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A WATER-SOLUBLE Mg^{2+}-ATPase FROM ERYTHROCYTE MEMBRANES

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SUMMARY

A ouabain-insensitive ATPase activity associated with the water-soluble proteins of the human and bovine erythrocyte membrane is demonstrated by means of activity-staining in polyacrylamide gels. The ATPase activity from both sources had an absolute requirement for Mg^{2+}, activity becoming easily detectable at 0.2 mM Mg^{2+}. At low Mg^{2+} concentrations added Ca^{2+} appeared to decrease the intensity of the ATPase stain. The activity is unaffected by monovalent cations, does not hydrolyse *p*-nitrophenyl phosphate and is not inhibited by 2 : 4 dinitrophenol. The ATPase has an apparent molecular weight of approximately 100 000 as determined by electrophoresis in acrylamide gels containing dodecyl sulphate.

INTRODUCTION

The ATPase activities of mammalian erythrocyte membranes have been studied extensively by numerous workers [1–6]. Some of these activities have been implicated in the biochemical mechanism of ion exchange across the red cell membrane. Post et al. [1], Schatzmann and Rossi [6], Dunham and Glynn [2], Onishi [7], Guidotti [8], Rosenthal et al. [9] suggest, however, that at least one of these activities is associated with "contractile" proteins present in the membrane. Further, Rosenthal et al. [9] describe the low ionic strength solubilisation of a group of fibril-forming human erythrocyte membrane proteins associated with a Ca^{2+}-dependent ATPase activity. They speculate that this system is involved in the contraction of the intact ghost and that the fibrils contribute to membrane elasticity.

This report describes the solubilisation from human and bovine erythrocyte membranes of a group of proteins associated with a Mg^{2+}-dependent ATPase and the demonstration of this activity in polyacrylamide gels. The effects of other ions on this activity are also examined. The ATPase activities solubilised from human or bovine membranes are shown to have identical ionic requirements which are dissimilar to those described by Rosenthal et al. [9].

METHODS

Preparation of membranes

Bovine blood, obtained from the Homebush Abattoirs, Sydney, was collected into citrate/glucose anticoagulant (150 ml/l), chilled in ice and transported to the laboratory for use within 48 h. Fresh human packed cells in citrate anticoagulant were supplied by the Red Cross Blood Bank, Sydney. The cells were collected by centrifugation at $3000 \times g$ in a Sorvall RC-5 Superspeed Refrigerated Centrifuge. The plasma and "buffy" coat were removed by aspiration and the cells washed 3 times in 0.95 % NaCl buffered to pH 7.5 with 10 mM Tris · Cl.

Membranes were prepared from the washed cells essentially by the method of Dodge et al. [10] but with hemolysis being performed in 10 mM Tris · Cl, pH 7.5, rather than in phosphate buffer. This eliminated traces of phosphate which could precipitate with lead used in subsequent activity staining procedures. The membrane preparation thus obtained was creamy-white in appearance.

Preparation of water-soluble proteins

The washed membrane suspension was diluted 1 : 1 with a solution of 0.1 mM EDTA (pH 7.5) and dialysed against 20 volumes of the same solution for 24 h. The contents of the dialysis tubing were centrifuged at $40\,000 \times g$ for 30 min to yield an insoluble residue and a protein-rich supernatant. The residue was reextracted with fresh buffer as described above and the two supernatants thus collected were pooled. The pooled supernatant solution was centrifuged in a Beckman L2-65B Preparative Ultracentrifuge for 1 h at $100\,000 \times g$ in order to remove membrane fragments. The pellet obtained was discarded and the protein solution stored in the refrigerator until required.

The procedures for the preparation of membranes and water-soluble proteins were normally carried out at 0–4 °C. In one experiment, however, dialysis of human erythrocyte membranes was carried out at 22 °C, in order to duplicate the extraction conditions used by Rosenthal et al. [9].

