Large-scale isolation of human erythrocyte Ca^{2+} -transport ATPase

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A rapid procedure for preparing large quantities of purified erythrocyte Ca^{2+} -transport ATPase is presented. The method involves: (1) fast preparation of calmodulin-deficient, essentially haemoglobin-free, erythrocyte membranes by molecular filtration using Pellicon filters; (2) solubilization of membrane proteins by deoxycholate; and (3) a batch procedure using calmodulin–Sephacrose 4B gel for purification of Ca^{2+} -transport ATPase.

Ca^{2+} -transport ATPase of human erythrocytes is responsible for the low intracellular Ca^{2+} concentration (Schatzmann, 1973). The activity of this enzyme is modulated by the Ca^{2+} receptor calmodulin in dependence on the cytoplasmic Ca^{2+} concentration.

The Ca^{2+} -dependent interaction of calmodulin with its effector enzymes can be used to purify these enzymes by means of calmodulin–Sephacrose conjugates, as was first shown by Watterson & Vanaman (1976) for phosphodiesterase. Such an affinity-chromatographic procedure was used successfully to purify erythrocyte Ca^{2+} -transport ATPase in a functional state (Gietzen *et al.*, 1980b). Calmodulin affinity chromatography is a powerful tool for purification of calmodulin-dependent enzymes, since calmodulin is easily accessible in large quantities and thus calmodulin–Sephacrose gel of sufficient binding capacity can be prepared. Since Ca^{2+} -transport ATPase is just a minor component of erythrocyte membrane protein (Gietzen *et al.*, 1980b), and since preparation of erythrocyte membranes by conventional centrifugation procedures is very time-consuming, large-scale purification of Ca^{2+} -transport ATPase requires an efficient preparation method for calmodulin-deficient erythrocyte membranes. More recently, Rosenberry *et al.* (1981) reported a large-scale preparation of erythrocyte membranes based on molecular filtration, with retention of biological activity of acetylcholinesterase. However, retention of biological activity of acetylcholinesterase is not a reliable measure for harmlessness of this procedure to more sensitive enzymes such as Ca^{2+} -transport ATPase.

The present paper describes a large-scale isolation technique for Ca^{2+} -transport ATPase of erythrocytes based on the combination of two

known procedures that had to be modified to meet the requirements of the intended preparation. The method makes use of (1) molecular filtration for the preparation of calmodulin-deficient erythrocyte membranes displaying a high specific Ca^{2+} -transport-ATPase activity and (2) a batch procedure using calmodulin–Sephacrose gel for purification of Ca^{2+} -transport ATPase from solubilized erythrocyte membrane proteins.

Experimental

Materials

All reagents were of highest purity available. Fresh human erythrocytes (group O, Rh⁺) were obtained from a local blood bank. Phosphatidylcholine was prepared from egg phospholipids (Singleton *et al.*, 1965) and calmodulin from bovine brain (Kakiuchi *et al.*, 1981). Potassium deoxycholate was recrystallized as described by Meissner *et al.* (1973), and Tween 20 (Sigma) was freed of contaminating metal ions by passing it before use through a Dowex 50 W (X8) ion-exchange column. Calmodulin–Sephacrose conjugate (10 mg of calmodulin/g of dry CNBr-activated Sepharose 4B) was prepared as described by Gietzen *et al.* (1980b).

Methods

Haemolysis. To 1 vol. of packed erythrocytes that had been washed three times in 150 mM-KCl/20 mM-Mops (4-morpholinepropanesulphonic acid) (pH 7.4) was added 1 vol. of a medium containing 50 mM-KCl, 20 mM-ascorbic acid, 1 mM-EDTA, 10 mg of Tween 20/ml, 2 mM-di-isopropyl phosphorofluoridate and 20 mM-Mops (pH 7.4). The erythrocytes were haemolysed by freezing this mixture at -80°C , followed by thawing of the haemolysate in a water bath at 15°C .

