasma membranes (A) and of the enzyme purified after the first gradient (B), fractions 2–6 of fig.1A, and after the second gradient (C), fractions 5–7 of fig.1B. Protein (5 μg) was applied to the gels, which were stained with Coomassie blue R 250 and scanned at 560 nm in a Gilford spectrophotometer with a linear transport accessory. Migration distance is expressed as molecular weight according to a calibration made with RNA polymerase, glucose oxidase, bovine serum albumin, α-chymotrypsinogen and cytochrome *c*.

It has been observed with a crude preparation of the enzyme that the 105 000 band becomes phosphorylated in the course of the reaction [15]. Therefore this polypeptide contains the active site in accordance with its enrichment during purification. A similar polypeptide represents 85% of the protein in the ATPase purified from the plasma membranes of *Schizosaccharomyces pombe* [10], although in this case phosphorylation of the enzyme has not been reported.

The (Na^+/K^+)-ATPase, the (Ca^{2+})-ATPase and the (H^+/K^+)-ATPase of animal cells also contain a major polypeptide of ~100 000 mol. wt which becomes phosphorylated in the course of the reaction [8]. This similarity between fungal and animal ATPases indicates a common evolutionary origin.

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