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

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Summary

A water-soluble Mg^{2+}-ATPase previously reported (White, M.D. and Ralston, G.B. (1976) Biochim. Biophys. Acta 436, 567—576) has been purified from human erythrocyte membranes. The purified enzyme has a molecular weight of 575 000; the apparent minimum molecular weight was 100 000, corresponding to a soluble protein of the component 3 region. The K_m value for ATP was 1 mM and apparent K_m for Mg^{2+} was 3.6 mM. By means of histochemical activity staining in acrylamide gels it was shown that the purified ATPase preparation could be inhibited by Cd^{2+} and Zn^{2+} salts, *p*-chloromercuribenzoate and *N*-ethylmaleimide, known inhibitors of membrane endocytosis.

Introduction

A number of ATPase activities have been reported to be associated with 'fibrillar proteins' present in the low ionic strength extract of erythrocyte membranes [1—5]. In a previous publication [6] we reported the presence of a water-soluble Mg^{2+}-ATPase activity. This activity appeared to be distinct from a Ca^{2+}-stimulated, Mg^{2+}-inhibited ATPase reported by Rosenthal et al. [1]. Kirkpatrick et al. [5] have reported that the water-soluble extract of erythrocyte membranes contained both Ca^{2+}- and Mg^{2+}-stimulated ATPase activities which could be separated from each other. Kirkpatrick et al. [5] have also indicated that Ca^{2+}-ATPase activity is associated with spectrin and a group of smaller peptides which cannot be removed under non-denaturing conditions. Avissar et al. [4] have also shown Ca^{2+}-ATPase activity associated with spectrin and other low molecular weight proteins. Other groups have not identified the

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protein(s) responsible for the respective ATPase activities reported.

The argument that the soluble proteins of the erythrocyte membrane resemble actomyosin has been based on solubility properties, apparent molecular weight of the major soluble proteins, amino acid analysis and the presence of ATPase activity in the extract. The argument may be more convincing if the proteins responsible for the various ATPase activities could be established.

We report the purification and partial characterization of the water-soluble Mg^{2+}-ATPase, confirming and extending our earlier observations on the divalent cation dependence and identity of the Mg^{2+}-ATPase. The purified Mg^{2+}-ATPase still displayed an absolute requirement for Mg^{2+} and ATP. This observation suggested that the ATPase may be involved in the process of endocytosis, which Penniston [7] had shown to be dependent on the presence of Mg^{2+} and ATP. Hayashi and Penniston [8] reported that such compounds as Cd^{2+} and Zn^{2+} salts, *p*-chloromercuribenzoate and *N*-ethylmaleimide could prevent endocytosis and ATP hydrolysis. The effect of these compounds on purified preparations of the Mg^{2+}-ATPase was tested by means of activity staining in polyacrylamide gels. The results obtained suggest a possible role for the Mg^{2+}-ATPase in membrane endocytosis.

The Ca^{2+}-ATPase activity known to be present in the low ionic strength of erythrocyte membranes [5] has been separated from the Mg^{2+}-ATPase. Preliminary studies have indicated that the protein(s) responsible for the Ca^{2+}-ATPase activity are quite distinct from the Mg^{2+}-ATPase protein. Spectrin appears to be involved in the Ca^{2+}-ATPase activity (White, M.D. and Ralston, G.B., unpublished results).

Methods

Preparation of water-soluble proteins. Erythrocyte membranes were prepared from fresh human packed cells as previously described [6]. However, the membranes were washed a total of three times; twice with 10 mM Tris-HCl buffer, pH 7.7, and once with 0.1 mM EDTA, pH 7.5. The membrane preparation thus obtained still retained much haemoglobin and appeared pink or light red in colour.

Water-soluble proteins were extracted from the pink membrane preparation by means of low ionic strength dialysis [6]. Aquacide III (Calbiochem) was used to concentrate the ultracentrifuged extract. During the preparation of membranes and extraction of water-soluble proteins the temperature was maintained in the range 0—4°C.