Protein estimation

The protein concentration of the supernatant was determined by the method of Lowry et al. [11] using crystalline bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in 4 % polyacrylamide gels with the use of the discontinuous buffer system of Davis [12], but without sample or spacer gels. In addition, the electrode buffers were stored at 4 °C and the lower electrode compartment was packed in ice during electrophoresis.

About 5 ml of the protein solution was dialysed against the cold upper electrode buffer for 1–2 h before electrophoresis. Sucrose and tracking dye (bromphenol blue) were added prior to layering approximately 0.2–0.4 ml of the sample onto each gel. Preliminary experiments revealed no alteration of the protein staining pattern nor of the ATPase activity in the presence of the dye. The total amount of protein applied to each gel was kept nearly constant. Electrophoresis was carried out at 100 V (1–2 mA/gel) for 15 min until the proteins had migrated into the top of the gel and then at

200 V (4–6 mA/gel) until the tracking dye was approximately 5 mm from the bottom of the gel.

Electrophoresis in the presence of 1 % sodium dodecyl sulphate was carried out essentially according to the method of Fairbanks et al. [13].

Electrophoresis in a continuous 4–26 % polyacrylamide gel gradient was carried out as described by Margolis and Kenrick [14] and with the aid of commercially available gradient gel slabs ("Gradipore", Sydney). Approximately 40 μl of the protein solution was applied to each sample space of the gel and electrophoresed overnight in the cold in Tris/EDTA/borate buffer, pH 8.3. After electrophoresis the gel was sliced into two parts: one half was stained with Coomassie blue and the other for ATPase activity as described below.

Detection of ATPase activity

After electrophoresis, the gels were removed from the tubes and washed in cold 20 mM Tris · maleate pH 9.0 for 15–30 min. Each gel was incubated overnight at room temperature in 15 ml of 20 mM Tris · maleate, pH 9.0, containing 1 mM ATP and 1 mM $PbCl_2$ together with divalent cations at final concentrations of 0–5 mM as indicated in Table I. Monovalent cations, ouabain, *p*-nitrophenyl phosphate and 2 : 4 dinitrophenol, where required, were added at the concentrations indicated in Table I.

At the end of the incubation period the gels were washed in distilled water for at least 1 h to remove excess lead. The gels were then immersed in a solution of 0.5 % sodium sulphide and usually within 5–10 min the ATPase activity was manifested as a dark brown precipitate of lead sulphide in the gel. These gels were photographed within a short period of time as they expanded even during overnight storage, becoming soft and unmanageable.

In order to locate the protein responsible for the ATPase activity, a thin wire was pushed through the lead sulphide band and the gel was then stained with Coomassie blue as detailed below.

Detection of proteins

The gels were first fixed by immersion in 10 % isopropanol and 10 % acetic acid and after 10 min were stained by replacing the fixing solution with approximately 0.0025 % Coomassie blue in 10 % acetic acid for 6–8 h. Excess stain was removed by rinsing overnight in the same solution used to fix the gels. Sodium dodecyl sulphate-containing gels were first washed overnight with 25 % isopropanol and 10 % acetic acid prior to staining, in order to remove the sodium dodecyl sulphate.

Proteins were also visualised by first immersing the gels in 2 M HCl for 2 min and then in 0.003 % 8-anilino-1-naphthalene sulphonic acid, and observing the fluorescent bands in an ultraviolet light box.

Gel filtration

The supernatant after 100 000 $\times g$ centrifugation was concentrated by dialysis against Aquacide II and was passed through a column of Bio-gel A-15 m at room temperature in a buffer consisting of 0.15 M NaCl, 20 mM sodium phosphate, 5 mM EDTA and 5 mM mercaptoethanol, pH 7.5. Elution, at a rate of 25 ml/h was monitored at 280 nm in a Hitachi-Perkin Elmer spectrophotometer, model 124D.B. The

TABLE I

EFFECT OF DIVALENT CATIONS ON THE FORMATION OF VISIBLE ATPase BANDS

All tubes contained 1 mM ATP and 1 mM lead nitrate in 20 mM Tris · maleate (pH 9.0). *Divalent cations and/or other reagents were added at the final concentrations indicated. After overnight incubation at room temperature the gels were washed and stained with 2% ammonium sulphide. The formation of a faint but detectable brown ATPase band is indicated thus, + ; and a darker band, ++. The results shown represent duplicate observation and were identical for human and bovine specimens. No evidence of enzymic hydrolysis of *p*-nitrophenyl phosphate was noted.

