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ACTIN-ACTIVATED ATPase FROM HUMAN ERYTHROCYTES

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SUMMARY

A fibrillar protein complex, possessing ouabain-insensitive Ca^{2+}-ATPase activity was isolated from human erythrocyte membranes by using a low ionic strength extraction procedure. Mg^{2+}-ATPase activity was revealed upon addition of rabbit skeletal muscle actin, thus demonstrating the presence of a myosin-like protein in the crude extract of the erythrocyte membrane. Upon sodium dodecylsulfate gel electrophoresis, the extract showed mainly the doublet of subunit molecular weight bands of 230 000 and 210 000, and more than 10 faster moving bands. Gel filtration of the erythrocyte membrane extract on Sepharose 4B furnished 4 fractions. Fraction I, containing the doublet and 80 000, 60 000 and 46 000 subunit molecular weight bands was 5-fold purified with respect to Ca^{2+}-ATPase activity, but was devoid of actin-activated Mg^{2+}-ATPase activity. Fraction II, containing only the doublet, was devoid of Ca^{2+} and actin-activated Mg^{2+}-ATPase activity. The 210 000 subunit molecular weight protein could be phosphorylated in the presence of Mg^{2+} in the crude extract and Fraction I but not in Fraction II.

INTRODUCTION

Myosin, actin and tropomyosin, the principal constituents of the mechano-chemical apparatus of muscle contraction, have been extracted from several non-muscular systems [1–7]. The presence of contractile proteins in the erythrocyte membrane has been suspected on the basis of the ATP and divalent cation-dependent erythrocyte shape change [8,9]. Attempts to identify such a system in the erythrocyte led to controversial structural and functional data. In the present study we were able to isolate and partially purify a fibrillar protein complex possessing ouabain-insensitive Ca^{2+}-and Mg^{2+}-ATPase activity, the latter being further activated by the addition of skeletal muscle actin.

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MATERIALS

Sepharose 4B was purchased from Pharmacia Fine Chemicals AB. [γ-^{32}P] ATP (spec. act. 15.9 Ci/mmole) was purchased from Radiochemical Centre, Amersham. Ouabain, ATP, adenosine 3'-5'-monophosphoric acid (cyclic AMP), dithiothreitol and bovine serum albumin were from Sigma Chemical Company.

METHODS

Extraction and purification of fibrillar proteins from human erythrocyte membrane. Erythrocyte membrane suspension and crude membrane extract were prepared according to Fairbanks *et al.* [10], with the following modifications. Washed erythrocytes were introduced into 10 vol. of 5 mM sodium phosphate buffer (pH 8.0) and extraction was carried out in 0.5 mM EDTA-Tris (pH 8.0). The crude extract was precipitated at 50 % ammonium sulfate saturation and the precipitate dissolved in a solution containing 0.3M NaCl, 10^{-3}M EDTA and 0.05 M Tris (pH 8.0). The solution was exhaustively dialyzed against the same buffer, according to Marchesi et al. [11], and subsequently against 10^{-3} M EDTA, 0.05 M Tris (pH 8.0). It was then applied to a Sepharose 4B column equilibrated with the latter buffer. Protein peaks estimated by absorption at 280 nm were pooled and concentrated by ultrafiltration on Diaflo membranes. All operations were carried out at 4 °C. 0.5 mM dithiothreitol was present at all stages of preparation.

Preparation of rabbit skeletal muscle proteins. Myosin was prepared according to Holtzer and Lowey [12], actin according to Kendrick-Jones et al. [13] and tropomyosin according to Hartshorne and Mueller [14].

ATPase assay. Fractions to be assayed were exhaustively dialyzed against 0.05 M Tris (pH 8.0). ATPase activity was measured at 37 °C in a system containing 25 mM Tris (pH 8.0), 0.4 mM ouabain, 2.5 mM ATP and, when used, 2.5 mM $MgCl_2$, 10 mM $CaCl_2$, 0.6 M KCl and 2 mM EDTA. The reaction was stopped by addition of 1 ml of cold 20 % trichloroacetic acid to the 2 ml reaction volume. The inorganic phosphate liberated was determined by the method of Ames [15]. Results are given in nmoles P_i/mg protein/h.

Protein concentration. The method of Lowry et al. [16] was used.

Sodium dodecylsulfate gel electrophoresis. The method of Weber and Osborn [17] was used with a modification in the processing of the sample, which was left in boiling water for 1 min in the presence of sodium dodecylsulfate and β-mercaptoethanol, both at 1 % concentration. Samples and gels contained 1 mM EDTA in order to inhibit protease activity [10]. 5 % polyacrylamide gels were used. Subunit molecular weights of the different bands were calculated on the basis of their mobility. Electrophoretic standards included myosin, actin and tropomyosin from rabbit muscle, and bovine serum albumin.

