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Calmodulin Affinity Chromatography Yields a Functional Purified Erythrocyte (Ca^{2+} + Mg^{2+})-Dependent Adenosine Triphosphatase

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The (Ca^{2+} + Mg^{2+})-dependent ATPase of human erythrocyte membranes was solubilized with deoxycholate and purified by calmodulin affinity chromatography to yield a functional enzyme. The method gave an enzyme purified 207-fold as compared with that of the erythrocyte membranes. The molecular weight of the ATPase was in the range 135000–150000, as revealed by a single major band after electrophoresis on dodecyl sulphate/polyacrylamide gels. The isolated enzyme was highly sensitive to calmodulin, since the activity was increased about 9-fold. At 37°C and in the presence of calmodulin the purified ATPase had a specific activity of 10.1 μmol/min per mg of protein. Triton X-100 or deoxycholate stimulated the calmodulin-deficient enzyme in a concentration-dependent fashion whereby the calmodulin-sensitivity was lost. The purification method is suitable for studying the lipid-specificity of the ATPase, since the lipids can easily be exchanged without a significant loss of activity. A purification procedure described by Niggli, Penniston & Carafoli [(1979) *J. Biol. Chem.* **254**, 9955–9958] resulted in an enzyme that indeed was pure but was lacking a predominant feature, namely the modulation by calmodulin.

Human erythrocytes maintain a low intracellular Ca^{2+} concentration, and this is of great physiological importance since an elevated intracellular Ca^{2+} concentration disturbs most functions of the erythrocyte (Orringer & Parker, 1973). According to Schatzmann (1973), the (Ca^{2+} + Mg^{2+})-dependent ATPase is responsible for the active Ca^{2+} extrusion across the plasma membrane.

Molecular characteristics of the enzyme such as the subunit structure or the amino acid sequence even of the active centre have not hitherto been described. To investigate these properties and to study Ca^{2+} transport and its regulation on the molecular level, attempts have been made to solubilize and purify the ATPase. Solubilization of the intrinsic membrane protein was first achieved with Triton X-100 (Wolf & Gietzen, 1974) and more recently with deoxycholate (Gietzen *et al.*, 1979). It was shown that solubilization with deoxycholate requires a membrane preparation stabilized with small amounts of Tween 20 (Gietzen *et al.*, 1980).

Abbreviations used: Tos-Lys-CH_2Cl, 7-amino-1-chloro-3-L-tosylamidoheptane-2-one; Mops, 4-morpholinepropanesulphonic acid; KI unit, kallikrein-inhibitor unit (Trautschold *et al.*, 1967).

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The ATPase could be partially purified by mixed-micelle gel chromatography to yield a preparation with three different polypeptides (Wolf *et al.*, 1977). The protein with a molecular weight of approx. 145000 was phosphorylatable (Wolf *et al.*, 1977). A partially purified enzyme, solubilized with deoxycholate, has been reconstituted to form membranous vesicles, capable of energized Ca^{2+} transport, with a protein/lipid ratio similar to that of biological membranes (Gietzen *et al.*, 1979, 1980).

(Ca^{2+} + Mg^{2+})-dependent ATPase and Ca^{2+} transport of erythrocyte membranes have been shown to be regulated by a cytoplasmic factor, calmodulin, which combines with the enzyme when the cytoplasmic Ca^{2+} concentration rises (Gopinath & Vincenzi, 1977; Hinds *et al.*, 1978; Jarrett & Penniston, 1978). The interaction of the enzyme with calmodulin has been used to purify the ATPase by calmodulin affinity chromatography. During the completion of the present work, a study by Niggli *et al.* (1979) appeared that reported the purification of (Ca^{2+} + Mg^{2+})-dependent ATPase with a procedure methodologically similar to ours, but the purified enzyme had lost a predominant feature, namely the modulation by calmodulin. Thus that preparation is unsuitable for studying the interaction with cal-

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modulin. The present paper describes a procedure for obtaining a purified enzyme with a higher specific activity and that can be stimulated by calmodulin about 9-fold.