Polyacrylamide gel electrophoresis. Disc gel electrophoresis in the absence of detergents was carried out in 4% polyacrylamide gels with the use of a discontinuous buffer system. The gel buffer was 0.375 M Tris-HCl, pH 9.0. The electrode buffer was 0.375 M Tris, 0.1 M glycine, pH 9.4, and contained 0.5 mM thioglycollic acid. Electrophoresis was carried out at room temperature at 100 V (4—6 mA/gel).

Disc gel electrophoresis in the presence of 1% sodium dodecyl sulphate (SDS) was carried out as described by Fairbanks et al. [9]. Electrophoresis in 3—26% continuous gradients of polyacrylamide gel was carried out as described by Margolis and Kenrick [10]. Gels were stained with Coomassie blue.

Histochemical activity staining. After electrophoresis, the detergent-free gels were incubated overnight at room temperature in 15 ml of 1 mM ATP, 1 mM Pb^{2+}, 5 mM Mg^{2+} and 20 mM Tris-maleate, pH 9.0. Various reagents were added at the final concentrations indicated in Tables II and III. After incubation the gels were washed for at least 1 h with distilled water and stained with 0.5% Na_2S. The ATPase activity was manifested as a dark-brown precipitate of PbS in the gel [6]. The intensity of the stained gel was quantitated by means of scanning in a Gilford 240DB spectrophotometer fitted with a linear transport accessory.

Colorimetric assay of ATPase activity. ATPase activity was measured in solution by determining inorganic phosphate released from ATP, as described by Ames [11]. The reaction volume was 500 μl and contained up to 200 μg protein in 0.05 M Tris-HCl buffer, pH 9.0. ATP concentration was maintained at 2 mM unless otherwise indicated. Mg^{2+} was added at the final concentrations indicated in Fig. 6. Incubation was carried out at 30°C and the reaction stopped by the addition of 0.1 ml 20% SDS.

Analytical ultracentrifugation. Analytical ultracentrifugation experiments were performed at 20°C in a Spinco model E analytical ultracentrifuge fitted with both schlieren and Rayleigh interference optics. Equilibrium experiments were performed on purified protein solutions by means of the meniscus-depletion method of Yphantis [12] at rotor speeds of 10 000 rev./min.

Results

Purification of the Mg^{2+}-ATPase

The procedure for the isolation and purification of the Mg^{2+}-ATPase was monitored by noting the intensity of the protein band E in detergent-free gels [6] at each fractionation step. The steps in the purification procedure were also followed by determining the specific activity of various fractions. The results of a representative experiment are shown in Table I.

The concentrated water-soluble protein extract was chromatographed on a 600 ml BioGel A15m column (gel filtration I). The buffer used was 0.05 M

TABLE I

PURIFICATION SCHEME FOR THE Mg^{2+}-ATPase

The table summarizes a typical experiment carried out for the purification of the water-soluble Mg^{2+}-ATPase. Activity in the presence of 5 mM Mg^{2+} was determined colorimetrically (see Methods). The protein concentration was determined by using the method of Lowry et al. [19].

	Total volume (ml)	Total protein (mg)	Mg^{2+}-ATPase activity (nmoles P_i/mg protein per h)	Total activity (nmol P_i/h)	Purification factor	Yield (%)
Crude water-soluble proteins	1810	1330	7	9310	1	100
Gel filtration I	375	220	24	5280	3	57
30% ammonium sulphate fraction	24	91	52	4732	7	51
Ion-exchange chromatography	6.5	3.9	1027	3954	147	43
Gel filtration II	7.0	1.02	1880	1918	270	21

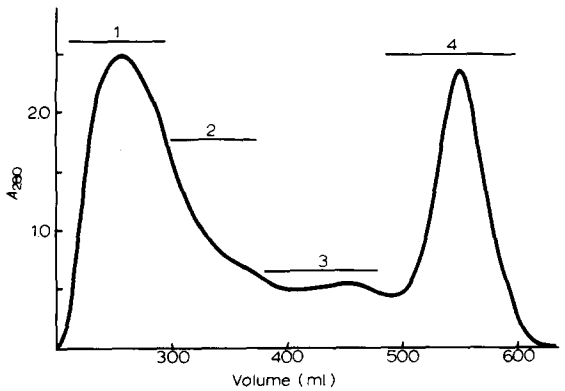


Fig. 1. Gel filtration I. A concentrated water-soluble protein extract was chromatographed at room temperature on a BioGel A15m agarose column. Fraction 3 was enriched in the band E (Mg^{2+}-ATPase) protein; this protein solution was used for ammonium sulphate fractionation.