Final concentration of divalent cations (mM)		Additional reagents	Band formation
Calcium	Magnesium		
0.0	0.0		–
0.2	0.0		–
0.5	0.0		–
2.0	0.0		–
5.0	0.0		–
0.2	0.5		+
0.5	0.5		+
1.0	0.5		+
2.0	0.5		+
5.0	0.5		+
0.0	0.2		+
0.0	0.5		+
0.0	1.0		++
0.0	2.0		++
0.0	5.0		++
0.5	0.2		+
0.5	1.0		++
0.5	2.0		++
0.5	5.0		++
0.0	0.5	2 mM dinitrophenol	+
0.0	0.5	5 mM *p*-nitrophenyl phosphate	–
0.0	0.5	50 mM NaCl	+
0.0	0.5	10 mM KCl	+
0.0	0.5	50 mM NaCl+10 mM KCl	+
0.0	0.5	50 mM NaCl+10 mM KCl +0.5 mM ouabain	+
0.0	0.5	0.5 mM ouabain	+

* When *p*-nitrophenyl phosphate was a substrate, ATP was not included in the incubation mixture.

material emerging in the 120–200 ml fraction was pooled and reconcentrated by dialysis against Aquacide II.

RESULTS

The protein content of the ultracentrifuged extracts was usually between 0.4 and 0.8 mg/ml. Electrophoresis of the extract at a loading of less than 100 μg protein

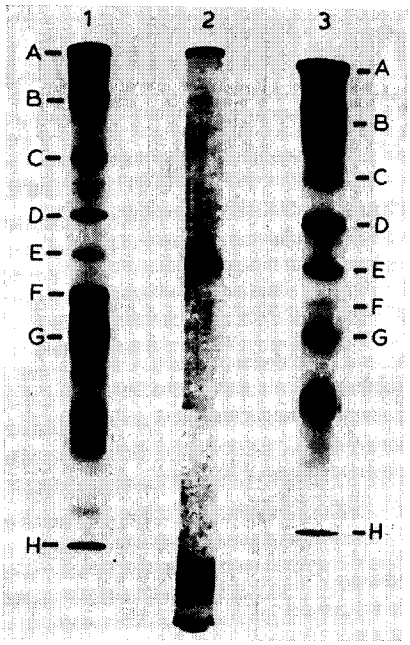

Fig. 1. Electrophoretic patterns of human and bovine soluble membrane proteins. Gels were run without detergents as described in methods, and after staining with Coomassie blue the protein bands were labelled A–H in order of increasing mobility. Gel 1, human preparation. Gel 3, bovine preparation. The relative staining intensities of protein bands within a gel were variable between different preparations, but in all cases ATPase activity corresponded to band E. Gel 2 shows the ATPase activity staining pattern for the human preparation.

yielded a pattern of Coomassie blue-staining bands arbitrarily designated A to H (Fig. 1). The pattern of well-defined bands was fairly reproducible from preparation to preparation, but the relative intensities of the bands were rather variable. Overloading tended to distort the pattern and to increase the staining at the top of the gel. As can be seen in Fig. 1, the differences between the human and bovine Coomassie blue-staining patterns are due mainly to varying amounts of protein present in each band, rather than the presence or absence of major components. In most preparations, both human and bovine, the ATPase staining method resulted in a single band at a position corresponding to the protein band 1E and 3E (Fig. 1). ATPase activity was detectable up to at least 1 week after preparation and the pattern was unaffected by dialysis of the extract against the electrode buffer prior to electrophoresis.

