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EXTRACTION AND LOCALIZATION OF A $(Ca^{2+}+Mg^{2+})$-STIMULATED ATPase IN HUMAN ERYTHROCYTE SPECTRIN

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SUMMARY

(1) A water soluble $(Ca^{2+}+Mg^{2+})$-activated ATPase has been extracted with 0.1 mM EDTA and 0.1 mM ATP from human erythrocyte membranes.

(2) The specific activity of the extracted protein is increased 4- to 6-fold in comparison with untreated ghosts.

(3) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [γ-^{32}P] ATP-labeled erythrocyte membranes shows that the $(Ca^{2+}+Mg^{2+})$-activated ATPase is located in the "spectrin" region (M_r 220 000–240 000). The radioactivity of these high molecular peptide bands is decreased markedly after the extraction of this ATPase at low ionic strength.

INTRODUCTION

In 1961, Dunham and Glynn [1] observed an ATPase activity in human red cell membranes which is stimulated by low concentrations of Ca^{2+} and Mg^{2+}. Evidence for a vectorially active transport of Ca^{2+} from the inside of the erythrocyte to the surrounding medium was given by Schatzmann and Vincenzi [2] and Weiner and Lee [3]. Since then many efforts have been made to identify and to isolate this enzyme [4, 5].

In the present communication, we describe the radioactive labelling of human erythrocyte ghosts with [γ-^{32}P]ATP in the presence of Ca^{2+} and Mg^{2+}. The electrophoretic peptide pattern of sodium dodecyl sulfate-lysed ghosts shows that ^{32}P is found in the highest molecular weight bands (Fig. 2). This protein fraction can be identified with the "spectrin" component studied by Marchesi and others [6, 7] and comprises 30–40 % of the total membrane protein [8, 9]. It is possible to extract these high molecular spectrin-peptides at low ionic strength [6, 11].

In our experiment, we removed most of the spectrin from human erythrocyte ghosts in the presence of 0.1 mM EDTA and 0.1 mM Mg^{2+}-ATP. The determination

Abbreviations: EGTA, Ethyleneglycol-bis(β-aminoethylether)-*N*,*N*'-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid.

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of the $(Ca^{2+}+Mg^{2+})$-dependent ATPase activity before and after this extraction showed that more than 70 % of the activity is lost from the membrane during the applied procedure. The "extracted" ATPase activity proved to be related to the spectrin in the supernatant, because its specific activity increased 4- to 6-fold in comparison with untreated ghosts.

The ATPase described in our paper is not indentical with the so-called "spectrin-fractions", but this enzyme seems to be associated with the spectrin peptides.

METHODS

Erythrocyte ghosts were prepared from freshly drawn human blood (O/Rh^+). We followed the method of Steck et al. [10], but utilized N-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulfonic acid (HEPES) buffer instead of sodium phosphate buffer. "Spectrin" was extracted by incubating 1 ml of packed red blood cell membranes with 10 ml of a medium containing 0.1 mM EDTA and 0.1 mM Mg^{2+}-ATP, titrated to pH 8 by KOH. After 1 h incubation at 30 °C, the ghosts were centrifuged for 1 h at 200 000 $\times$ *g*. One part of the pellet and the supernatant containing "spectrin I" were removed. "Spectrin II" was extracted from the other part of the pellet by repeating the above-mentioned incubation procedure. Spectrin I and spectrin II pellets were kept at 4 °C and investigated separately.

Protein determinations were done fluorimetrically as described by Fairbanks et al. [11], using a Perkin-Elmer spectrofluorimeter MPF-3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in a Shandon electrophoresis apparatus at 20 °C. We applied the general procedures in ref. 11, but routinely utilized 3 mm diameter gels at 5 mA/gel. Acrylamide concentration was 6 %, crosslinked with 0.16 % *N*, *N'*-methylene-bisacrylamide. Prior to electrophoresis, the protein samples were lysed in 4 % sodium dodecyl sulfate, reduced with β-mercaptoethanol (1 %) and made 10 % in sucrose. Pyronin Y was added as tracker dye. After electrophoretic separation of the applied material, the gels were stained with Coomassie Blue as described by Fairbanks et al. [11].

ATPase assay system I

ATPase activities were measured at 30 °C by coupling the production of ADP to NADH oxidation with lactate dehydrogenase and pyruvate kinase. ATP hydrolysis was followed by the decrease in absorption at 366 nm, in an Eppendorf spectrophotometer.