Preparation of erythrocyte membranes by centrifugation. All steps of the preparation of erythrocyte membranes and purified Ca^{2+} -transport ATPase were performed at 0–4°C. Membrane-bound erythrocyte Ca^{2+} -transport ATPase, deficient in calmodulin, was prepared on the basis of the procedure described by Gietzen *et al.* (1980a), modified as follows: the wash solution, termed 'W1' below, used for the first three washes after haemolysis contained 50 mM-KCl, 10 mM-ascorbic acid, 0.5 mM-EDTA and 5 mM-Mops (pH 7.0). The wash solution ('W2') used for the next two washes was identical with solution W1, except that EDTA was omitted. The final pellet of erythrocyte membranes was suspended in an equal volume of a buffer consisting of 1 M-sucrose, 200 mM-KCl, 1 mM-MgCl₂, 10 mM-dithiothreitol and 10 mM-Mops (pH 7.0) and stored in a liquid-N₂ refrigerator until use.

Preparation of erythrocyte membranes by molecular filtration. Calmodulin-deficient erythrocyte membranes, displaying Ca^{2+} -transport-ATPase activity, were prepared by removing haemoglobin and other soluble proteins from plasma membranes by means of a Millipore Pellicon Cassette System in series with a 3 litres/min peristaltic pump. The operation of the Pellicon Cassette System has been described in detail (Rosenberry *et al.*, 1981). The Cassette contained 10 ft² (0.93 m²) of Pellicon HVLP filter (pore size 0.5 µm) in 20 sheets. In contrast with Rosenberry *et al.* (1981), we chose this type of filter because of lower costs and higher efficiency. It must be stressed that this type of filter does not tolerate any back pressure at the retentate channel.

In typical membrane preparations 1 vol. of haemolysate was diluted with 9 vol. of solution W1. The sample was maintained at this 10 vol. level for constant volume wash, during which 10 vol. of solution W2 was continuously added. The membrane suspension then was concentrated to 1.5 vol., and the concentrated sample was washed with additional 3.5 vol. of solution W2 under constant-volume wash conditions. After washing, the membrane suspension was concentrated down to 0.35 vol. and flushed out of the system with 400 ml of solution W2. Membranes were recovered by sedimentation of this sample at 37000 g for 60 min and stored as described above.

Maximally 2 litres of haemolysate (equivalent to 1 litre of packed cells) were processed at once. Then the filters were soaked in and flushed with 0.1 M-NaOH for 20–30 min to restore a high filtration rate before the next 2 litres of haemolysate were worked up. The final centrifugation of the membrane suspension obtained from the Pellicon System was started while the filters were soaking.

Solubilization and purification of Ca^{2+} -transport

ATPase. Solubilization of erythrocyte Ca^{2+} -transport ATPase was performed as described by Gietzen *et al.* (1980b) by using deoxycholate at a detergent/protein ratio of 0.08–0.1 (w/w) in a medium consisting of 200 mM-sucrose, 300 mM-KCl, 0.1 mM-MgCl₂, 0.5 mM-CaCl₂, 10 mM-dithiothreitol, 1 mM-di-isopropyl phosphorofluoridate, 0.1 mM-phenylmethanesulphonyl fluoride, 0.05 µg of pepstatin A/ml, 0.2 mg of phosphatidylcholine/ml and 10 mM-Mops (pH 7.0). For processing the amount of membranes obtained from 2 litres of packed cells, two Beckman type 35 rotors were used simultaneously.