Phosphorylation of extracted proteins. The procedure of Williams [18] was modified as follows. Protein (0.2–0.4 mg) was incubated for 1 h at 37 °C in a medium containing 25 mM Tris (pH 8.0), 2.5 mM $MgCl_2$ or 10 mM $CaCl_2$, 62.5 μM [γ-^{32}P] ATP (10^6–10^7 cpm) and, when used, 1 μM cyclic AMP, in a total volume of 0.2 ml. The reaction was stopped by adding 0.03 ml of a mixture containing 10 % sodium dodecylsulfate, 10 % β-mercaptoethanol and 10 mM EDTA, and heating at 100 °C

for 1 min. The mixture was exhaustively dialyzed against 0.1 % β-mercaptoethanol, 0.1 % sodium dodecylsulfate, 1 mM EDTA and 0.01 M sodium phosphate buffer (pH 7.0). Aliquots of 0.05 ml were layered on 5 % sodium dodecylsulfate–polyacrylamide gels [17] containing 1 mM EDTA (5.9 mm internal diameter × 100 mm length) and electrophoresis was carried out at 15 mA/gel for 4.5 h. The gels were then fractionated on a Savant auto-gel divider [19]. Samples were collected and counted in a Packard Model 3380 Tricarb Spectrometer.

Electron microscopy. Crude extract and fractions (0.2–0.4 % concentration) were dialyzed against 0.05 M $CaCl_2$, 0.05 M Tris (pH 8.0). The turbid suspensions were layered over 200 mesh formvar–carbon coated grids and negatively stained with 1 % uranyl acetate.

RESULTS

Characterization of crude erythrocyte membrane extract

The crude extract of erythrocyte membranes, analyzed by sodium dodecylsulfate gel electrophoresis, showed a slow-moving doublet and several low molecular weight bands (Fig. 1 CE). Subunit molecular weights of the doublet proteins were 230 000 and 210 000, the latter being slightly higher than that of rabbit myosin heavy chain, as observed in the electrophoretic pattern of crude extract and myosin applied to the same gel. Noteworthy was the presence of 46 000 subunit molecular weight band, the mobility of which was identical to that of muscle actin, as ascertained by the electrophoretic pattern of crude extract and actin applied to the same gel.

The presence in the crude extract of proteins with subunit molecular weight close to that of rabbit myosin and actin led us to search for a muscle-type ATPase activity. At low ionic strength ($I = 0.05$) a Ca^{2+}-activated ATPase activity with a range of 20–90 nmole P_i/mg protein/h ($n = 20$) was found in the crude extract.

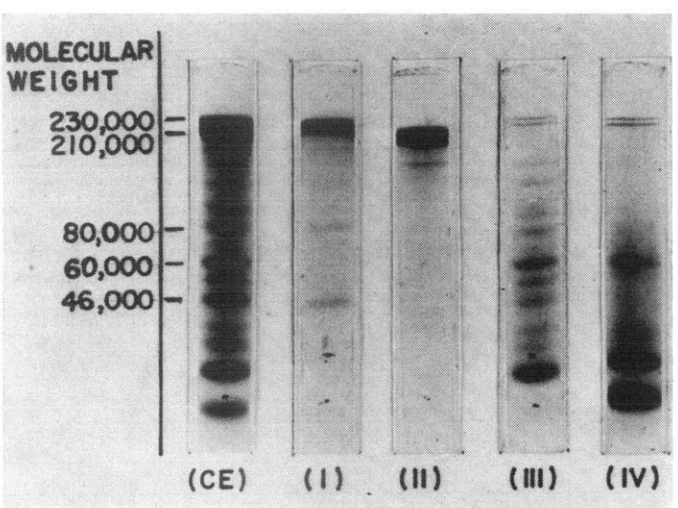

Fig. 1. Sodium dodecylsulfate gel electrophoresis of erythrocyte membrane crude extract and Sepharose 4B fractions. (CE) crude extract; I, II, III and IV, Sepharose 4B pooled fractions.