The assay volume was 1 ml, containing 0.6 ml of 30 mM HEPES, pH 7.0, 200 mM KCl, 10 mM $MgCl_2$, 0.3 mM NADH, 0.8 mM phosphoenolpyruvate, 7.5 units pyruvate kinase, 18 units lactate dehydrogenase, 0.1 mM $CaCl_2$ and 0.4 ml distilled water including the protein sample. The reaction was started by adding 10 μl of a 1 M ATP solution, pH 7, to the reaction mixture. Control experiments were done in the same buffer containing 0.5 mM EGTA instead of 0.1 mM $CaCl_2$.

ATPase assay system II

To study the Mg^{2+}-dependence of this ATPase, one cannot use Assay system I, because pyruvate kinase requires Mg^{2+}. Therefore, we incubated ghosts and spectrin II in 15 mM HEPES, pH 7, containing 5 mM $MgCl_2$ or 0.1 mM $CaCl_2$ or

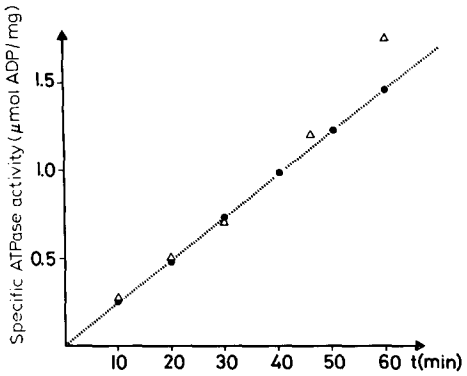

Fig. 1. Specific ATPase activity of human erythrocyte membranes measured with assay system I (●) and assay-system II (△). For details, see Methods. Protein concentration was 50 μg/ml.

both or 1 mM EGTA at 35 °C. After different times, the samples were boiled for 10 min to stop the enzyme activity. The denatured protein was removed by centrifugation and the generated ADP in the supernatant was determined according to Bergmeyer [22]. Within a reaction time of 30 min, the values for the generated ADP obtained with method II are identical with those from ATP assay system I which is based on the oxidation of NADH (Fig. 1). This proves that only the ATPase activity is rate limiting in our test system, using pyruvate kinase and lactate dehydrogenase to oxidize NADH. The pyruvate kinase activity is not inhibited by the Ca^{2+} concentrations used in our experiments.

To determine the Ca^{2+} concentration ($K_{0.5}$) which gives the half maximal reaction rate of the isolated spectrin I fraction, we incubated the extracted material in the presence of different Ca^{2+} concentrations ranging from $0.5 \cdot 10^{-6}$ M to $2 \cdot 10^{-5}$ M. The Mg^{2+} concentration was kept constant at 5 mM (Fig. 3). The ATPase activity at each Ca^{2+} concentration was measured with ATPase assay system I. The protein concentration was 90 μg/ml sample volume.

For our autoradiograms, we prepared ghosts and "ghosts-spectrin II" in the absence of ATP. These samples were incubated 15 min at 30 °C in 1 ml of 5 mM HEPES, pH 7.5, 1.67 μM [γ-^{32}P]ATP (ammonium salt) (15 Ci/mmol), 10 μM $MgCl_2$, 0.5 mM $CaCl_2$ and 50 mM choline chloride. Control experiments were performed in a medium without Mg^{2+} or without Ca^{2+} or containing 1 mM EDTA instead of $CaCl_2$.

After the incubation, the membranes were washed in 5 mM Tris · HCl, pH 7.5, and centrifuged for 1 h at 200 000 × g. Subsequently we added sodium dodecyl sulfate and β-mercaptoethanol to the pellets and electrophoresed these samples as described above. The gels were sliced longitudinally and autoradiographed on Kodirex films (Kodak). After development, the autoradiograms were scanned with a densitometer to determine the area under the radioactive peak.

RESULTS

After the incubation of erythrocyte ghosts in the above-mentioned medium,

TABLE I

Values in this table were obtained with ATPase assay system I (see Methods). Mean values are taken from 8 identically performed experiments.

	Specific ATPase activity (μmol P_i/mg per h)	Total activity (μmol P_i/h)	Protein concentration (mg)	Purification	Yield (%)
Ghosts	1.59	7.9	5.0	1.0	100
Ghosts-spectrin I	0.95	3.4	3.6		
Ghosts-spectrin II	0.46	1.5	3.3		
Spectrin I	1.38	1.6	1.2	0.9	20
Spectrin II	7.31*	0.7	0.1	4.6	9

* Highest value, 12 μmol P_i/mg per h.

about 20–25 % of the total protein is found in the supernatant [11]. Concomitantly, the specific $(Ca^{2+}+Mg^{2+})$-ATPase activity of the ghosts is decreased to 60 % of the initial value (Table I). Sodium dodecyl sulfate gel electrophoresis (Fig. 2) shows