The ionic conditions required to produce a detectable lead sulphide band are detailed in Table I. The results shown represent duplicate observations. No difference between human and bovine preparations could be detected in the ionic requirements for the ATPase activity. Omission of ATP or its replacement by CTP, GTP or ADP in the incubation medium resulted in the loss of the lead sulphide band. No evidence of enzymic hydrolysis of *p*-nitrophenyl phosphate was noted. The intensity of the ATPase band in the presence of Na^+ and/or K^+ was no different from that in their absence. $(Na^+ + K^+)$-ATPase activity, if at all present, is probably not detectable by this technique as it is easily inhibited by Pb [18]. Table I also shows that calcium

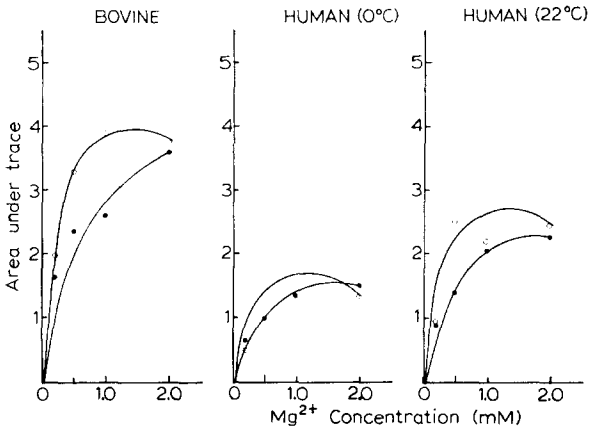

Fig. 2. Intensity of the ATPase stain as a function of Mg^{2+} concentration. 100 μl of the water-soluble protein solution was applied to each gel and electrophoresed for 30 min. The washed gels were incubated overnight for ATPase activity in the presence of divalent cations and then stained with ammonium sulphide as described in methods. The ATPase band was scanned in a Gilford Spectrophotometer with a linear transport accessory. The area under the densitometer trace of the ATPase stains was plotted in arbitrary units as a function of Mg^{2+} concentration in the absence of Ca^{2+} (open circles) and in the presence of 0.5 mM Ca^{2+} (closed circles). The bovine preparation was made near 0 °C; the human preparations were made both near 0 °C and at 22 °C.

could neither replace magnesium, nor exert marked stimulation or inhibition in the presence of magnesium.

In another series of experiments electrophoresis was carried out at 100 V (1–2 mA/gel) for only 30 min so that the proteins which entered the gel separated into very sharp, distinct bands. These gels were incubated and stained for ATPase activity and then scanned in a Gilford Spectrophotometer model 240. The area under the densitometer trace of the lead sulphide band in each gel was plotted on an arbitrary scale against the corresponding Mg^{2+} concentration. The results, shown in Fig. 2, indicate that the ATPase activity was stimulated by very low Mg^{2+} concentrations and was maximal at about 1 mM Mg^{2+}. Half-maximal stimulation occurred near 0.2 mM Mg^{2+}. The effect of added Ca^{2+} was to decrease the intensity of staining at low Mg^{2+} concentrations only, suggesting simple displacement of the Mg^{2+} by Ca^{2+}. While the relative intensities of activity staining varied with the samples shown, presumably due to different concentrations of the ATPase in the different samples, the shape of the Mg^{2+}-stimulated ATPase activity curve remained the same.

In order to determine whether the presence of Pb^{2+} altered the specificity for divalent cations or inhibited the Mg^{2+}-ATPase, a series of incubations for ATPase activity was carried out in the absence of Pb^{2+}. In this case the gels were briefly rinsed at the end of the incubation period and immediately stained with phosphomolybdate reagent. ATPase activity, manifested as a dark blue band on a lighter background, showed the same specificity for divalent cations.