Tris-HCl, pH 7.7, containing 0.1 M NaCl, 5 mM EDTA and 5 mM mercaptoethanol. Elution of protein at a flow rate of 30—40 ml/h per cm^2 was monitored at 280 nm in a Varian Superscan spectrophotometer. The protein solution eluting in fraction 3 (Fig. 1) was found to contain most of the band E protein. This fraction was adjusted to 15% (w/v) ammonium sulphate by the slow addition of the solid to the gently stirred protein solution. After standing for 15 min at room temperature, the turbid material was collected by centrifugation at 8000 $\times g$ for 10 min. The pellet, in which spectrin and actin were the major components, was discarded. The supernatant was adjusted to 30% (w/v) ammonium sulphate and the precipitate allowed to flocculate overnight in the cold. This precipitate, containing the Mg^{2+}-ATPase, was resuspended in and then dialyzed against 0.05 M Tris-HCl buffer, pH 8.3, containing 0.25 M

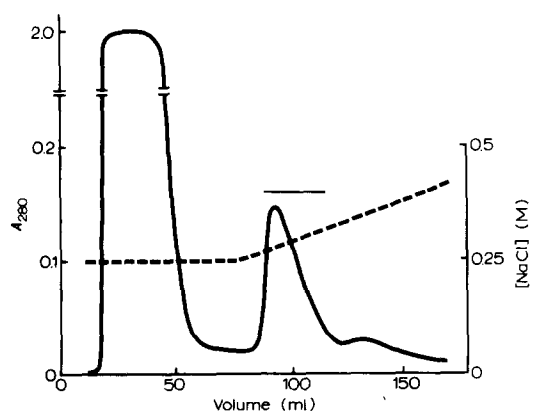


Fig. 2. Ion-exchange chromatography. The protein solution obtained after ammonium sulphate fractionation was equilibrated with cold column buffer and applied to a DEAE-Sephadex A-25 column. Adsorbed protein was eluted (———) by means of a linear gradient of 0.25—0.5 M NaCl (- - - - -). The Mg^{2+}-ATPase was eluted in a peak near 0.3 M NaCl; the fractions indicated by the bar were pooled and concentrated.

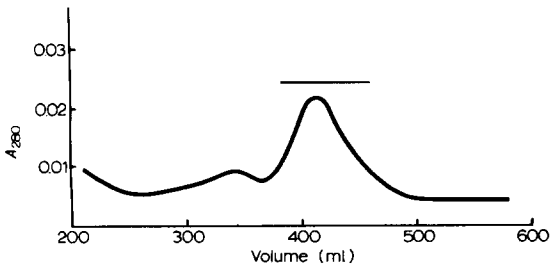


Fig. 3. Gel filtration II. The concentrated protein solution after ion-exchange chromatography was rechromatographed on the BioGel column used before (gel filtration I, Fig. 1). The enzyme eluted in a single peak corresponding to fraction 3 (Fig. 1). The fractions indicated by the bar were pooled, concentrated and called the purified Mg^{2+}-ATPase preparation.