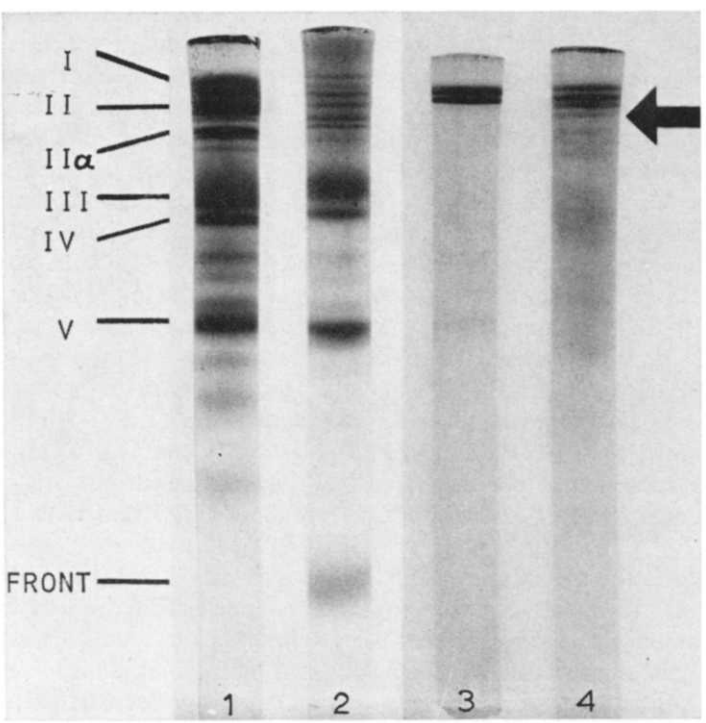

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of (1) normal ghosts (20 μg protein loaded), (2) "ghosts-spectrin II" (25 μg protein loaded) identical with "ghosts-spectrin I", (3) "spectrin I" (3 μg applied), (4) "spectrin II" (3 μg applied). Separated peptide bands are stained with Coomassie Blue. Migration is from top to bottom. The "spectrin" region comprises peptide components I, II and IIa. Other details are given in text.

that the extracted protein, which we call "spectrin I", is derived from the high molecular spectrin region of the ghosts. It consists of two broad peptide bands with a specific activity of 86 % of the untreated ghosts. The second extraction liberates only 3 % of the ghost protein, but reduces the membrane-bound ATPase activity to 29 %. The soluble proteins in the supernatant, called "spectrin II", are electrophoretically separated into several fine bands, some of which are identical with the "spectrin I" peptides, but in addition one can find very weak bands of lower molecular weight (Fig. 2, indicated by an arrow). It is interesting that this "spectrin II" fraction containing only 3 % of the ghosts protein exhibits the highest specific ($Ca^{2+}+Mg^{2+}$)-ATPase activity, namely 400–600 % compared with the intact ghosts. Comparison of the absolute activity values given in Table I excludes a possible activation of the solubilized ATPase.

Without the addition of 0.1 mM Mg^{2+}-ATP during the extraction procedures, the ATPase activity is unstable [4]. In general, only 50 % of the initial activity could be recovered after one day.

Mg^{2+} plays an important role in the activation of the soluble ATPase which is extracted with the "spectrin II" fraction. The ATPase activity with 0.1 mM $CaCl_2$ or 5 mM $MgCl_2$ alone amounted to only 0.7 and 1.2 μmol ADP/mg per h, respectively. In the presence of both ions (0.1 mM $CaCl_2$ plus 5mM $MgCl_2$) the activity increased to 3.7–4.2 μmol ADP/mg per h. These values were taken from three identically performed experiments.

We determined the $K_{0.5}$ for Ca^{2+} of the solubilized ATPase which is associated with the "spectrin I" fraction by plotting ATPase activity vs Ca^{2+} concentration (Fig. 3). Half saturation is obtained at $0.9 \cdot 10^{-6}$ M Ca^{2+}. For intact ghosts, we obtained a $K_{0.5}$ for Ca^{2+} of $2 \cdot 10^{-6}$ M. In contrast to the solubilized enzyme, the membrane-bound ($Ca^{2+}+Mg^{2+}$)-activated ATPase is inhibited at Ca^{2+} concentrations higher than 50 μM. In addition, the erythrocyte membrane binds calcium at different sites which are not related to the ATPase activity and which interfere with the determination of the $K_{0.5}$ at Ca^{2+} concentrations below 1.0 μM. Therefore, the