TABLE I

ATPase ACTIVITIES OF ERYTHROCYTE MEMBRANE CRUDE EXTRACT AND FRACTION I

Experiment No.	Fraction	ATPase activity (nmoles P_i/mg protein/h)			
		Ca^{2+} ($I = 0.05$)	Ca^{2+} ($I = 0.6$)	Mg^{2+} ($I = 0.05$)	Mg^{2+} + actin* ($I = 0.05$)
1	Crude extract	44	65	27	44
	Fraction I	100	125	0	0
2	Crude extract	90	165	33	124
	Fraction I	290	400	0	0
3	Crude extract	24	50	24	43
	Fraction I	67	125	0	0

* Weight ratio of erythrocyte fraction to rabbit actin was 2:1. Actin alone was devoid of ATPase activity.

No Mg^{2+}-ATPase, or only low Mg^{2+}-ATPase activity, with a range of 0–56 nmole Pi/mg protein/h ($n = 10$) could be demonstrated. In representative experiments (Table I) the Ca^{2+}-activated ATPase activity was increased 1.5- to 2-fold at high ionic strength ($I = 0.6$). Although in the crude extract, no EDTA-activated ATPase activity specific for myosin was found, the presence of a Ca^{2+}-activated ATPase activity higher than the Mg^{2+}-activated one seemed to suggest the presence of free myosin-like protein. Indeed, in representative experiments at low ionic strength the Mg^{2+}-activated ATPase activity was increased 2- to 3-fold by the addition of rabbit actin (Table I).

Characterization of Sepharose 4B fractions

Fractionation of the crude extract from erythrocyte membrane by gel filtration on Sepharose 4B furnished 4 fractions (Fig. 2). Fraction I, first eluted, showed on

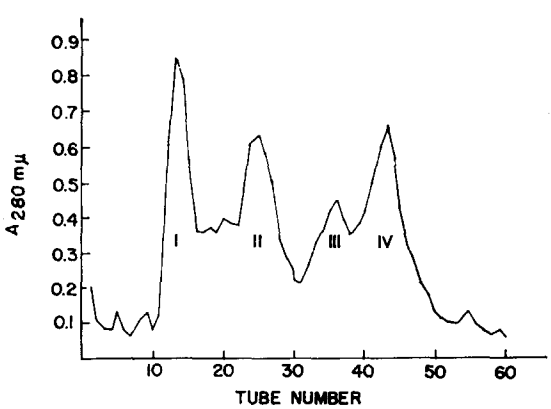

Fig. 2. Partial purification of crude ATPase on Sepharose 4B column. 80 mg of crude extract were applied to a 3 × 40 cm column of Sepharose 4B. Elution was carried out with 1 mM EDTA, 0.5 mM dithiothreitol and 0.05 M Tris (pH 8.0). Flow rate was 15 ml/h. 6 ml effluent were collected per tube. I, II, III and IV, protein peaks at 280 nm.

sodium dodecylsulfate gel electrophoresis (Fig. 1) as major bands the doublet, and three minor bands of subunit molecular weight 80 000, 60 000 and 46 000. Only Ca^{2+}-activated ATPase activity, increasing with rising ionic strength ($I = 0.6$), was demonstrated in this fraction. It showed a 3- to 5-fold purification. Addition of rabbit actin did not cause activation of Mg^{2+}-ATPase in this fraction (Table I). Fraction II was devoid of ATPase activity and showed on sodium dodecylsulfate gel electrophoresis only the doublet (Fig. 1). Fractions III and IV were devoid of ATPase activity and showed mainly fast-moving bands on sodium dodecylsulfate gel electrophoresis (Fig. 1).

Electron microscopy

In the presence of 0.05 M $CaCl_2$, the crude extract (Fig. 3) as well as fraction I formed fibrillar structures. No fibrillar structures were found in fractions III and IV. Electron micrographs of fraction II were inconclusive.

Phosphorylation experiments

The crude extract as well as fractions I and II were incubated with radioactive ATP and Mg^{2+} under the conditions described in Methods. A radioactive peak was observed in the crude extract and in fraction I but not in fraction II. Mobilities of the radioactive peak were compared with those of the stained 230 000 and 210 000 subunit molecular weight proteins in 13 different experiments. The mean of the differences in mobility between the radioactive peak and the 210 000 subunit molecular weight band is 0.00115 ± 0.005 (mean $\pm$ standard error of differences) and the

