Abundance of the Ca²⁺-pumping ATPase in pig erythrocyte membranes

Clement O. BEWAJI* and Enitan A. BABABUNMI

Department of Biochemistry, University of Ibadan, Ibadan, Nigeria, and Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

The Ca²⁺-pumping ATPase (Ca²⁺-ATPase) was purified from human and pig erythrocyte membranes by calmodulin affinity chromatography in the presence of phosphatidylcholine. The amount of enzyme present in pig erythrocytes is at least 7 times greater than that isolated from human erythrocyte ghosts. However, the properties of the enzyme from the two species are similar in many respects.

INTRODUCTION

The activities of ATP-dependent Ca²⁺ pumps in plasma membranes are fundamental to the messenger functions of Ca²⁺, since these enzymes regulate cytosolic Ca²⁺ concentrations. The Ca²⁺-ATPase from human erythrocytes has been purified and characterized in many laboratories (Gietzen *et al.*, 1980; Niggli *et al.*, 1981; Al-Jobore & Roufogalis, 1981). The activity of the enzyme can be stimulated by calmodulin, acidic phospholipids and unsaturated fatty acids, and by limited proteolysis (Niggli *et al.*, 1981; Stieger & Schatzmann, 1981).

The understanding of the molecular events produced by various effectors on the erythrocyte and other plasma-membrane Ca²⁺-ATPases, as well as the overall mechanism of plasma-membrane Ca²⁺ transport, would require a knowledge of the primary structure of the enzyme. Such studies have been hindered by the paucity of the enzyme, which represents less than 0.1% of the total protein of the human erythrocyte membrane (Gietzen & Kolandt, 1982). As a result, much effort has been put into devising methods for obtaining sufficient quantities of the enzyme for molecular studies (Rosenbery *et al.*, 1981; Gietzen & Kolandt, 1982).

We have previously reported that the specific activity of this enzyme in intact pig erythrocyte membranes is an order of magnitude higher than in some other mammalian erythrocytes examined (Bewaji *et al.*, 1985). In the present study, we have purified the Ca²⁺-ATPase from pig erythrocytes and reconstituted it into liposomes. We report that the enzyme is at least 7 times more abundant in pig than in human erythrocyte membranes.

METHODS

Preparation of proteins

Calmodulin was prepared from bovine brain by the procedure described by Guerini *et al.* (1984). The Ca²⁺-ATPase was purified from human and pig erythrocyte membranes, in the presence of phosphatidylcholine, essentially as described by Niggli *et al.* (1981). The pig erythrocyte Ca²⁺-ATPase was reconstituted into asolectin liposomes as described by Niggli *et al.* (1982).

Assays

ATPase activities were determined at 37 °C as described by Niggli *et al.* (1981). Protein was determined by

Table 1. Balance sheet for the purification of Ca²⁺-ATPase from human erythrocytes

Solubilization and isolation of the Ca²⁺-ATPase were performed as described by Niggli *et al.* (1981). ATPase activity was determined at 37 °C and a free Ca²⁺ concentration of 10 µM. When present, the assay medium contained 3 µg of calmodulin (CaM)/ml.

Fraction	Total protein (mg)	Protein yield (%)	Ca ²⁺ -ATPase			
			Total activity (µmol/min)		Specific activity (µmol/min per mg)	
			No CaM	+ CaM	No CaM	+ CaM
1. Ghosts	189	100	1.51	9.45	0.008	0.05
2. Triton-X-100-solubilized ghosts	41.6	22	3.74	9.98	0.09	0.24
Column:						
3. Peak eluted with Ca ²⁺ buffer	35.9	19	1.11	1.29	0.031	0.36
4. Peak eluted with EDTA buffer	0.17	0.09	0.44	1.22	2.6	7.2

Abbreviation used: Ca²⁺-ATPase, Ca²⁺-pumping ATPase.

* Present address: Department of Biochemistry, University of Ilorin, Ilorin, Nigeria.

Table 2. Balance sheet for the purification of Ca²⁺-ATPase from pig erythrocytes

Experimental conditions are as stated in the legend to Table 1.

Fraction	Total protein (mg)	Protein yield (%)	Ca ²⁺ -ATPase			
			Total activity (μmol/min)		Specific activity (μmol/min per mg)	
			No CaM	+ CaM	No CaM	+ CaM
1. Ghosts	500	100	10.5	56	0.021	0.112
2. Triton-X-100-solubilized ghosts	145.5	29.1	56.7	157.14	0.39	1.08
Column:						
3. Peak eluted with Ca ²⁺ buffer	138	27.6	9.25	9.94	0.067	0.072
4. Peak eluted with EDTA buffer	3.6	0.72	16.02	34.7	4.45	9.64

the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard. Where there are interfering materials such as Hepes, dithiothreitol and Triton X-100, the protein was first precipitated with deoxycholate and trichloroacetic acid as described by Bensadoun & Weinstein (1976).