Experimental

Materials

All reagents were of highest purity available. Trasylol was obtained from Bayer A.G., Leverkusen, Germany. CNBr-activated Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden; dialysis tubing with a 12000-dalton cut-off was from A. H. Thomas, Philadelphia, PA, U.S.A.; Biobeads SM2 were from Bio-Rad, München, Germany.

Dialysis tubing was treated before use as described previously (Meissner & Fleischer, 1974). Potassium deoxycholate was recrystallized as described by Meissner *et al.* (1973). Phosphatidylcholine was prepared from egg phospholipids (Singleton *et al.*, 1965). Erythrocyte membrane lipids were extracted by the method of Folch *et al.* (1957). Erythrocyte calmodulin was prepared as described previously (Jarrett & Penniston, 1978) and bovine brain calmodulin by the procedure of Watterson *et al.* (1976).

Calmodulin affinity column. The purity of the affinity ligand was assessed by dodecyl sulphate/polyacrylamide-gel electrophoresis. A 25 mg portion of purified bovine brain calmodulin was coupled to 2.5 g of CNBr-activated Sepharose 4B in accordance with the manufacturer's instructions. Approx. 11 mg of calmodulin was immobilized on the Sepharose gel, as revealed by determination of the uncoupled protein. The actual capacity of the column was never determined. In our experiments 0.4–2 mg of purified ATPase was bound to the column without a sign of saturation. On taking in account that about 30% of the immobilized ligand is coupled in a way that it still can interact with the ATPase (a value found for several other proteins) and considering molecular weights of 17000 for calmodulin and 140000 for the ATPase, a binding capacity of the column of approx. 25 mg of ATPase seems to be reasonable. The calmodulin–Sepharose gel was packed into a column 10 cm long × 9 mm diam.

Preparation of calmodulin-deficient erythrocyte membranes. Membrane-bound human erythrocyte $(Ca^{2+} + Mg^{2+})$-dependent ATPase suitable for solubilization with Triton X-100 was prepared by the method of Jarrett & Penniston (1978). A membrane-bound enzyme, free of calmodulin, that can be solubilized with deoxycholate was prepared on the basis of the iso-osmotic freeze-haemolysis procedure (Gietzen *et al.*, 1980) modified as follows: the buffer used for haemolysis contained no $CaCl_2$ but 1 mM-EDTA and 25 mg of Tween 20/ml (final concentrations). Also, in the buffer for washing the membranes, $CaCl_2$ was replaced by 1 mM-EDTA for three washes after haemolysis. For the next three washes $CaCl_2$ was omitted.

Several different buffer solutions were used for affinity chromatography. Solution A contained 100 mM-KCl, 0.1 mM-$CaCl_2$, 100 KI units of Trasylol/ml, 0.5 mg of phosphatidylcholine/ml, 1.25 mg of Tween 20/ml and 0.5 mg of Triton X-100/ml. Solution B contained 300 mM-KCl, 1 mM-EDTA, 0.5 mg of phosphatidylcholine/ml, 0.5 mg of Tween 20/ml and 0.5 mg of Triton X-100/ml. Solution C contained 100 mM-KCl, 0.1 mM-$CaCl_2$, 1 mg of erythrocyte lipids/ml and 0.4 mg of potassium deoxycholate/ml. Solution D contained 300 mM-KCl, 1 mM-EDTA, 1 mg of erythrocyte lipids/ml and 0.4 mg of potassium deoxycholate/ml. Solution E contained 100 mM-KCl, 0.1 mM-$CaCl_2$, 0.5 mg of phosphatidylcholine/ml, 20 μg of Tween 20/ml and 0.5 mg of Triton X-100/ml. Solution F contained 300 mM-KCl, 1 mM-EDTA, 0.5 mg of phosphatidylcholine/ml, 10 μg of Tween 20/ml and 0.5 mg of Triton X-100/ml. Solution G contained 100 mM-KCl, 0.1 mM-$CaCl_2$, 0.5 mg of phosphatidylcholine/ml, 0.5 mg of Tween 20/ml and 5 mg of Triton X-100/ml. Solution H contained 300 mM-KCl, 1 mM-EDTA, 0.5 mg of phosphatidylcholine/ml, 0.5 mg of Tween 20/ml and 5 mg of Triton X-100/ml. All solutions contained in addition 200 mM-sucrose, 1 mM-$MgCl_2$, 5 mM-dithiothreitol and 20 mM-Mops buffer, pH 7.0, and were sonicated for 10 min with a Branson Sonifier B12 at setting 6. It is important for the purity of the resulting enzyme to sonicate solution A until it is no longer turbid. Before use all solutions were centrifuged at 100000 $g_{av.}$ for 45 min at 0°C to remove insoluble material and metallic particles that come from the tip of the sonicator.