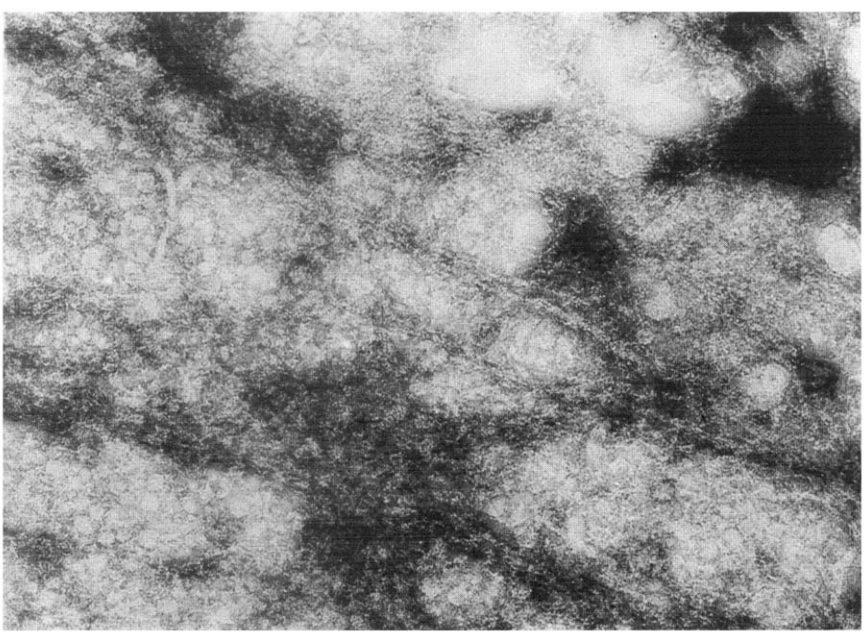

Fig. 3. Electron micrograph. Fibrillar structures from erythrocyte membrane crude extract (magnification $\times$ 80 000).

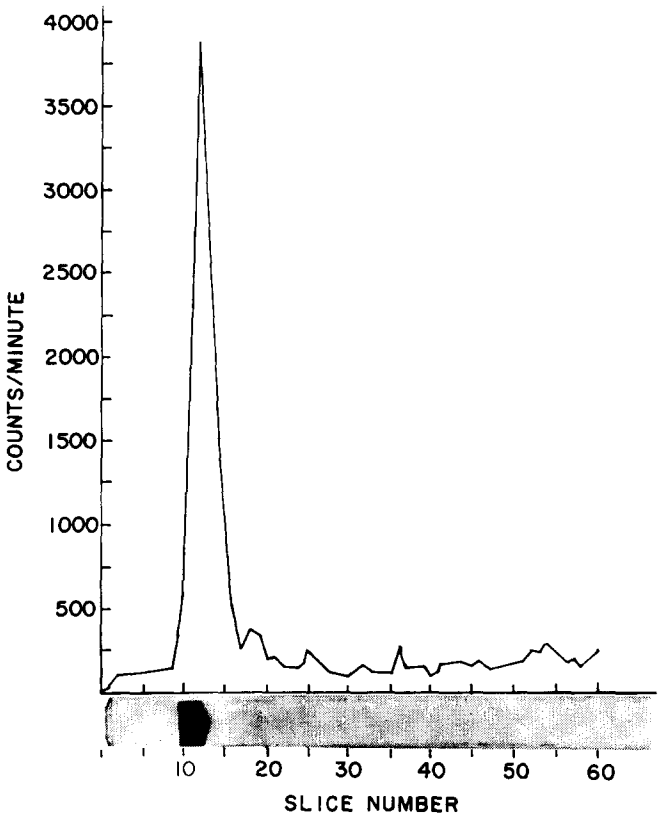

Fig. 4. Phosphorylation of fraction I. Sodium dodecylsulfate gel electrophoresis of phosphorylated fraction I in the presence of Mg^{2+}, fractionated on a Savant auto-gel divider. Control gel was stained with coumassie blue. Conditions as described under Methods.

95 % confidence limit for the mean of the difference in mobility is −0.00975 and + 0.01205. For the 230 000 subunit molecular weight band, the mean of the differences in mobility is 0.0342 ±0.00438 and the 95 % confidence limit for the mean of the difference in mobility is +0.0247 and +0.0437. Thus, the radioactive peak is consistent with the 210 000 subunit molecular weight band and differs significantly ($P < 0.0001$) from the 230 000 subunit molecular weight band. A representative experiment is illustrated in Fig. 4. Labeling was not observed in the absence of Mg^{2+} or when Mg^{2+} was substituted by Ca^{2+}. 1 μM cyclic AMP added to the reaction mixture did not affect labeling either of fraction I or fraction II.