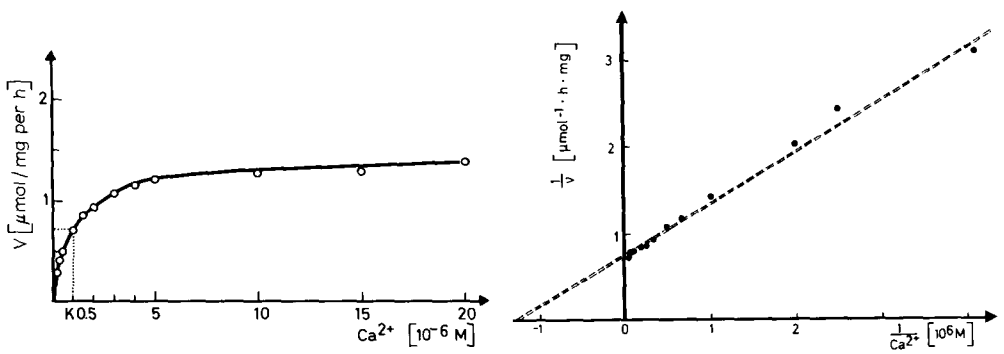

Fig. 3. (A) Ca^{2+} saturation curve of the isolated "spectrin I" fraction. Half maximal reaction rate is obtained at $0.9 \cdot 10^{-6}$ M Ca^{2+} ($K_{0.5}$). The specific ATPase activities were measured with ATPase assay system I. Mean values were taken from six identically performed experiments. (B) Plot of $1/Ca^{2+}$ concentration vs 1/I.U. per mg. The value for K_s is $0.8 \cdot 10^{-6}$ M. Experimental conditions are as in Fig. 3A. The line was fitted by the least squares method.

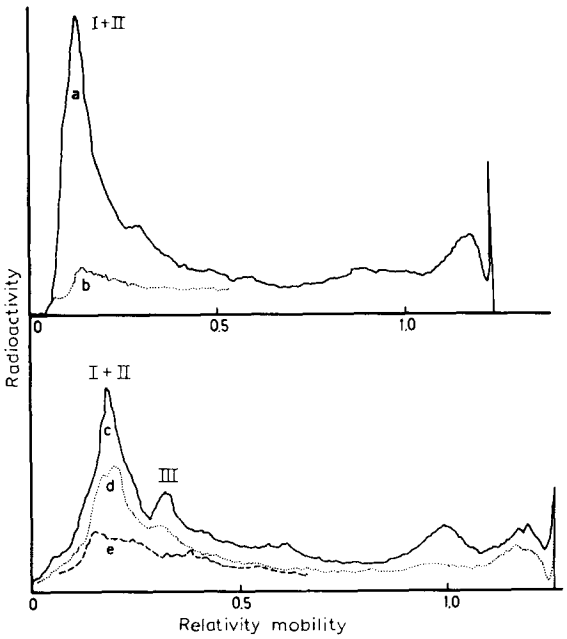

Fig. 4. Densitometric scans of the autoradiographic negatives exposed to gels which have been sliced longitudinally after the electrophoretic separation of (a) ghosts incubated with [γ-^{32}P]ATP and Ca^{2+} and Mg^{2+}. The area of the radioactive peptides I+II is taken as 100 %. (b) Ghosts incubated with [γ-^{32}P]ATP and EDTA in the absence of Ca^{2+} and Mg^{2+}. The area of the radioactive peaks I+II is 10.5 %. (c) Ghosts incubated with [γ-^{32}P]ATP and Mg^{2+}. The area of the radioactive peaks I+II is 45 %. (d) Ghosts incubated with [γ-^{32}P]ATP and Ca^{2+}. The area of the radioactive peaks I+II is 32 %. (e) Ghosts after extraction of "spectrin II", incubated with [γ-^{32}P]ATP and Ca^{2+} and Mg^{2+}. The area of the radioactive peaks of I+II is 20 %.

given $K_{0.5}$ for intact ghosts is valid only for Ca^{2+} concentrations between 1.0 and 50 μM.

Fig. 4 shows the electrophoretic distribution of radioactive $^{32}P_i$ bound to erythrocyte membranes after incubation with [γ-^{32}P]ATP and Ca^{2+} and/or Mg^{2+}. In agreement with Knauf et al. [18], we could demonstrate that almost all of the radioactivity is found in the high molecular weight peptides of the erythrocyte ghost. The relative mobility of this radioactive peak can be related to peptide band I+II of the spectrin region (Fig. 2). In the absence of Ca^{2+} and Mg^{2+} (replaced by EDTA), the radioactivity in this region amounts to only 10 % of the value found with Ca^{2+} and Mg^{2+}. With Mg^{2+} alone, in addition to peptide band I+II another radioactive peak appears, which shows the same relative mobility as band III in Fig. 2. Ca^{2+} alone reduces the radioactivity of the labelled bands to 32 % of the value found with Ca^{2+} and Mg^{2+}. In addition we found that, after the second spectrin extraction, the radioactivity of the ghosts decreased in these high molecular weight peptides by more than 75 %. The radioactive peak at the front could be due to labelled lipids and unbound $^{32}PO_4$ [20].

