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SOME PROPERTIES OF THE PURIFIED (Ca^{2+} + Mg^{2+})-ATPase FROM HUMAN RED CELL MEMBRANES

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Summary

The (Ca^{2+} + Mg^{2+})-ATPase from red cell membranes, purified by means of a calmodulin-containing affinity column according to the method of Gietzen et al. (Gietzen, K., Tejčka, M. and Wolf, H.U. (1980) Biochem. J. 189, 81—88) with either phosphatidylcholine or phosphatidylserine as phospholipid is characterized. The phosphatidylcholine preparation can be activated by calmodulin, while the phosphatidylserine preparation is fully activated without calmodulin. The enzyme shows a biphasic ATP dependence with two K_m values of 3.5 and 120 μM. The enzyme is phosphorylated by ATP in the presence of Ca^{2+} only.

The membranes of erythrocytes contain an ATP-fueled outwardly directed Ca^{2+} pump which keeps the intracellular Ca^{2+} concentration as low as 10^{-7} M [1—3]. In disrupted red cell membranes its activity is apparent as (Ca^{2+} + Mg^{2+})-stimulated ATPase. It can be stimulated within the membrane by the activator protein calmodulin [4—7]. The strong interaction between calmodulin and the Ca^{2+}-transporting protein mediated by Ca^{2+} has been used to isolate the enzyme by means of a calmodulin-containing affinity column [8,9]. The isolated protein shows (Ca^{2+} + Mg^{2+})-ATPase activity [8,9] and transports Ca^{2+} [10].

The present paper describes some properties of the isolated (Ca^{2+} + Mg^{2+})-ATPase obtained by method A of Gietzen et al. [8]. This procedure yields

Abbreviations: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetra-acetic acid; Mops, 4-morpholinepropanesulfonic acid.

a highly purified enzyme, more than 90% of the protein being made up by (Ca^{2+} + Mg^{2+})-ATPase (see Fig. 3). The protein was harvested from the column in an eluant containing 0.05% (w/v) of a phospholipid, 0.05% (w/v) Triton X-100 and 0.05% (w/v) Tween 20. In the presence of phosphatidylcholine and excess calmodulin, the specific activity of the enzyme was 10—15 μmol P_i/mg protein per min at 37°C immediately after elution from the affinity column. The enzyme is very unstable, having a half-life of about 1—2 days at 0°C. However, addition of Ca^{2+} and calmodulin to a final concentration of 50 μM free Ca^{2+} and 5 μg/ml calmodulin increases the half-life to about 1 week.

Regarding the phospholipid requirement, phosphatidylcholine and phosphatidylserine were compared. Phosphatidylcholine was product No. 5763 and phosphatidylserine product No. 6641 of Sigma Chemical Co. They were tested for purity by thin-layer chromatography on silica gel plates developed in $CHCl_3/CH_3OH/H_2O$ (14 : 6 : 1, v/v). For detection, iodine vapour, treatment with 25% $HClO_4$ or staining with bromocresol green was used. Phosphatidylcholine contained a minor non-identified contaminant and phosphatidylserine two unknown compounds in trace amounts. Free fatty acids were below the detectability of the method (4 μg), when 0.5 mg phosphatidylserine was chromatographed, i.e., less than 1% (w/w). The protein was eluted from the column with different buffers containing 0.5 mg/ml of either phosphatidylcholine or phosphatidylserine. Fig. 1A and C shows the Ca^{2+} dependence of the ATPase in the presence of phosphatidylcholine or phosphatidylserine. With phosphatidylcholine the enzyme displays high affinity (K_{Ca} = 3.3 μM) in the presence and a low affinity (K_{Ca} = 16.4 μM) in the absence of calmodulin. However, if phosphatidylcholine is replaced by phosphatidylserine the (Ca^{2+} + Mg^{2+})-ATPase has approximately the same activity as in phosphatidylcholine plus calmodulin at all Ca^{2+} concentrations and addition of calmodulin achieves no further stimulation. The effect is due to phosphatidylserine itself, since its contaminants, isolated by thin-layer chromatography, were without effect when added to the phosphatidylcholine preparation of the enzyme in amounts equal to those present in the experiments with the phosphatidylserine form of the enzyme. Furthermore, free fatty acids (palmitic acid and stearic acid) exceeding the worst possible contamination in phosphatidylserine by a factor 100 were without any effect when added to the phosphatidylcholine preparation of the enzyme. The apparent K_{Ca} value in the phosphatidylserine system is 3.7 μM which is similar to that of 3.3 μM in the phosphatidylcholine system with calmodulin. In the phosphatidylcholine system the enzyme shows the same affinity for Mg^{2+} in the presence or absence of calmodulin (K_{Mg} = 35 μM; Fig. 1B), but a higher V value with (13.7 μmol P_i/mg protein per min) than without (5.2 μmol P_i/mg protein per min) calmodulin. The phosphatidylserine enzyme (Fig. 1D) again is fully activated at all Mg^{2+} concentrations with and without calmodulin and shows a slightly higher affinity for Mg^{2+} (with a K_{Mg} of 18 μM) than the phosphatidylcholine enzyme. (The abscissa in Fig. 1 represents free Ca^{2+} and free Mg^{2+} and K values are calculated for free metal concentrations.)