DISCUSSION

A variety of methods have been used in the search for muscle-like proteins in human and animal erythrocyte membranes [20–28]. However, thus far, in none of the reported studies on erythrocytes were the criteria sufficient for the definition of a muscle-like contractile system [28]. High ionic strength extraction from erythrocyte membranes resulted either in ATPase activity of the same order of magnitude as

that of skeletal muscle myosin [20, 21] or in a much lower one [22]. Solubility properties and ion requirements for ATPase activities differed in each study. Procedures of low ionic strength extraction from erythrocyte membranes resulted in preparations in which ATPase activity was not always observed. Ouabain-insensitive ATPase with different ion requirements has been found in crude low ionic strength erythrocyte extracts [23–25]. No mention was made of ATPase activity in fractions resulting from gel filtration of such extracts [11, 25–27]. In one study [27], the actomyosin nature of a gel filtered fraction obtained from a low ionic strength extract of human erythrocyte membrane was suggested mainly on the basis of amino acid analysis and sodium dodecylsulfate gel electrophoresis pattern. In the present study on human erythrocyte membrane, using a sensitive method for the estimation of inorganic phosphate, we demonstrated Ca^{2+}-ATPase activity in a crude low ionic strength extract. The Ca^{2+}-ATPase activity was of the same order of magnitude as that found by Rosenthal et al. [23] in crude human erythrocyte membrane extract, but much lower than that of either human platelet [4] or rabbit smooth and skeletal muscle myosin [29].

The specific activation by rabbit muscle actin of the Mg^{2+}-ATPase in human erythrocyte membrane extract, observed in the present study, may indicate the presence of a myosin-like system. The 46 000 subunit molecular weight band found in the sodium dodecylsulfate gel pattern of the crude erythrocyte extract, which is identical to the subunit molecular weight of muscle actin, suggests the presence of actin in the erythrocyte. Noteworthy in this respect is the isolation by Shainoff [30] of an actinopeptide from thrombin-treated human erythrocyte ghosts. The low, 2- to 3-fold, activation of the erythrocyte Mg^{2+}-ATPase by rabbit actin is similar to the activation of platelet and polymorphonuclear leucocyte myosin by rabbit muscle actin [4, 5].

Among the 4 fractions obtained by Sepharose 4B gel filtration of the crude human erythrocyte extract, only the first eluted, fraction I, containing 230 000, 210 000, 80 000, 60 000 and 46 000 subunit molecular weight proteins, exhibited Ca^{2+}-ATPase activity. The absence of Mg^{2+}-ATPase activity in fraction I, also in the presence of rabbit actin, could be due to the absence in this fraction of a cofactor protein which was present in the crude extract. Such a cofactor, in analogy to the amoeba contractile system [2], might be required for actin-activation of erythrocyte Mg^{2+}-ATPase activity. Noteworthy is the observation that fraction II, showing on polyacrylamide gel electrophoresis only the pure doublet (230 000–210 000 subunit molecular weight) was devoid of both Mg^{2+}- and Ca^{2+}-ATPase activities. It may be argued, therefore, that one of the three faster moving bands in fraction I is a protein cofactor required for the Ca^{2+}-ATPase activity of this fraction, or else that none of the doublet-bands in the crude erythrocyte extract and in fractions I and II is myosin-equivalent.

Electron microscopy of the crude low ionic strength erythrocyte extract and of fraction I in the presence of Ca^{2+} showed fibrillar structures similar to those found by Rosenthal et al. [23] and by Marchesi et al. [11]. Whether these structures are related to erythrocyte actin or myosin is not known. No myosin-like thick filaments could be identified.

It has recently been shown that a myosin light chain from muscle [31] and platelets [32] is phosphorylated in the presence of a protein kinase. Unlike these systems, in our erythrocyte membrane preparations phosphorylation took place in the "heavy" 210 000 subunit molecular weight protein from the crude extract

and fraction I but not from fraction II. This may be taken to indicate that protein kinase is present in the low ionic strength crude extract and fraction I, which were also found to be ATPase active. An influence of the ATPase activity upon the phosphorylation or vice-versa might be considered. Cyclic AMP seems to saturate the kinase, since exogenous addition of the nucleotide did not increase phosphorylation. The same subunit molecular weight protein is known to be phosphorylated in the whole erythrocyte membrane [18, 33]. The occurrence of phosphorylation of the 210000 subunit molecular weight protein in the erythrocyte extract and fraction I indicates that the phosphorylation does not require a special organization of this protein in the erythrocyte membrane.

Since actin is known to activate myosin ATPase specifically, the present observations seem to leave little doubt as to the existence of a myosin-like protein in the erythrocyte. More work is needed to identify the myosin-like component present in low ionic strength extract of the human erythrocyte membrane.

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