Methods

Solubilization of the ATPase. Solubilization of the $(Ca^{2+} + Mg^{2+})$-dependent ATPase was achieved by using Triton X-100 or potassium deoxycholate at 0°C in a medium consisting of 200 mM-sucrose, 300 mM-KCl, 1 mM-$MgCl_2$, 0.5 mM-$CaCl_2$, 10 mM-dithiothreitol, 1 mM-Tos-Lys-CH_2Cl, 100 KI units of Trasylol/ml, 0.2 mg of phosphatidylcholine/ml and 10 mM-Mops buffer, pH 7.0. The final protein concentration in the solubilizing medium was approx. 6–8 mg/ml. The mixture was kept on ice for about 10 min before separation of the solubilized material from the insoluble membrane fragments by centrifugation at 140000 $g_{av.}$ for 60 min.

Purification of the ATPase. Deoxycholate-solubilized material was treated as follows. Before each use the calmodulin–Sepharose column was equilibrated with approx. 50 ml of solution A at a flow rate of 120 ml/h. The deoxycholate extract (solubilized material) was then applied to the column at a

flow rate of 45 ml/h followed by 200 ml of solution A at a flow rate of 120 ml/h. Finally the flow rate was decreased to 45 ml/h and the purified ATPase was eluted from the column with solution B. The column was operated at 2–4°C and the effluent was fractionated as indicated in Fig. 2. For longer periods between usage the column was stored at 4°C in a medium containing 1% (w/v) penicillin, 1% (w/v) streptomycin, 0.5 M-NaCl and 50 mM-Mops buffer, pH 7.0.

Triton X-100-solubilized material was treated as follows. When low Triton X-100 concentrations in the buffers were used, solution B was sometimes replaced by solution F. Otherwise the procedure was the same as described for deoxycholate-solubilized material. With high concentrations of Triton X-100 in the buffers, solutions A and B had to be replaced by solutions G and H respectively.

Elution of the ATPase in different detergent/lipid environments. The procedure described above for deoxycholate-solubilized material was followed up to the point at which 200 ml of solution A had been passed through the column. For elution of the ATPase in a deoxycholate/erythrocyte-lipids environment, 50 ml of solution C was passed through the column at a flow rate of 120 ml/h before the ATPase was eluted at a decreased flow rate (45 ml/h) with solution D. Replacement of solutions C and D by solutions E and F respectively resulted in a purified enzyme dispersed in a Triton X-100/phosphatidylcholine system.

Assays. ($Ca^{2+} + Mg^{2+}$)-dependent ATPase activity was determined at 36 μM-$CaCl_2$ and 30°C, unless otherwise specified, as described previously (Arnold *et al.*, 1976) in the presence and in the absence of erythrocyte calmodulin. Before the reaction was started the enzyme was incubated for 5 min at 30°C without or with 10 times the amount of calmodulin needed for half-maximal activation.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. To avoid interference by Triton X-100 and dithiothreitol, the protein was precipitated and further washed as described (Bensadoun & Weinstein, 1976) before the procedure of Lowry *et al.* (1951) was used.