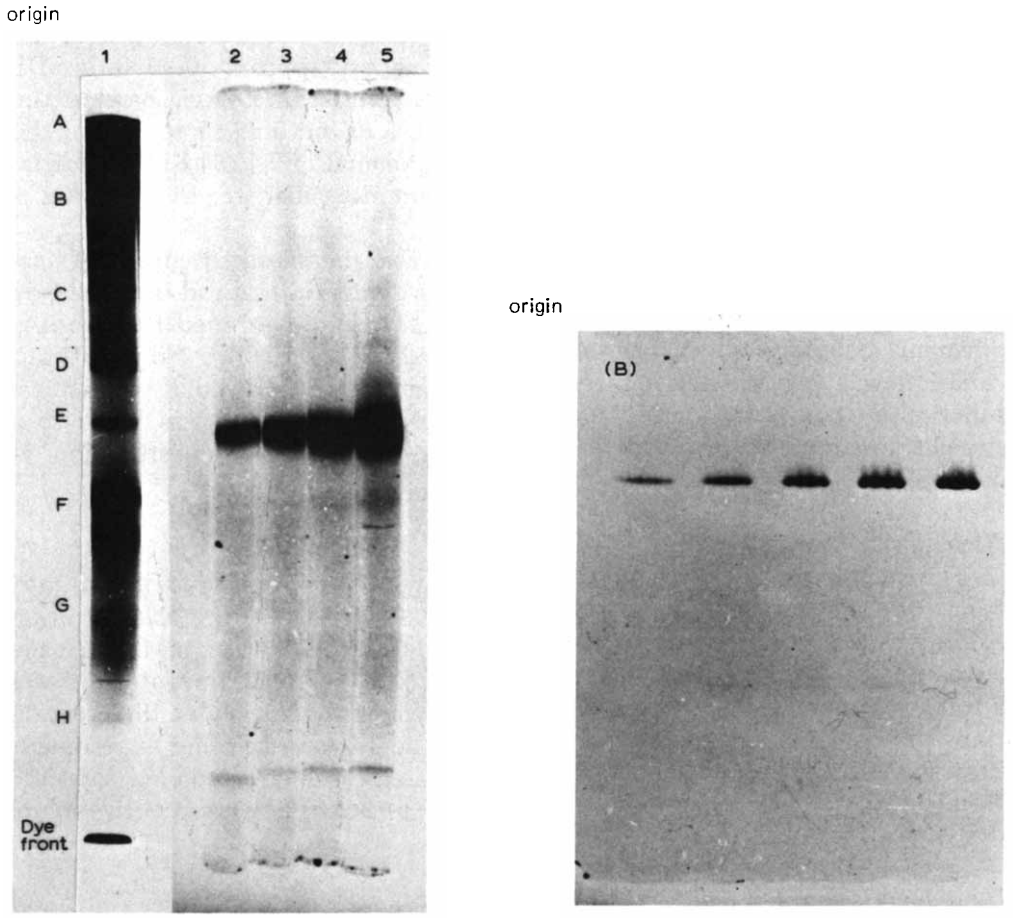


Fig. 4. Electrophoresis of the purified ATPase. The purified Mg^{2+}-ATPase was electrophoresed in detergent-free gels which were stained with Coomassie blue. (A) Gel 1 represents the crude water-soluble extract electrophoresed in disc gels, pH 9.4. The bands are labelled A—H in order of increasing mobility [6]. Gels 2—5 represent the purified Mg^{2+}-ATPase applied in increasing amounts up to 20 μg to several similar gels. (B) Up to 5 μg of the same purified ATPase preparation was applied to each of several sample wells in polyacrylamide gradient gels, pH 8.3. Only a single band was seen after electrophoresis at the two different pH values.

NaCl. The protein solution was applied to a DEAE-Sephadex A-25 column equilibrated with the same buffer and maintained at 0—4°C. Adsorbed protein was eluted at a flow rate of 20 ml/h per cm^2 with a 140 ml linear gradient of 0.25 M—0.5 M NaCl; the Mg^{2+}-ATPase eluted in a peak near 0.3 M NaCl (Fig. 2). The fractions indicated by the bar in Fig. 2 were pooled, concentrated and rechromatographed on the BioGel A15m column (gel filtration II). The Mg^{2+}-ATPase eluted from the second gel filtration column in a single peak corresponding to fraction 3 (Fig. 3). These fractions were pooled, concentrated by dialysis against Aquacide II (Calbiochem) and called the purified Mg^{2+}-ATPase preparation.