In contrast with our earlier report (Gietzen *et al.*, 1980b), Ca^{2+} -dependent binding of Ca^{2+} -transport ATPase to calmodulin–Sephacryl gel was not achieved by column chromatography, but by a batch procedure which allows fast processing of large volumes of solubilized material. Before each use 100 ml of calmodulin–Sephacryl gel (containing 350 mg of immobilized calmodulin) was washed, on a fritted glass filter connected to a water aspirator, with 200 ml of buffer A containing 100 mM-KCl, 200 mM-sucrose, 0.1 mM-MgCl₂, 0.1 mM-CaCl₂, 0.1 mM-di-isopropyl phosphorofluoridate, 0.1 mM-phenylmethanesulphonyl fluoride, 5 mM-dithiothreitol, 0.5 mg of phosphatidylcholine/ml, 1.25 mg of Tween 20/ml, 0.5 mg of Triton X-100/ml and 10 mM-Mops (pH 7.0). Usually 700 ml of deoxycholate extract (solubilized material) was mixed with 100 ml of calmodulin–Sephacryl gel in a bottle and the mixture was rotated end-over-end for 30 min. Protein not bound to the gel in the presence of Ca^{2+} was separated from it on a fritted glass filter. Subsequently non-specifically adsorbed protein was removed by washing the calmodulin–Sephacryl gel with 2–2.5 litres of buffer A. To elute the purified ATPase in a minimum volume of buffer, the gel was then transferred in buffer A into a column of 4 cm diam., which was connected to a u.v. monitor. Purified Ca^{2+} -transport ATPase was eluted in a single peak exclusively after EGTA-containing buffer B (identical with buffer A except that CaCl₂ was replaced by 1 mM-EGTA and the concentration of Tween 20 was decreased to 0.5 mg/ml) was applied to the column. After elution of the purified protein from the column, CaCl₂ (final concn. 1.1 mM) was added.

Concentration and storage of purified Ca^{2+} -transport ATPase. Ca^{2+} -pump protein was concentrated with minimal loss of activity by means of Amicon XM100 ultrafiltration membranes. The purified protein was stored in a liquid-N₂ refrigerator, in buffer B supplemented with CaCl₂ to give a free Ca^{2+} concentration of 0.1 mM, 250 mM-glycerol and, when present, 0.15 mg of calmodulin/mg of ATPase.

Assays. ATPase activities were determined at

37°C as described by Gietzen *et al.* (1981). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. To avoid interference by detergents and dithiothreitol, protein was precipitated by mixing 1 vol. of protein solution with 5 vol. of a solution containing 12% (w/v) of trichloroacetic acid and 30% (v/v) of propan-2-ol. The mixture was kept on ice for about 30 min before the precipitate was sedimented by centrifugation at 50000g for 20 min at 0°C. The pellet then was washed with 2 ml of a solution consisting of 10% (w/v) of trichloroacetic acid and 25% (v/v) of propan-2-ol and centrifuged again. The final pellet was redissolved in 10 mM-NaOH before the procedure of Lowry *et al.* (1951) was used.

Results and discussion

Calmodulin-deficient erythrocyte membranes were prepared by a centrifugation procedure or by molecular filtration. The two methods and the properties of the erythrocyte membranes obtained are compared in Table 1. Both types of membranes display an acceptable activity of (Na⁺ + K⁺)-transport ATPase, though the preparation conditions were not designed for this ATPase but for obtaining optimum Ca²⁺-transport ATPase activity. The use of the Pellicon Cassette System, as compared with the centrifugation procedure, results not only in a higher yield of Ca²⁺-transport ATPase activity/litre of packed cells but also in membranes that exhibit a significantly higher specific activity of this enzyme (determined in the presence of calmodulin) because of a more pronounced sensitivity to calmodulin. Specific Ca²⁺-transport ATPase activity of erythrocyte membranes prepared by the Pellicon System is considerably higher than that of membrane pre-

parations from other laboratories (Farrance & Vincenzi, 1977; Niggli *et al.*, 1981; Scharff, 1981). More important than the gain in specific activity of membrane-bound Ca²⁺-transport ATPase is the fact that the Pellicon System allows preparation of erythrocyte membranes from 5 litres of packed cells within 8–9 h, whereas the centrifugation procedure is much more time-consuming (Table 1). Furthermore, the molecular-filtration apparatus is capable of handling even larger quantities of haemolysate per day by increasing the number of filter sheets.