Dodecyl sulphate/polyacrylamide-gel electrophoresis. The protein was precipitated and washed as described for the protein determinations. The pellet was solubilized in a solution containing 10 mM-NaOH, 10 mM-dithiothreitol and 1% (w/v) sodium dodecyl sulphate and heated for 10 min in a boiling-water bath. The protein sample was mixed with 2 M-sucrose to give a final concentration thereof of 500 mM and then applied to polyacrylamide gels (5% acrylamide). Gel electrophoresis was performed as described by Weber & Osborn (1969). Fixation, staining and destaining of gels were done by the procedure of Fairbanks *et al.* (1971).

Dialysis. Deoxycholate-containing purified ATPase preparations were dialysed for 4 h at 2–4°C and a buffer/sample ratio of at least 1000 : 1. The dialysis buffer consisted of 400 mM-KCl, 1.5 mM-$MgCl_2$, 0.1 mM-$CaCl_2$, 1 mM-EDTA, 10 mM-cysteine, 250 mM-sucrose and 7.5 mM-Mops buffer, pH 7.0.

Removal of Triton X-100. Samples of purified ATPase containing Triton X-100 were treated with Biobeads SM2 as described by Chiesi *et al.* (1978) to remove the detergent.

Results

Effect of detergents on the ATPase activity

Triton X-100 has a great influence on the ($Ca^{2+} + Mg^{2+}$)-dependent ATPase activity of calmodulin-deficient erythrocyte membranes as well as of the solubilized material, and obviously the stimulation of the ATPase by calmodulin is altered by it (Fig. 1). Treatment of a calmodulin-depleted erythrocyte membrane suspension with increasing amounts of Triton X-100 results in an increase of the ATPase activity (when determined without calmodulin). Up to a detergent/protein ratio of 0.1 : 1

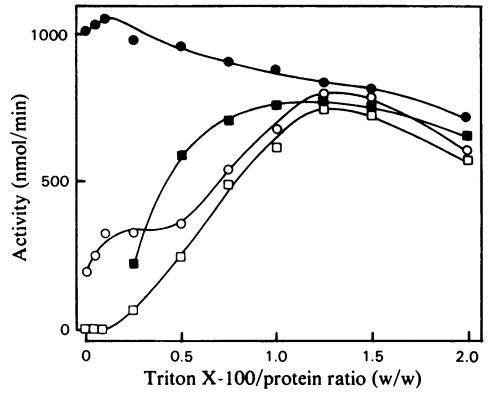

Fig. 1. *Effects of Triton X-100 and calmodulin on membrane-bound and solubilized ($Ca^{2+} + Mg^{2+}$)-dependent ATPase*

Equal samples of membrane-bound and calmodulin-deficient ($Ca^{2+} + Mg^{2+}$)-dependent ATPase were treated with increasing amounts of Triton X-100, and the solubilized material was obtained by removal of the insoluble membrane fragments by centrifugation. The ATPase activity of the membrane suspension as well as of the solubilized material was determined in the presence and absence of erythrocyte calmodulin at 30°C. ●, Membrane suspension with calmodulin; ○, membrane suspension without calmodulin; ■, solubilized material with calmodulin; □, solubilized material without calmodulin.

(w/w) it is an activation of only the membrane-bound ATPase, since no activity is found in the solubilized material. From the point where solubilization of the ATPase starts, up to about a ratio of 0.5 : 1 the activity remains constant and then starts to increase again. The activation caused by Triton X-100 reaches its maximum at a Triton X-100/protein ratio of about 1.25 : 1 (w/w). At this ratio the ATPase activity is about 4 times higher than the value in the absence of Triton X-100. At ratios above 1.25 : 1 (w/w) Triton X-100 has an inactivating effect on the ATPase. When the activity of the membrane suspension is determined in the presence of calmodulin a slight increase of the activity is obtained at low detergent/protein ratios, but increasing the Triton X-100 concentration leads to a slight decrease of the ATPase activity. At a detergent/protein ratio of about 1.25 : 1 (w/w) the two lines representing the activity of the membrane suspension with and without calmodulin coincide, indicating that the Triton X-100-stimulated enzyme cannot be further activated by addition of calmodulin.