Homogeneity and molecular weight of the Mg^{2+}-ATPase

The Mg^{2+}-ATPase was examined for homogeneity in detergent-free disc gels, pH 9.4 (Fig. 4A), and gradient slab gels, pH 8.3 (Fig. 4B); only a single band was seen in these gels. The mobility of the band on the gradient gels in the absence of detergents indicated a molecular weight of approx. 600 000. In SDS gels (Fig. 5), the purified ATPase was seen to consist of a single band at the trailing edge of the component 3 region. From a calibration curve relating the apparent minimum molecular weights of components 1—7 [13] to their migration distance in SDS gels, the apparent minimum molecular weight of the Mg^{2+}-ATPase was estimated to be approx. 100 000.

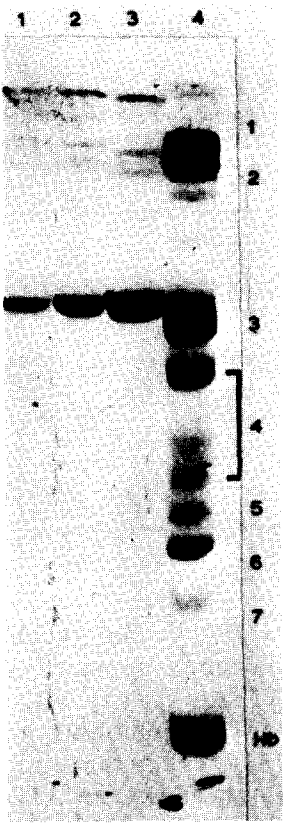
The molecular weight of the Mg^{2+}-ATPase was measured by means of analytical ultracentrifugation, using the meniscus-depletion method described by Yphantis [12]. The molecular weight of the ATPase, determined from several different experiments, was estimated to be 575 000 ± 20 000. No significant variation was seen in weight average or number average molecular weight as a function of concentration, so the entire plot of log J vs. r^2 was fitted to a straight line by an unweighted least-squares method for the estimation of molecular weight.

Michaelis constants for the Mg^{2+}-ATPase

Using a colorimetric assay, the K_m values for ATP and Mg^{2+} were determined from the Lineweaver-Burk plots shown in Fig. 6. In Fig. 6A the concentration of Mg^{2+} was maintained at 20 mM; the K_m value for ATP was 1 mM. When the apparent K_m for Mg^{2+} was determined (Fig. 6B) the ATP concentration was 2 mM, in order to maintain low blank absorbance values. Under these conditions the apparent K_m for Mg^{2+} was found to be 3.6 mM. In other experiments (not shown) Ca^{2+} at 4 mM was found to increase the apparent K_m for Mg^{2+} from 3.6 to 6.3 mM, suggesting that Ca^{2+} may be acting as a competitive inhibitor of the Mg^{2+}-ATPase.

Histochemical activity staining

Some of the properties of the Mg^{2+}-ATPase previously demonstrated by means of activity staining in disc gels [6] were confirmed by using a purified preparation of the Mg^{2+}-ATPase. The activity stain was not detected in those gels which were incubated without Mg^{2+} or ATP or when Ca^{2+} replaced Mg^{2+} in the incubation mixture. Neither ADP nor GTP could substitute for ATP in the incubation medium.