Purified ATPase was prepared from deoxycholate-solubilized erythrocyte membranes by a batch procedure using calmodulin–Sephacryl gel. This procedure is more efficient than column chromatography, since it allows fast processing of large volumes of solubilized material. In Table 2, a balance sheet of a typical experiment for the isolation procedure of Ca²⁺-transport ATPase is given. Erythrocyte membranes obtained from 2 litres of packed cells yield approx. 10 mg of purified Ca²⁺-transport ATPase, displaying high specific activity of 15–17 µmol/min per mg of protein. This represents a 50–70% increase in specific activity as compared with our earlier report (Gietzen *et al.*, 1980b), and 300% increase as compared with the preparation of Niggli *et al.* (1981). The gain in specific activity might be due to the rapid procedure of both steps, membrane preparation as well as isolation of the purified enzyme. Solubilization and purification of Ca²⁺-transport ATPase on a scale as shown in Table 2 or even twice as much (provided that sufficient rotor capacity is available) can be completed within 4 h. In terms of protein, the yield of purified Ca²⁺-transport ATPase could be doubled as compared with our previous report (Gietzen *et al.*, 1980b), because the ATPase in the solubilized state displayed a higher responsiveness to calmodulin, and

Table 1. *Preparation and properties of erythrocyte membranes*

Erythrocyte membranes were prepared either by a centrifugation procedure or by molecular filtration in a Pellicon Cassette System: 10 litres of haemolysate are equivalent to 5 litres of packed cells. For further details see the Experimental section. The yield is expressed as activity/litre of packed cells measured in the presence of calmodulin. When present, the assay medium contained 30 nM-calmodulin. The values for yield and specific activities represent mean values ± s.d. Statistical analysis was performed by using Student's *t* test for evaluation of significance: the values marked with * are significantly different from each other (*P* < 0.01). One 'day' is a working day of 8–9 h.

Procedure	Time required for processing 10 litres of haemolysate (days)	Ca ²⁺ -transport ATPase		(Na ⁺ + K ⁺)-transport ATPase
		Yield (µmol/litre of packed cells)	Sp. activity (± calmodulin) (nmol/min per mg of protein)	Sp. activity (nmol/min per mg of protein)
Centrifugation	5	180	(–) 16 (+) 67 ± 7.1 (<i>n</i> = 8)*	7.5
Pellicon	1	250	(–) 17 (+) 85.1 ± 8.8 (<i>n</i> = 5)*	7.8

Table 2. *Balance sheet: purification of Ca²⁺-transport ATPase*

Solubilization and isolation of erythrocyte Ca²⁺-transport ATPase was performed as described in the Experimental section. Ca²⁺-transport ATPase activity was determined at 37°C and a free Ca²⁺ concentration of 36 μ M as described by Gietzen *et al.* (1981). When present, the assay medium contained 30 nM-calmodulin. In analogy to the column procedure, the protein not bound to the calmodulin–Sephacryl gel in the presence of Ca²⁺ is referred to as 'Ca²⁺ peak'. The protein eluted with the EGTA-containing buffer B is termed 'EGTA peak'.

Fraction	Volume (ml)	Protein (mg)	Activity (μ mol/min)		Activation by calmodulin (fold)	Sp. activity + calmodulin (μ mol/min per mg of protein)
			–Calmodulin	+Calmodulin		
Erythrocyte membranes	800	5258	93.0	468	5.03	0.089
Deoxycholate extract	690	549	90.4	280	3.10	0.510
Ca ²⁺ peak	680	518	62.7	68.3	1.09	0.132
EGTA peak	150	10.2	21.6	161.2	7.46	15.8

as a result of this more ATPase could be retained by the affinity column. Decreasing MgCl₂ in the buffer used for solubilization and in buffer A from 1 mM (Gietzen *et al.*, 1980a) to 0.1 mM likewise contributed to the better yield. This is in agreement with the fact that millimolar concentrations of Mg²⁺ enhance the dissociation of calmodulin from the ATPase (Scharff, 1980).

