Biochimica et Biophysica Acta, 596 (1980) 472—475

BBA Report

BBA 71439

A WATER-EXTRACTABLE Ca^{2+}-ATPase FROM ERYTHROCYTE MEMBRANES

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(Received October 4th, 1979)

Key words: Ca^{2+}-ATPase; Spectrin; Water extraction; (Erythrocyte membrane)

Summary

The Ca^{2+}- and Mg^{2+}-stimulated ATPase activities present in low ionic strength extracts of erythrocyte membranes have been separated from each other. The Ca^{2+}-ATPase appears to be associated with particulate material which could be sedimented by high-speed centrifugation. The pellet obtained was composed mainly of components 1, 2, 4.5, 5 and 7. A soluble protein from the band 3 region, known to be responsible for the Mg^{2+}-ATPase activity, was not detected in this pellet.

The low ionic strength extract of erythrocyte membranes has been reported to contain three ATPase activities which were either Ca^{2+}-stimulated, Mg^{2+}-stimulated or Ca^{2+}-stimulated, Mg^{2+}-inhibited [1]. Kirkpatrick et al. [1] attributed the Ca^{2+}-stimulated ATPase to a partially-purified spectrin preparation which contained a number of unidentified peptides: they were also of the opinion that the Ca^{2+}-ATPase activity was an entity distinct from the Mg^{2+}-ATPase activity. Avissar et al. [2] have reported the presence of Ca^{2+}-ATPase activity in a preparation containing spectrin and three other proteins of molecular weights 80 000, 60 000 and 46 000. In a previous publication [3], we identified the protein responsible for the Mg^{2+}-ATPase activity. Subsequent studies (White, M.D. and Ralston, G.B., unpublished data) have resulted in the purification and partial characterization of the Mg^{2+}-ATPase. The present report describes the isolation of the Ca^{2+}-ATPase to a fraction distinct from the Mg^{2+}-ATPase and containing spectrin and three other major proteins. This Ca^{2+} ATPase activity could be inhibited by Mg^{2+}.

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Fresh human packed cells were washed three times in cold 10 mM Tris-HCl buffer, pH 7.7, containing 0.95% NaCl and 20 mM glucose. The buffy coat was aspirated off at each step. The cells were hemolysed at 0--4°C in 10 mM Tris-HCl buffer, pH 7.7, washed twice in the same buffer and once more with 0.1 mM EDTA, pH 7.5. This membrane preparation which retained much hemoglobin was used to prepare water-soluble proteins. The suspension of pink membrane was dialysed at low temperature against 0.1 mM EDTA, pH 7.5, for at least 16 h. Water-soluble proteins were collected by low-temperature centrifugation at 100 000 × *g* for 1 h.

ATPase activities were measured colorimetrically in duplicate by determining inorganic phosphate released from ATP in a final reaction volume of 500 μl. Divalent cations were added at concentrations of 5 or 10 mM to solutions containing 2 mM ATP in 0.05 M Tris-HCl buffer, pH 9.0. The final concentration of protein in the assay mixture was 2—5 mg/ml. Gel electrophoresis in the presence of 1% sodium dodecyl sulphate was carried out as described by Fairbanks et al. [4].

The concentrated water-soluble extract was subjected to gel filtration on BioGel A15m agarose beads equilibrated with 0.1 M NaCl, 5 mM EDTA and 5 mM mercaptoethanol in 0.05 M Tris-HCl buffer, pH 7.7. The eluted proteins were divided into four fractions, A—D, as shown in Fig. 1; fraction D contained mainly hemoglobin. Fractions A, B and C were concentrated to approx. one-fifth of their original volumes and then examined for the presence of both Ca^{2+}- and Mg^{2+}-stimulated ATPase activities (Table I). Fraction A was usually turbid, in spite of ultracentrifugation of the water-soluble proteins prior to the gel filtration step. Therefore, fraction A was recentrifuged at 100 000 × *g* for 1 h. Both the supernatant (A_s) and the pellet (A_p) were then examined for ATPase activity.

Colorimetric assay of the concentrated water-soluble protein extract

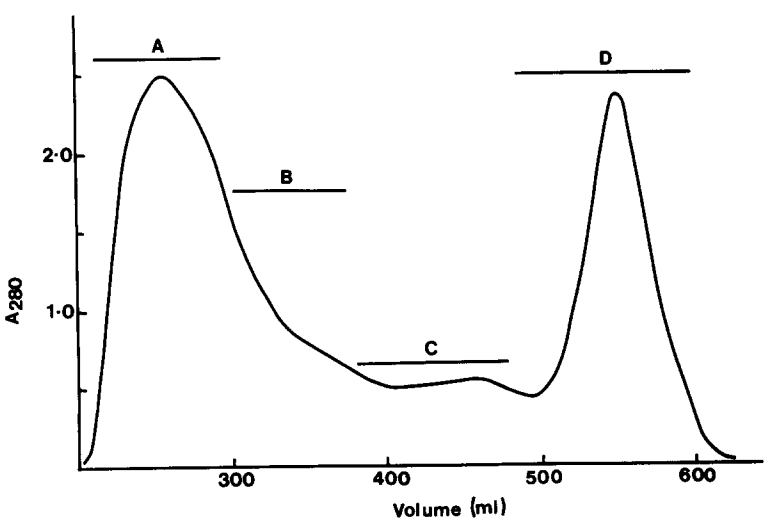

Fig. 1. Gel filtration. The concentrated water-soluble extract was chromatographed on a BioGel A15m column. Four fractions A—D of approximately equal volumes were pooled as shown. Fractions A, B and C were examined for Ca^{2+}- and Mg^{2+}-stimulated ATPase activities.