The experiment in which extraction was performed at 22 °C showed that the temperature of extraction has no effect on the ionic requirement of the ATPase, nor on the positions of the Coomassie blue-staining bands. However, the top of the gel was not heavily stained. When the extract was run on a sodium dodecyl sulphate

gel it was apparent that some of the high molecular weight proteins had been broken down during dialysis. The ATPase activity, however, remained intact.

In order to examine the possibility that physical separation of the proteins might alter the ionic requirements of the ATPase, a series of experiments was performed in which electrophoresis was stopped at the end of 10 min. In this way, the proteins of the extract entered the gel but were not distinctly separated. After incubation with ATP and staining for ATPase activity, it was observed that the ATPase still displayed the absolute requirement for Mg^{2+}.

One series of experiments was run with a very light protein load (less than 50 μg protein/gel) in order to examine the possibility that proteins possessing ATPase activity may not have entered the heavily-loaded gels because of entrapment at the

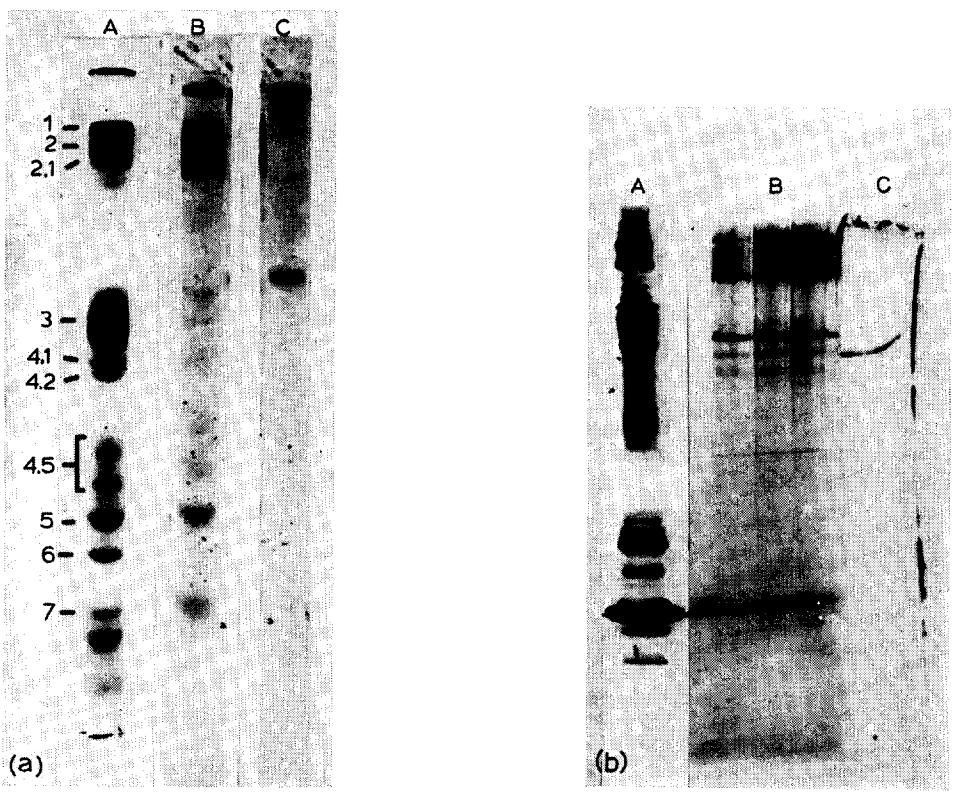

Fig. 3. The identity of the ATPase band on gel electrophoresis. (a). The location of the ATPase on sodium dodecyl sulphate-containing gels. Gels were run and the protein bands numbered according to the nomenclature of Fairbanks et al. [13]. A, human erythrocyte ghosts; B, 120–200 ml fraction from gel filtration containing at least two bands in the component 3 region and significant amounts of spectrin and component 5. C, ATPase band which was cut from a gel run in the absence of sodium dodecyl sulphate and rerun on the sodium dodecyl sulphate system. The major component had a mobility in the '3' region. (b). The location of the ATPase on Gradipore gels. 40 μl of the 120–200 ml fraction from gel filtration was applied to each of several sample spaces on the gel (B & C). For comparison, a dilute solution of plasma proteins was also examined (A). After electrophoresis overnight one portion of the gel (C) was stained for ATPase activity and the remainder was stained with Coomassie blue.