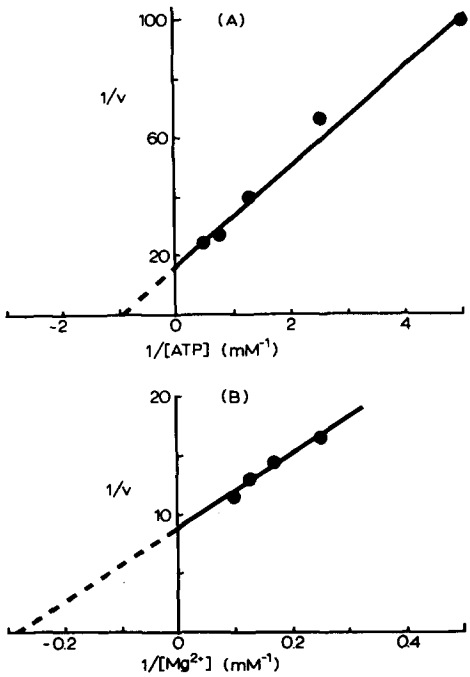


Fig. 5. SDS gel electrophoresis. A purified preparation of the Mg^{2+}-ATPase was applied to gels 1—3 after heating with 1% SDS in the presence of mercaptoethanol. A sample of erythrocyte membranes treated in the same way was applied to gel 4. The gels contained 1% SDS and were electrophoresed as described by Fairbanks et al. [9]. The Mg^{2+}-ATPase was seen to consist of a single band in the component 3 region. The apparent minimum molecular weight of this band was estimated to be 100 000.

Fig. 6. Michaelis constants for the ATPase. Using a colorimetric assay, the K_m values for ATP and Mg^{2+} were determined from the Lineweaver-Burk plots shown. (A) Plot for the Mg^{2+}-ATPase when Mg^{2+} was maintained at 20 mM, in order to saturate the enzyme and ATP. The value of K_m for ATP obtained under these conditions was 1 mM. (B) ATP was maintained at 2 mM in order to obtain low absorbance values in the blanks. The apparent K_m for Mg^{2+} was 3.6 mM.

Furthermore, it was shown that F^- and 2,4,6-trinitrobenzenesulphonate did not appear to affect the intensity of the activity stain. However, Cd^{2+} and Zn^{2+} salts caused a substantial decrease in the intensity of the ATPase activity stain. The effect of various concentrations of these and other reagents on the ATPase activity stain were quantitated by means of scanning in a densitometer. The results of these studies are shown in Table II, where the intensity of the activity stain is shown as a percentage of the control, which was a gel incubated in the presence of Mg^{2+} and ATP but no other reagent. Table II shows a group of reagents which appeared to be inhibitors of the ATPase. These inhibitors were tested at concentrations near those used by Hayashi and Penniston [8] in their study of the inhibitor of endocytosis. Cd^{2+} and Zn^{2+} salts appeared to inhibit

TABLE II

EFFECT OF INHIBITORS

All gels were incubated overnight at room temperature in 15 ml of 1 mM ATP, 1 mM Pb^{2+}, 5 mM Mg^{2+} and 20 mM Tris-maleate, pH 9.0. Zn^{2+}, Cd^{2+}, *N*-ethylmaleimide and *p*-chloromercuribenzoate were added to the incubation mixture at the final concentration indicated. The numbers represent the intensity of the ATPase stain in the presence of the inhibitor as a percentage of the intensity of the control (no additional reagent).

Inhibitor	Concentration (mM)	% of control
$CdCl_2$	0.5	5.1
	1.0	0
$Zn(CH_3CO_2)_2$	0.15	37.4
	0.3	19.8
	0.6	11.5
N-Ethylmaleimide	0.5	11.9
	1.0	0
p-Chloromercuribenzoate	0.1	46.5
	0.2	53.5

the ATPase at these concentrations; however, neither Cd^{2+} nor Zn^{2+} could replace Mg^{2+} in the incubation medium. Whilst *N*-ethylmaleimide appeared to cause a substantial decrease in the intensity of the ATPase activity stain, *p*-chloromercuribenzoate did not appear to be as effective an inhibitor. All these reagents were shown by Hayashi and Penniston [8] to simultaneously inhibit endocytosis of the erythrocyte membrane and an Mg^{2+}-dependent ATPase.

On the other hand, another group of reagents had little or no effect on the intensity of the ATPase activity stain. F^-, a glycolytic inhibitor, was used by Ben-Bassat et al. [14] to block vacuole formation by other agents. However, 4 mM F^- decreased the activity of the purified Mg^{2+}-ATPase by approx. 20% only. 2,4,6-trinitrobenzenesulphonate, which reacts with the amino groups of lysine, caused a 30% decrease in the ATPase activity stain only at the relatively high concentration of 4 mM but had little effect at concentrations less than this. Colchicine, which has been reported to cause aggregation of membrane proteins [15], appeared to have no effect on the activity of the Mg^{2+}-ATPase. None of these reagents prevented membrane endocytosis [8].