The curves representing the ATPase activity of the solubilized material in the presence and absence of calmodulin (Fig. 1) reach a peak value at about a detergent/protein ratio of 1.25 : 1 (w/w). At low detergent concentrations the activation of the solubilized material by calmodulin is more than 300%. With an increased Triton X-100 concentration the activation by calmodulin is subsequently lost.

Deoxycholate used on membranes obtained by iso-osmotic freeze-haemolysis results in a similar pattern except that detergent/protein ratios are decreased by a factor of 0.16 (results not shown). Thus both detergents stimulate the ATPase in a concentration-dependent fashion whereby the calmodulin-sensitivity is lost (cf. Table 3).

Purification procedure

For best results, on the stability and purity of the resulting enzyme, a deoxycholate or Triton X-100 concentration solubilizing two-thirds of the initial ATPase activity should be used. This is achieved at a detergent/protein ratio of 0.08 : 1 for deoxycholate and of 0.5 : 1 (w/w) for Triton X-100.

Affinity chromatography of human erythrocyte ($Ca^{2+} + Mg^{2+}$)-dependent ATPase on a column of calmodulin covalently coupled to Sepharose 4B makes use of the fact that a calmodulin-depleted enzyme binds to calmodulin in the presence of Ca^{2+} and dissociates in the absence of Ca^{2+} (Farrance & Vincenzi, 1977; Scharff & Foder, 1977). The elution pattern of such a column is shown in Fig. 2 (by recording the absorbance at 280nm).

Most proteins of the deoxycholate or Triton X-100 extract pass directly through the column and are eluted in the first peak. After extensive elution

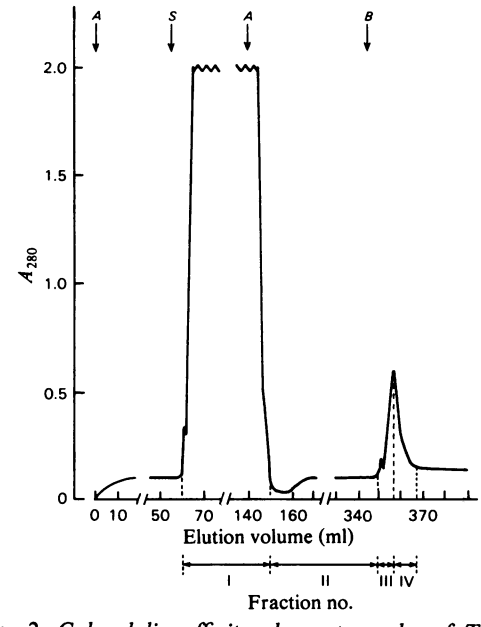

Fig. 2. *Calmodulin affinity chromatography of Triton X-100- or deoxycholate-solubilized erythrocyte membranes*

Arrows *A* mark the start of application of buffers containing 0.1 mM-$CaCl_2$ (solution A or G). Arrow *B* indicates the application of buffers containing 1 mM-EDTA (solution B, F or H), whereas arrow *S* marks the start of application of the solubilized material. For further details see the Experimental section.

with a buffer containing 0.1 mM-$CaCl_2$, the ATPase that is bound to the column is eluted in a second peak only after a buffer containing 1 mM-EDTA (solution B, D, F or H) is applied to the column. The absorbance of the second peak is mainly caused by an increased concentration of mixed micelles containing the ATPase.