DISCUSSION

Our autoradiograms are in agreement with the findings of Williams [12]. He describes that two proteins of bovine erythrocyte ghosts have been phosphorylated with [γ-^{32}P]ATP and have been isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. One protein (M_r 98 000) has been identified as the intermediate of the ($Na^+ + K^+$)-activated ATPase. The other phosphorylated protein (M_r 220 000) was characterized as "apparently unrelated to the ($Na^+ + K^+$)-ATPase" and we assume that this protein is identical with our ($Ca^{2+} + Mg^{2+}$)-ATPase. In addition, Duffy et al. [13] demonstrated that strongly bound ^{45}Ca was confined to two high molecular weight proteins, located in the spectrin region, but no relation to a possible ATPase activity was investigated.

Bramley et al. [19] prepared human erythrocyte ghosts in media of different osmolarities and found a loss of ($Ca^{2+} + Mg^{2+}$)-ATPase activity when ghosts prepared at 80 imosM were resuspended in 0–10 imosM buffers. As no activity could be detected in the sediment or supernatant after centrifugation, these workers explained their finding by inactivation rather than solubilization.

The high specific activity of our "spectrin II" fraction, which comprises less than 10 % of the total spectrin protein, leads to the assumption that only a few bands, or even one single band, located in this region, can be identified with the ($Ca^{2+} + Mg^{2+}$)-dependent ATPase and therefore the actual specific activity is probably much higher. This assumption is supported by the results of several authors, showing that the spectrin peptides are heterogeneous and not in a monomeric state [15–17].

Our experiments with [γ-^{32}P]ATP clearly demonstrate that this membrane-bound ATPase is stimulated by Ca^{2+} and Mg^{2+} and that it is located in the spectrin region, because the relative migration of the main peaks in our autoradiograms are identical with bands I, II and IIa shown in Fig. 2.

Phosphorylation of erythrocyte protein by an endogenous protein kinase is described by Roses and Appel [21], who found that transfer of phosphate from [γ-^{32}P]ATP is stimulated by cyclic AMP and inhibited by calcium. Most of the erythrocyte peptides are phosphorylated under their conditions, which are clearly different from those existing in our system.

A Ca^{2+}-dependent, Mg^{2+}-inhibited ATPase, which is associated with a group of erythrocyte membrane proteins that from fibrils and can be solubilized at low ionic strength, is reported by Rosenthal et al. [5]. The specific activity of their enzyme is more than 100 times lower than the activity of the ($Ca^{2+} + Mg^{2+}$)-activated ATPase reported in this paper. It is tempting to speculate that these myosin-like proteins are involved in a contractile system which controls the mechanical characteristics of the erythrocyte membrane, as myosin-correlated ATPase activity is stimulated by Ca^{2+} and inhibited by Mg^{2+}. In contrast to this, the ATPase discussed in this paper is activated by Mg^{2+} and Ca^{2+}. Therefore our enzyme could be identical with the ($Ca^{2+} + Mg^{2+}$)-dependent ATPase which is involved in the outward directed Ca^{2+} transport of the human erythrocyte [1,2].

The $K_{0.5}$ of Ca^{2+} is $0.9 \cdot 10^{-6}$ M for the solubilized ATPase and $2 \cdot 10^{-6}$M for the intact erythrocyte membrane. The total intracellular Ca^{2+} concentration of human erythrocytes is about $4 \cdot 10^{-5}$ M [2], but as the free intracellular Ca^{2+}

concentration is suggested to be much lower (in the range of 10^{-7} M) the (Ca^{2+} + Mg^{2+})-activated ATPase is probably not saturated under these conditions.

Our (Ca^{2+} + Mg^{2+})-stimulated ATPase from human erythrocyte ghosts shows the great advantage of being extractable without the use of detergents like deoxycholate. Thus, avoiding perturbation of lipid-lipid interactions due to residual detergent molecules, it should be possible to build this protein into liposomes or black films and to study the role of this soluble ATPase in the Mg^{2+}-activated Ca^{2+} transport of the erythrocyte membrane. This matter is under investigation.

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