top of the gel. Again, only Mg^{2+}-ATPase activity was detected and no evidence of ATPase activity in the absence of Mg^{2+} was noted on top of the gel. Incubation in the presence of 2 M KCl [15] or at pH 7.5 did not alter the ionic requirements of the ATPase.

To identify the protein(s) responsible for the ATPase activity, the crude supernatant was concentrated to about 5 mg/ml by dialysis against Aquacide II and approx. 6 ml of this solution was subjected to gel filtration on Bio Gel A-15 m. Fractions of the effluent were examined for ATPase activity, which was found to reside in the fractions between 120–200 ml elution volumes ($0.3 < K_d < 0.7$). The peak of activity was found near 160 ml elution volume ($K_d \approx 0.45$) close to the elution position of ferritin ($K_d = 0.50$), suggesting a molecular weight in the vicinity of 500 000. The material in the 120–200 ml fraction was pooled, reconcentrated as before, and electrophoresed in acrylamide gels. Gel filtration removed some of the high molecular weight proteins which did not enter 4 % gels, thus permitting slightly heavier loading. After fluorescent staining in disc gels, the band containing the ATPase activity was cut out and reelectrophoresed in a gel containing dodecyl sulphate. The major polypeptide species had an apparent molecular weight of about 100 000 (Fig. 3a).

Electrophoresis on a Gradipore gel of the concentrated 120–200 ml fraction from gel filtration gave the pattern shown in Fig. 3b. The ATPase was located by

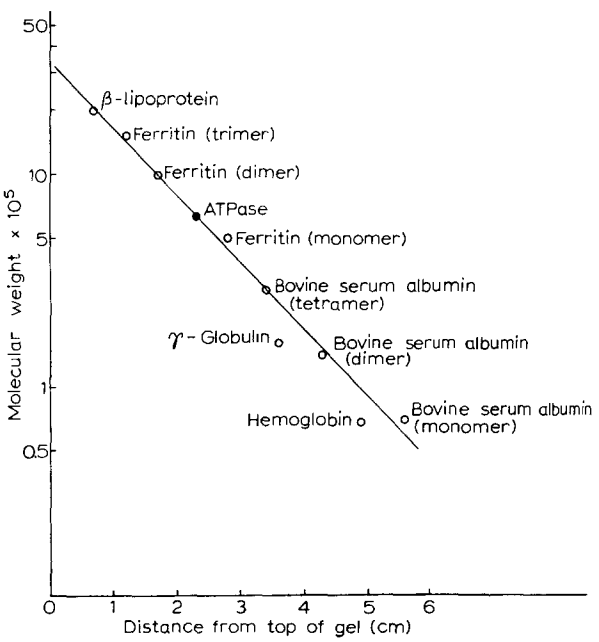

Fig. 4. Electrophoretic mobility as a function of molecular weight. 40 μl samples of standard proteins (1 mg/ml) and plasma proteins were electrophoresed overnight on a Gradipore gel as described in methods. 40 μl of the 120–200 ml fraction from gel filtration was run on the same gel. The migration distance of each protein band was measured and plotted as a function of molecular weight. The position of the Mg^{2+}-ATPase, located by activity staining, is indicated. The molecular weight thus determined was about 640 000 daltons.

means of activity staining, and its position on the gel is indicated. A series of proteins of known molecular weights, including serum proteins, haemoglobin, and ferritin was also examined on the same gel. These proteins were used to construct a calibration curve of molecular size as a function of migration distance (Fig. 4). From this calibration curve, an apparent molecular weight of the ATPase was estimated as 640 000.