Discussion

The purification procedure described for the Mg^{2+}-ATPase resulted in a 270-fold purification of the enzyme, with a final yield of approx. 20%. This isolation procedure used a membrane preparation which had not been washed free of haemoglobin. This was because extensive washing of the membranes resulted in a lower yield of the ATPase activity, probably due to fragmentation of the membrane. However, substantial amounts of Mg^{2+}-ATPase activity could also be recovered from white or creamy membrane preparations. In addition, there did not appear to be any detectable Mg^{2+}-ATPase protein present in the haemolysates. These observations suggest that the Mg^{2+}-ATPase is not a cytoplasmic

component which becomes adsorbed to the membrane during preparation. Substantial amounts of the activity appeared to be lost after the first gel filtration step. This may be due to surface denaturation of the proteins during concentration of large volumes of the crude water-soluble extract. Purified preparations of the Mg^{2+}-ATPase were stored frozen after dialysis against 0.05 M Tris-HCl buffer, pH 9.0. The relatively high pH values were chosen so that colorimetric assay conditions approximated those of the histochemical activity staining studies.

Preliminary studies on the Mg^{2+}-ATPase activity present in crude extracts of water-soluble erythrocyte membrane proteins relied heavily on the use of histochemical activity staining techniques [6]. In this study we have obtained a purified preparation of the ATPase, and have shown that the properties of the enzyme have not been altered during the isolation procedure. By means of histochemical activity staining it was shown that the ATPase still displayed a absolute requirement for Mg^{2+}; Ca^{2+} and other divalent cations did not appear to replace Mg^{2+} in the incubation mixture. The purified Mg^{2+}-ATPase could not utilize ADP or GTP when these nucleotides replaced ATP in the incubation medium.

Low concentrations of Pb^{2+} (1 mM) were previously shown not to alter the ionic requirements of the Mg^{2+}-ATPase [6]. However, the presence of Pb^{2+} during detection of enzymic activity is not always desirable, as Pb^{2+} has been shown to be an inhibitor of the ATPase associated with the $(Na^+ + K^+)$ pump [16]. In addition, the histochemical activity staining technique does not permit accurate kinetic data to be obtained. We have therefore shown, by means of a colorimetric assay, that the purified ATPase still displayed an absolute requirement for Mg^{2+}. Ca^{2+}, tested at similar concentrations to Mg^{2+}, was unable to stimulate the ATPase. We have also shown that Ca^{2+} may be a competitive inhibitor of the Mg^{2+}-stimulated ATPase. The values of K_m for ATP and apparent K_m for Mg^{2+} reported here were obtained at a relatively high pH, but are in reasonable agreement with the values for maximum activity reported by Kirkpatrick et al. [5] for crude preparations of the Mg^{2+}-ATPase. The specific activity of the ATPase is approx. 2 μmol of inorganic phosphate/mg protein per h, which is of the same order of magnitude for myosin-ATPase activity [21].

Examination of the purified Mg^{2+}-ATPase in SDS gels revealed the presence of a single band; no minor band other than faint traces of spectrin was detected even at higher protein loadings on these gels. This therefore confirmed the earlier observations that the Mg^{2+}-ATPase contained six apparently identical subunits. The previously reported value of 600 000 for the molecular weight of the native ATPase in crude preparations was obtained by means of gel electrophoresis. This value appears to have been a slight overestimation, but is nevertheless within 4% of the value now obtained by means of analytical ultracentrifugation.

Reagents such as Zn^{2+} and Cd^{2+} salts, *N*-ethylmaleimide and *p*-chloromercuribenzoate have been shown to inhibit endocytosis and an Mg^{2+}-ATPase activity apparently associated with the phenomenon [7,8]. Furthermore Hayashi and Penniston [17] have shown that protein components responsible for endocytosis lay on the inner face of the membrane and required an active ATPase protein. The above reagents were capable of inhibiting the purified

Mg^{2+}-ATPase used in this study. In addition, another group of reagents such as F^-, 2,4,6-trinitrobenzenesulphonate and colchicine did not inhibit endocytosis [8] or the purified Mg^{2+}-ATPase. These observations suggest that the Mg^{2+}-ATPase solubilized from the membrane at low ionic strengths may be an ATPase involved in the process of endocytosis.