A summary of a single experiment, in which deoxycholate was used as a solubilizing agent, is given in Table 1. The fraction of solubilized ATPase activity that can be activated by calmodulin appears exclusively during elution in the presence of EDTA (fraction IV), the activity not retained in the presence of Ca^{2+} (fraction I) being refractory to calmodulin. Absence of calmodulin-sensitivity from the enzyme activity in fraction I may reflect damage by the detergent or be due to part of the solubilized enzyme not having lost its complement of calmodulin. The purified enzyme, collected in fraction IV, is stimulated 8.9-fold by calmodulin. In the presence of calmodulin, the purified ATPase has a specific activity of 6.64 or 10.1 μmol/min per mg of protein at 30°C or 37°C respectively. This rep-

Table 1. *Characteristics of the fractions in the course of the purification procedure*

Erythrocyte membranes were prepared by using the iso-osmotic freeze-haemolysis procedure (see the Experimental section) and were solubilized with deoxycholate. The insoluble residue was removed by centrifugation and re-suspended in the same buffer as used for solubilization. The deoxycholate extract was applied to the affinity column. Elution was carried out with the solutions A and B and the effluent was fractionated as indicated in Fig. 2. The concentrations of detergents and lipids in the assay medium for determination of the ATPase activity were $\leqslant 50\,\mu g$/ml and $\leqslant 25\,\mu g$/ml respectively. For further details see the Experimental section.

Fraction	Volume (ml)	Protein (mg)	Activity (μmol/min) −Calmodulin	Activity (μmol/min) +Calmodulin	Activation by calmodulin (fold)	Sp. activity + calmodulin (μmol/min per mg of protein)
Erythrocyte membranes	80	512	7.08	16.28	2.30	0.032
Deoxycholate extract	72	41	4.91	7.46	1.52	0.182
Pellet	8	464	2.35	3.17	1.35	0.007
Fraction I	90	36	3.37	3.38	1.00	0.094
Fraction II	200	3.1	—	—	—	—
Fraction III	7	0.21	—	0.17	—	0.81
Fraction IV	10	0.55	0.41	3.65 (5.56*)	8.90	6.64 (10.1*)

* The values in parentheses were determined at 37°C.

resents a 207-fold purification of the ATPase as compared with erythrocyte membranes. The purified enzyme exhibits no Ca^{2+}-independent Mg^{2+}-dependent ATPase activity, as compared with approx. 5% of the total ($Ca^{2+} + Mg^{2+}$)-dependent ATPase activity of the erythrocyte membranes used.

In fraction IV 22% of the initial ATPase activity of the erythrocyte membranes (determined in the presence of calmodulin) or 49% of the total ATPase activity loaded on the affinity column is recovered.

The percentage of total erythrocyte membrane protein recovered in fraction IV, containing the purified ATPase, is about 0.1%. Since the insoluble pellet and fraction I contain about the same ATPase activity as fraction IV, a reasonable estimate of the amount of ($Ca^{2+} + Mg^{2+}$)-dependent ATPase seems to be 0.3% of the total membrane protein of the erythrocyte.

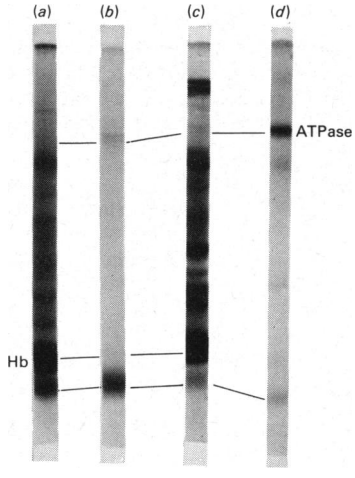

Fig. 3. *Dodecyl sulphate/polyacrylamide-gel electrophoresis of the solubilized material and purified fraction of different experiments*

Gel electrophoresis was performed as described in the Experimental section. The gels were cut below the tracking dye, Pyronin Y. (*a*) Triton X-100 extract from membranes prepared by the method of Jarrett & Penniston (1978); (*b*) fraction IV of affinity chromatography was obtained from Triton X-100-solubilized material by using the elution buffers with a low Triton X-100 concentration [solutions A and F (or B)]; (*c*) deoxycholate extract from membranes obtained by the iso-osmotic-freeze-haemolysis procedure (Gietzen *et al.*, 1980); (*d*) fraction IV of affinity chromatography obtained from deoxycholate-solubilized material by using the elution buffers A and B. Hb is the symbol for haemoglobin.