TABLE I

ATPase ACTIVITIES OF WATER-EXTRACTED PROTEINS

Fractions A, B and C from gel filtration were examined for ATPase activity in the presence of 10 mM Ca^{2+} or Mg^{2+}. The turbid fraction, A, was separated into a soluble fraction, A_s, and a pellet fraction, A_p, by centrifugation. The whole water-soluble extract, W, was also examined.

Fraction	Specific activity (nmol P_i per mg protein per h)	
	Ca^{2+}-ATPase	Mg^{2+}-ATPase
W	15	7
A	42	4
A_s	16	—
A_p	107	—
B	6	5
C	4	25

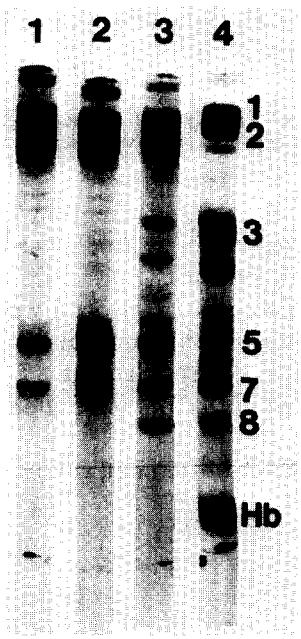

Fig. 2. Gel electrophoresis. Gels containing 1% sodium dodecyl sulphate were electrophoresed as described by Fairbanks et al. [4]. Gel 1 represents the fraction A pellet; gel 2 is the same pellet after washing with buffer. Gel 3 shows the crude water-soluble extract and gel 4 the total membrane protein.

showed a very low Mg^{2+}-stimulated ATPase activity: fractions A and B contained little or none of this activity. However, fraction C contained the highest specific activity: this fraction was used to isolate and purify the Mg^{2+}-ATPase (White, M.D. and Ralston, G.B., unpublished results).

The concentrated water-soluble extract contained a Ca^{2+}-stimulated ATPase of specific activity 15 nmol P_i per mg protein per h in the presence of 10 mM Ca^{2+} alone. At this concentration the Ca^{2+} also caused the protein solution to become turbid. The Ca^{2+}-ATPase activity could be inhibited by 5 mM Mg^{2+} to a level of 4 nmol P_i per mg protein per h. Fractions B and C were almost devoid of Ca^{2+}-ATPase activity. However, fraction A, the void volume material, had the highest specific activity in the presence of Ca^{2+}. Almost all the Ca^{2+}-ATPase activity could be recovered in the pellet obtained

after centrifugation of fraction A at 100 000 × *g* for 30—60 min. The specific activity of the resuspended pellet was in excess of 100 nmol P_i per mg protein per h, about six times greater than in the supernatant.

The pellet obtained after centrifugation of fraction A was examined in sodium dodecyl sulphate gels, as shown in gel 1, Fig. 2. Gel 3 shows the crude water-soluble extract and gel 4 the total membrane protein. The major components of the pellet appear to be spectrin, actin and components 4.5 and 7. Component 4.1 appears to be absent from that fraction. Washing the pellet caused an apparent enrichment of components 4.5, 5 and 7, probably due to dissociation and removal of spectrin (gel 2). Most of the Ca^{2+}-ATPase activity remained with the washed pellet.

The demonstration of Ca^{2+}-ATPase activity in fraction A, which was shown to be devoid of significant Mg^{2+}-ATPase activity, suggests that these two activities are due to two distinct enzymes. This is further confirmed by the absence of any detectable component 3 proteins associated with the Ca^{2+}-ATPase proteins. It therefore appears likely that the Ca^{2+}-ATPase activity is not the result of the modification of the ionic requirements of the Mg^{2+}-ATPase by association with other proteins. Fraction B of the gel filtration is composed almost exclusively of spectrin and was shown to contain only minimal Ca^{2+}-ATPase activity: it is, therefore, unlikely that spectrin alone is responsible for this activity. Our observations implicate components 4.5, 5 and 7 in the Ca^{2+}-ATPase activity: this study cannot, however, exclude the possibility that spectrin, in association with one or more of the above components, accounts for the ATPase activity. The Ca^{2+}-stimulated ATP hydrolysis reported in this study may thus represent the activity of a protein kinase-protein phosphatase system in which spectrin may have a role, or alternatively, hydrolysis of ATP associated with a dynamic spectrin-actin network.

The Ca^{2+}-ATPase appears to be associated with a group of proteins excluded from a column of exclusion limit 15 000 000. Further, this activity could be sedimented by high-speed centrifugation, suggesting that the ATPase activity was particulate. These observations also explain the failure to detect Ca^{2+}-ATPase activity by means of the histochemical activity staining studies previously described [3]. Membrane fragments, which were not pelleted during the initial high-speed centrifugation of water-soluble proteins, may account for the Ca^{2+}-ATPase activity. However, examination of the pellet obtained after centrifugation of fraction A in sodium dodecyl sulphate gels showed that component 3, the major transmembrane protein, was not present. This suggests that membrane fragments or vesicles are not likely to be responsible for the Ca^{2+}-ATPase activity.

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