Finally, Fig. 2 shows the ATP-dependent activation. The curve turns out to be biphasic as reported earlier for intact membranes [11—13]. Our experi-

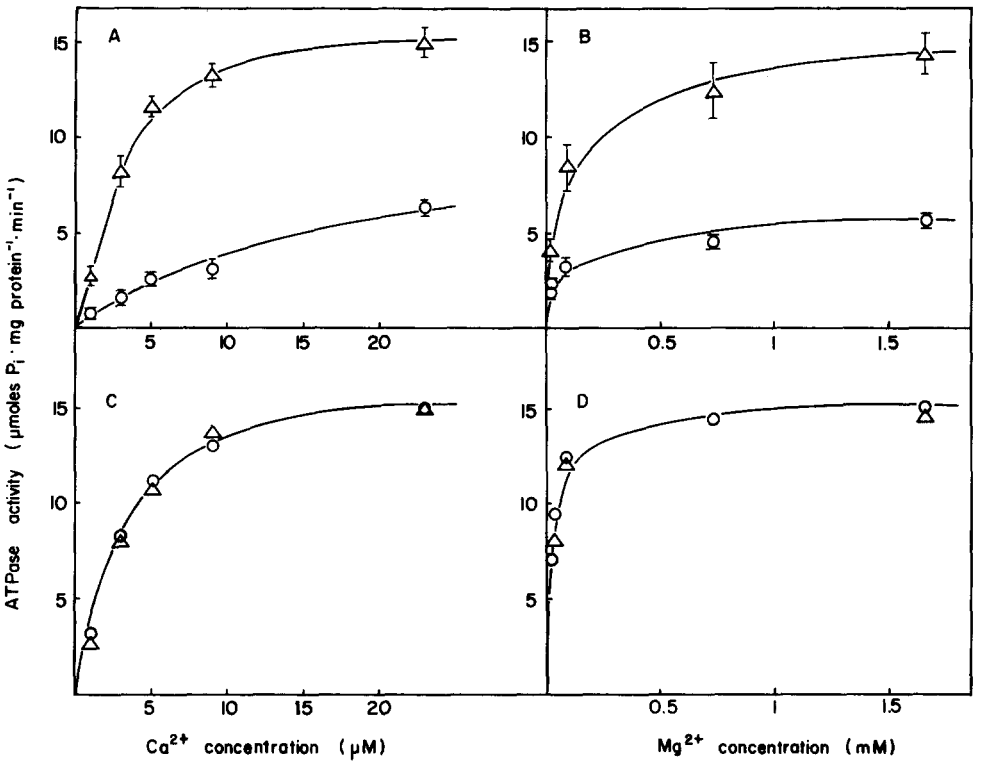

Fig. 1. Ca^{2+} dependence (A,C) and Mg^{2+} dependence (B,D) of the isolated (Ca^{2+} + Mg^{2+})-ATPase in the presence of phosphatidylcholine (A, B) or phosphatidylserine (C,D) with (△) and without (○) calmodulin. The assay medium (3 ml) contained (mM): KCl, 120; imidazole hydrochloride, 30 (pH 7.0 at 37°C); $MgCl_2$, 4 (in A and C) or variable; Tris-EGTA, 1; $CaCl_2$, 1.05 (in B and D) or variable; Na_2ATP, 2; bovine brain calmodulin, 1 μg (if present); and 0.2 ml enzyme solution (approx. 5 μg protein). Incubation was carried out for 10 min at 37°C. Inorganic phosphate was determined according to the method of Fiske and Subbarow [15]. Points: mean of four (A) or three (B) experiments ± S.E., viz., mean of two (C,D) experiments.

ments yield for the high affinity site a K_m of 3.5 μM ATP_{tot} with a maximal rate of 1.0 μmol P_i/mg protein per min and for the low-affinity site a K_m of 120 μm ATP_{tot} with V = 12.6 μmol P_i/mg protein per min. The maximal rate elicited by the high-affinity site is therefore about 8% of that corresponding to the low-affinity site.