SDS/polyacrylamide-gel electrophoresis

The electrophoretic system used was that described by Laemmli (1970). Samples (50 μl) containing about 20 μg of erythrocyte ghost protein or 6–7 μg of the purified Ca²⁺-ATPase were mixed with 25 μl of a buffer containing 30 mM-sodium phosphate, pH 7.0, 30 % (v/v) glycerol, 7.5 % (w/v) SDS, 10 mM-dithiothreitol and 0.05 % (w/v). Bromophenol Blue as tracking dye, boiled for 3 min and applied to the gels; 10%-polyacrylamide gels were routinely used. The gels were stained with Coomassie Brilliant Blue R-250 and destained with 7.5% acetic acid/20 % methanol (both v/v).

RESULTS AND DISCUSSION

The balance sheets for the purification of Ca²⁺-ATPase from human and pig erythrocyte membranes are shown in Tables 1 and 2. These results clearly demonstrate that the enzyme is more abundant in pig than in human erythrocyte membranes. The yield of the enzyme from human erythrocyte membranes is 0.09% of the total membrane proteins, compared with 0.7% from pig erythrocytes. Fig. 1 also shows a Coomassie Blue-stained polyacrylamide gel of the enzyme from pig erythrocyte membranes at various stages of purification. On SDS/10%-polyacrylamide slab gel, the enzyme shows a major band of *M_r* approx. 140000. A minor band, representing about 6% of the total proteins, is usually present at a position corresponding to *M_r* 90000. It is not clear whether the minor band is a subunit of the ATPase or a product of proteolytic degradation of the enzyme. After storage of the enzyme for a few days at –80 °C it usually forms aggregates, probably dimers, which could not be dissociated in the presence of SDS.

In other respects, the properties of the pig erythrocyte Ca²⁺-ATPase are similar to those of the human enzyme. It is highly sensitive to inhibition by orthovanadate; the inhibition constant, determined by a Dixon plot (Fig. 2),

Fig. 1. SDS/polyacrylamide-gel electrophoresis of pig erythrocyte Ca²⁺-ATPase at various stages of purification

Lanes 1–4 correspond to the different fractions in Table 2; 5–20 μg of protein from each fraction was dissolved in a sample buffer containing SDS as described in the Methods section and applied to a 10%-polyacrylamide slab gel. The gel was stained with Coomassie Brilliant Blue R-250.

is $1.6 \pm 0.2 \mu\text{M}$. The purified enzyme, when reconstituted into asolectin liposomes, transports Ca²⁺ into the vesicles with an initial rate of $1.43 \pm 0.11 \mu\text{mol/min per mg}$ of protein (Fig. 3). The steady-state rate of ATP hydrolysis was $3.14 \pm 0.08 \mu\text{mol/min per mg}$ of protein. This was stimulated about 4-fold after the addition of A23187 (Fig. 4).

The abundance of the Ca²⁺-ATPase in pig erythrocyte membranes reported here looks promising, because it

Fig. 2. Dixon plot for the inhibition of purified pig erythrocyte Ca^{2+} -ATPase by vanadate

ATP hydrolysis was measured at 37 °C with the coupled enzyme assay described by Niggli *et al.* (1981).

Fig. 3. Ca^{2+} transport by the purified Ca^{2+} -ATPase from pig erythrocytes after reconstitution into asolectin liposomes

Ca^{2+} transport was measured at 37 °C with a Ca^{2+} -selective electrode. The system was calibrated after completion of the uptake by the addition of 10 nmol of CaCl_2 .

raises the hope of obtaining enough of the enzyme for further molecular studies. However, it is not yet clear why pig erythrocytes have such a high content of this enzyme. Since it has been suggested that the Ca^{2+} pump in human erythrocyte membranes is functioning well below its capacity (Jarrett & Kyte, 1979; Roufogalis, 1979; Penniston, 1982), it would be interesting to find out the reasons for the apparent increase in the amount of pump protein in pig erythrocytes.

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Fig. 4. ATPase activity of the purified pig erythrocyte Ca^{2+} -ATPase after reconstitution into asolectin liposomes

ATP hydrolysis was measured at 37 °C by the coupled enzyme assay described by Niggli *et al.* (1981). The absorbance difference between 366 and 550 nm was monitored continuously in an Aminco dual-wavelength spectrophotometer.

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