Recently, Jarrett et al. [18] have shown that energy-dependent endocytosis and a low-affinity Ca^{2+}-ATPase were concurrently inhibited by two inhibitors, carbonyl cyanide *m*-chlorophenylhydrazone and *N*-naphthylmaleimide. Other inhibitors of endocytosis such as Cd^{2+} and Zn^{2+} salts or *p*-chloromercuribenzoate were not examined for concurrent inhibition of the Ca^{2+}-ATPase. Whilst the studies of Jarrett et al. [18] implicate a Ca^{2+}-ATPase activity in the phenomenon of endocytosis, their results do not preclude the involvement of other ATPase activities in endocytosis.

In the present study, a Ca^{2+}-stimulated, Mg^{2+}-inhibited ATPase activity was also shown, by means of colorimetric assay, to be present in the water-soluble extract of erythrocyte membranes. The Ca^{2+}-ATPase has been separated from the Mg^{2+}-ATPase and partially purified (White, M.D. and Ralston, G.B., unpublished results). This observation suggests that the Ca^{2+}- and Mg^{2+}-stimulated ATPase activities are due to different proteins. A single protein, of which the divalent cation requirements may be altered by the presence of other proteins, is not likely to account for both the ATPase activities.

It has been shown that spectrin is not responsible for the water-soluble Mg^{2+}-ATPase activity; this precludes any similarity between myosin and spectrin on the basis of associated ATPase activity. The Mg^{2+}-ATPase may be involved in the phenomenon of membrane endocytosis, though the mechanism by which this occurs is unknown. These studies do not, however, rule out the involvement of the spectrin-actin network in the mechanism of endocytosis.

Acknowledgements

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References

1 Rosenthal, A.S., Kregenow, F.M. and Moses, H.L. (1970) Biochim. Biophys. Acta 196, 254—262
2 Hoogeveen, J.T., Juliano, R., Coleman, J. and Rothstein, A. (1970) J. Membrane Biol. 3, 156—172
3 Weidekamm, E. and Brdiczka, D. (1975) Biochim. Biophys. Acta 401, 51—58
4 Avissar, N., De Wries, A., Ben-Shaul, Y. and Cohen, I. (1975) Biochim. Biophys. Acta 375, 35—43
5 Kirkpatrick, F.H., Woods, G.M., La Celle, P.L. and Weed, R.I. (1975) J. Supramol. Struct. 3, 415—425
6 White, M.D. and Ralston, G.B. (1976) Biochim. Biophys. Acta 436, 567—576
7 Penniston, J.T. (1972) Arch. Biochem. Biophys. 153, 410—412
8 Hayashi, H. and Penniston, J.T. (1973) Arch. Biochem. Biophys. 159, 563—569
9 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
10 Margolis, J. and Kenrick, K.G. (1968) Anal. Biochem. 25, 347—362
11 Ames, B.N. (1966) in Methods in Enzymology (Neufeld, E.F. and Ginsburg, V., eds.), Vol. 3, p. 115, Academic Press, New York
12 Yphantis, D.A. (1964) Biochemistry 3, 297—317
13 Steck, T.L. (1974) J. Cell Biol. 62, 1—19

14 Ben-Bassat, I.B., Bensch, K.G. and Schrier, S.L. (1972) J. Clin. Invest. 51, 1833—1944
15 Wilson, L., Bryan, J., Ruby, A. and Mazia, D. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 807—814
16 Jacobson, N.O. and Jorgensen, P.L. (1969) J. Histochem. Cytochem. 17, 443—453
17 Hayashi, H. and Penniston, J.T. (1974) Biochem. Biophys. Res. Commun. 61, 1—7
18 Jarrett, H.W., Reid, T.B. and Penniston, J.T. (1977) Arch. Biochem. Biophys. 183, 498—510
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Galand, M.B. and Luscher, E.F. (1965) Adv. Protein Chem. 20, 1—35