Electrophoresis of the purified Ca²⁺-transport ATPase on 5% polyacrylamide gels revealed that the enzyme consists of a single polypeptide (Fig. 1, gel a), of 140 000 mol.wt. (Gietzen *et al.*, 1980b). However, in the gel system of Laemmli & Favre (1973), with 10% polyacrylamide in the separating gel, the single band seen in the 5%-acrylamide system was resolved into two bands (Fig. 1, gel b). The doublet only can be made visible on high-resolution gels when not overloaded. This could mean that the enzyme consists of two different polypeptides, but it is also possible that the doublet is due to proteolytic degradation.

In theory, the ATPase when purified by a calmodulin–Sephacryl column should be free of calmodulin. Essentially it is so, since the preparation contains only 1 mol of EGTA-dissociable calmodulin/400 mol of ATPase as determined by radioimmunoassay by the method of Chafouleas *et al.* (1979). From this it can be concluded that erythrocyte Ca²⁺-transport ATPase has a basal activity even in the total absence of calmodulin.

Some additional properties of purified erythrocyte Ca²⁺-transport ATPase are given in Table 3. As was shown for the membrane-bound enzyme (Scharff, 1980), calmodulin also transforms the purified ATPase from a low-activity to a high-activity state and in addition from a low-affinity to a high-affinity state for Ca²⁺. Binding of calmodulin to the purified ATPase obviously stabilizes the enzyme, since the half-life at 0°C is increased from 1.2 to 6 days by the addition of calmodulin (Table 3). In the

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified Ca²⁺-transport ATPase. Protein of a typical purified preparation was concentrated before electrophoresis by precipitation as described by Gietzen *et al.* (1980b). Gel electrophoresis was performed as described by Weber & Osborn (1969), with disc (6 mm diam.) polyacrylamide gels with 5% (w/v) acrylamide (gel a). The same sample also was subjected to electrophoresis by the method of Laemmli & Favre (1973) in slab gels (1.5 mm thick), with a stacking gel of 3% and a separating gel of 10% acrylamide (gel b).

presence of Ca²⁺ and calmodulin, purified Ca²⁺-transport ATPase was found to survive at least five freeze–thaw cycles (freezing in liquid N₂, thawing at 15°C) without significant lowering of the specific activity and stability of the enzyme, whereas in the absence of calmodulin one freeze–thaw cycle caused

Table 3. *Properties of purified erythrocyte Ca^{2+} -transport ATPase*

Usually ATPase activity was determined at $36\text{ }\mu\text{M}$ - Ca^{2+} , 1 mM -MgATP and, when present, 30 nM -calmodulin. The K_m value is related to the concentration of MgATP. K_{Ca} and K_{CaM} refer to the concentrations needed for half-maximum stimulation by Ca^{2+} and calmodulin respectively. The half-life of the ATPase was determined in buffer B supplemented with CaCl_2 to yield a free Ca^{2+} concentration of $100\text{ }\mu\text{M}$ and, when present, with 0.15 mg of calmodulin/mg of Ca^{2+} -pump protein. The numbers are mean values for at least two different preparations and two determinations on each preparation.

Parameter	Purified ATPase ($\pm$ calmodulin)
Sp. activity ($\mu\text{mol/min per mg of protein}$)	($-$) 2.1 ($+$) 16.2
K_m (μM)	($+$) 57
K_{Ca} (μM)	($-$) 20 ($+$) 1.5
K_{CaM} (nM)	1
Half-life at 0°C (days)	($-$) 1.2 ($+$) 6

loss of 20–30% of the activity (results not shown).

The rapid and efficient isolation procedure of erythrocyte Ca^{2+} -transport ATPase described here should enable investigation of molecular characteristics (e.g. the amino acid sequence) that require large quantities of purified enzyme.

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