DISCUSSION

It is evident that the ATPase described here shows an absolute requirement for Mg^{2+}, while Ca^{2+} alone was unable to stimulate the ATPase activity. When Ca^{2+} was added at low Mg^{2+} concentrations, the ATPase activity appeared to be decreased, while at higher Mg^{2+} concentrations added Ca^{2+} appeared to be without effect.

The ATPase activity is similar to that reported by Hoogeveen et al. [15] and Rosenthal et al. [9] in that the activities are water-soluble and insensitive to ouabain. However, although Hoogeveen et al. [15] reported a requirement for Mg^{2+}, Rosenthal et al. [9] report that their ATPase activity is stimulated by Ca^{2+} and inhibited by Mg^{2+}. In all our experiments performed to determine whether an activity similar to that reported by Rosenthal et al. [9] was also present in our preparations, no Ca^{2+} stimulated ATPase could be detected. It appears unlikely that the ionic requirements are modified by interaction with other proteins, because similar results were obtained in the absence of clear separation of the proteins in the extract. However, it is conceivable that sufficient separation may take place in the brief period of electrophoresis to alter the ionic requirements of the ATPase.

Although Rosenthal et al. [9] extracted their enzyme at 22 °C and we routinely extracted at 4 °C, the difference in temperature cannot account for the difference in the ionic requirements of the ATPase, as we have observed similar results for 22 °C and 4 °C extracts. It is possible that the Ca^{2+}-ATPase reported by Rosenthal et al. [9] may not be able to enter the gels; however, very little stain was detectable at the origin in the presence of Ca^{2+}.

In our laboratory dodecyl sulphate gel electrophoresis patterns of both human and bovine erythrocyte ghosts at low loadings have consistently shown multiple bands in the 100 000 dalton region. At higher loadings of protein, these discrete zones merge and appear as a single zone, resembling that labelled by Fairbanks et al. [13] as "component 3". At least three minor polypeptide species from this molecular weight region are water-extractable, although the major component remains with the membranes. One of these minor proteins from the "component 3" region appears to be the Mg^{2+}-dependent ATPase.

A water-soluble ($Ca^{2+}+Mg^{2+}$)-dependent ATPase activity associated with the "spectrin" fraction, has been extracted from erythrocyte membranes with 0.1 mM EDTA and 0.1 mM ATP, as reported by Weidekamm and Brdiczka [16]. The activity, unstable in the absence of Mg^{2+}-ATP, was low, but detectable, in the presence of Mg^{2+} alone. However, only a small fraction of the total ($Mg^{2+}+Ca^{2+}$)-ATPase activity could be extracted, and about 70 % remained with the membrane [16]. In contrast, Quist and Roufogalis [17] showed that a high-affinity ($Ca^{2+}+Mg^{2+}$)-ATPase activity could be removed from the membrane by means of low ionic strength extraction, but that the supernatant from the extraction was devoid of activity. When the supernatant and membrane residues were remixed, however, activity was restored

to the mixture. These results suggest that the $(Ca^{2+}+Mg^{2+})$-ATPase activity is the result of the interaction of several protein species. These species may be subunits of a complex involved in the directed transport of Ca^{2+}, or they may be a combination of protein kinases and phosphatases.

The Mg^{2+}-ATPase reported in the present communication, however, is independent of the spectrin polypeptides, and migrates as a single species on electrophoresis. Furthermore, in acrylamide gels containing dodecyl sulphate, only a single major band was seen, indicating that the subunits are of similar molecular weight. Comparison of the apparent molecular weight in dodecyl sulphate gels with that estimated with Gradipore gels suggests that the active enzyme is a hexamer of about 600 000 daltons, comprised of six subunits of about 100 000 daltons.

The possible functional role of the Mg^{2+}-ATPase reported here must await further characterisation. Experiments to this end are currently in progress.

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