Properties of the ATPase

Table 2 shows that the conditions used during the purification procedure have a great influence on the properties of the purified ATPase, obtained in fraction IV. The highest specific activity and maximum calmodulin activation result from the use of deoxycholate as a solubilizing agent. The specific activity is $6.6\,\mu$mol/min per mg of protein at 30°C, and the ATPase is stimulated about 9-fold (mean of three preparations) by calmodulin. A polyacrylamide-gel electrophoretogram of the purified ($Ca^{2+} + Mg^{2+}$)-dependent ATPase is shown in Fig. 3 (gel *d*). The gel with the purified enzyme exhibits just one major band in the 135000–150000-mol.wt. region.

With the use of Triton X-100 for solubilization no satisfactory result could be achieved (Table 2). When low concentrations of Triton X-100 are used for elution, an enzyme activatable 6.2-fold with calmodulin is obtained, but the preparation is not pure, as revealed by a specific activity of $0.76\,\mu$mol/min per mg of protein and by dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3, gel *b*).

Table 2. *Effects of preparation conditions on the properties of the purified ATPase of fraction IV obtained from the affinity column*

Erythrocyte membranes suitable for Triton X-100 solubilization were prepared according to the method of Jarrett & Penniston (1978), whereas membranes used for solubilization with deoxycholate were prepared by using the iso-osmotic freeze-haemolysis method. For further details see the Experimental section.

		Purity of the ATPase		
Solubilizing agent	Buffers used for elution	Judged from gel electrophoresis (%)	Sp. activity + calmodulin (μmol/min per mg of protein)	Activation by calmodulin (fold)
Triton X-100	A and F (or B) with 0.05% (w/v) Triton X-100	10–20	0.76	6.2
	G and H with 0.5% (w/v) Triton X-100	~80	3.5	1.0
Deoxycholate	A and B with 0.05% (w/v) Triton X-100	~80	6.6	9

Table 3. *Properties of purified ATPase eluted in different detergent/lipid systems*

Elution was carried out in systems containing: (*a*) Tween 20, Triton X-100 and phosphatidylcholine (solution B); (*b*) deoxycholate and erythrocyte lipids (solution D); (*c*) mainly Triton X-100 and phosphatidylcholine (solution F). In all cases 72ml of deoxycholate extract was applied to the column. For experimental details see the Experimental section. ATPase activity was determined in the presence and absence of calmodulin at 30°C.

Detergent/ lipid system	Activity of fraction IV (±) calmodulin (μmol/min)	Activation by calmodulin (fold)	Sp. activity of fraction IV (±) calmodulin (μmol/min per mg of protein)
(*a*)	(−) 0.41 (+) 3.65	8.90	(−) 0.74 (+) 6.64
(*b*)	(−) 1.67 (+) 3.36	2.01	(−) 2.93 (+) 5.90
(*c*)	(−) 0.37 (+) 3.12	8.43	(−) 0.75 (+) 6.32

High concentrations (0.5%) of Triton X-100 in the elution buffers result in an enzyme that has an acceptable specific activity of 3.5μmol/min per mg of protein. The gel pattern of this preparation is similar to that of gel (*d*) (Fig. 3). The purified enzyme obtained by this procedure has a severe disadvantage in that it has lost the calmodulin-sensitivity (Table 2). This is due to the high Triton X-100 concentration used for elution (cf. Fig. 1), and the calmodulin stimulation cannot be restored even if Triton X-100 is removed by using Biobeads SM2.

Polyacrylamide-gel electrophoretograms of the Triton X-100- and deoxycholate-solubilized material are shown in Fig. 3 (gels *a* and *c*). The Triton X-100 extract contains a heavy band in the 10000-mol.wt. region (below the haemoglobin band) that is much less conspicuous in the deoxycholate extract. This protein is responsible for the moderate results achieved with Triton X-100-solubilized material (Table 2 and Fig. 3), since it cannot be separated from the ATPase with low Triton X-100 concentrations that are essential to obtain calmodulin-sensitive ATPase.

Exchange of the detergent/lipid environment

A deoxycholate-solubilized ATPase that has been separated from contaminating proteins can be eluted from the affinity column in different detergent/lipid environments (Table 3), since it is easy to change buffers when the ATPase is bound to the column. As shown in Table 3, the specific activities of the ATPase, determined in the presence of calmodulin, are nearly identical for the different detergent/lipid systems. The activation factors are high for the Tween 20/Triton X-100/phosphatidylcholine and the Triton X-100/phosphatidylcholine systems. When the enzyme is eluted in a deoxycholate/erythrocyte-lipids environment the activation by

calmodulin is diminished. Apparently the eluted enzyme is already largely activated by deoxycholate (Triton X-100 acts similarly at higher concentrations) and is therefore only slightly calmodulin-sensitive (see Fig. 1). Removal of the deoxycholate from the system by dialysis again leads to an ATPase highly sensitive to calmodulin, indicating that the activation of the ATPase by deoxycholate is a reversible process (results not shown).

Discussion

The purification of a functional ($Ca^{2+} + Mg^{2+}$)-dependent ATPase from human erythrocytes was achieved by calmodulin affinity chromatography and with deoxycholate as a solubilizing agent. In dodecyl sulphate/polyacrylamide-gel electrophoretograms of the purified enzyme preparation one single major band in the range 135000–150000 mol.wt. was visible; this value is slightly higher than that indicated by Niggli *et al.* (1979). This discrepancy can presumably be explained by partial proteolytic degradation of the preparation obtained by Niggli *et al.* (1979), as it has been shown that erythrocyte membranes contain a considerable proteinase activity (Wolf *et al.*, 1977). Wolf *et al.* (1977) gave evidence that the 145000-mol.wt. band of their partially purified preparation is phosphorylatable, thus representing the ATPase.

It is shown in the present study that when Triton X-100 is used as solubilizing agent it is difficult to obtain an enzyme that combines high purity with calmodulin-sensitivity. In contrast, when deoxycholate is used for solubilization the purified enzyme exhibits a high specific activity and is sensitive to calmodulin. The purified enzyme can be activated by calmodulin by 9-fold, whereas for the membrane-bound ($Ca^{2+} + Mg^{2+}$)-dependent ATPase factors below 4.7 have been reported (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1978; Hanahan *et al.*, 1978). This higher calmodulin-sensitivity of the purified enzyme is presumably due to a more complete calmodulin depletion.

The purification procedure described in the present paper has three different advantages as compared with the method of Niggli *et al.* (1979) in that: (1) the specific activity of the purified enzyme is about 3 times higher, which is apparently due to higher stability; (2) the detergent/lipid environment of the enzyme can easily be changed without a significant loss of activity; (3) most important, the purified enzyme is stimulated by calmodulin about 9-fold. Failure to recover calmodulin-sensitivity (Niggli *et al.*, 1979) may be due to the marked stimulation exerted by detergents (see Fig. 1).

The detergent/lipid environment can easily be changed while the ATPase is attached to the column, which allows reconstitution of the enzyme by removal of the detergent by using dialysis in the case of deoxycholate (Meissner & Fleischer, 1974; Gietzen *et al.*, 1979, 1980) or Biobeads SM2 in the case of Triton X-100 (Chiesi *et al.*, 1978). This may prove extremely useful for producing vesicles of different lipid/protein ratios according to the requirements of the experiment planned.

In addition, the method described in the present paper is suitable for studying the specific lipid requirement of the enzyme, for which discrepant results have been reported (Roelofsen & Schatzmann, 1977; Ronner *et al.*, 1977). Niggli *et al.* (1979) only obtained a good yield of purified ATPase if all buffers used for purification contained phosphatidylserine, whereas in the present study phosphatidylcholine-containing buffers were quite effective.

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