In Fig. 3 an SDS-polyacrylamide gel pattern (A) of the purified enzyme is shown. In addition to the main peak (130 000 daltons) there is a minor peak of approx. 90 000 daltons belonging probably to band 3 protein. After exposure of the ATPase to [γ-^{32}P]ATP in the presence of Ca^{2+} (B) or Ca^{2+} and Mg^{2+} (not shown), a peak of radioactivity reflecting protein phosphorylation appears exclusively at 130 000 daltons in precise coincidence with the major protein peak. The appearance of this radioactivity peak is completely suppressed when Ca^{2+} (removed by adding 2 mM EGTA to the medium) is replaced by 2 mM Mg^{2+} (C), showing that no Mg^{2+}-dependent protein phosphorylation occurs.

These results demonstrate that the purified enzyme recovered in a phos-

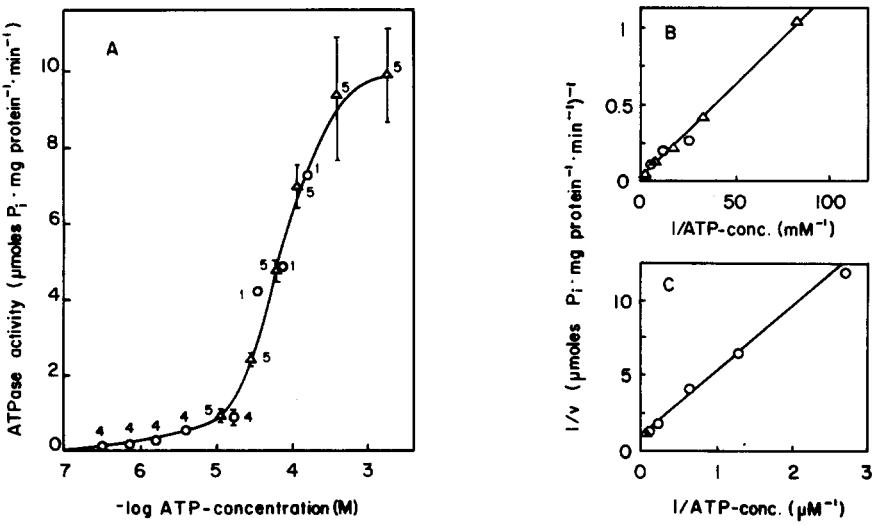

Fig. 2. (A) ATP dependence of the purified (Ca^{2+} + Mg^{2+})-ATPase. The ATPase assay was carried out with the following conditions: 0.3 ml incubation medium was incubated at 37°C for different times, so that not more than 20% of the ATP was split. The medium contained (mM): KCl, 120; $MgCl_2$, 4; imidazole hydrochloride, 30 (pH 7.0 at 37°C); Tris-EGTA, 0.5; $CaCl_2$, 0.52; 5—10 μl enzyme solution (approx. 0.1—0.2 μg protein); bovine brain calmodulin, 50 ng; and Na_2ATP (plus [γ-^{32}P]ATP, $9 \cdot 10^4$ cpm) in the concentration range of 0.5—200 μM (○). The reaction was terminated by adding 0.2 ml of solution containing 4% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 4 N H_2SO_4, 10% $HClO_4$ and 0.2 mM H_3PO_4. The tubes were cooled in ice, 0.8 ml isobutanol was added and the tubes shaken for 15 s. 0.5 ml of the organic phase was counted in a Packard Tricarb 460 C scintillation counter. The enzyme assay in the ATP concentration range between 0.02 and 2 mM (△) was carried out as described in Fig. 1. Abscissa: mean of the total ATP concentration. Points: mean ± S.E. (number of experiments near points, S.E. smaller than symbol not shown). (B) Lineweaver-Burk-plot of the values from (A) in the ATP-range of 12—1900 μM ATP and (C) in the range of 0.37—18 μM.

phatidylcholine medium behaves in a manner similar to that of the enzyme in a membrane preparation. Phosphorylation of the purified enzyme in the presence of Ca^{2+} and Mg^{2+} was shown before by Niggli et al. [9] and Carafoli et al. [10] but the action of Ca^{2+} alone was not tested. In their preparation, an additional minor radioactivity peak appeared at 205 000 daltons which was interpreted as being possibly a dimer of the (Ca^{2+} + Mg^{2+})-ATPase. The present experiment demonstrates that the protein is phosphorylated by ATP in the presence of Ca^{2+} in the absence of Mg^{2+}, which is in agreement with the result obtained with whole membranes [18]. The isolated enzyme shows affinities for Ca^{2+}, Mg^{2+} and ATP similar to those found in whole membranes. It is sensitive to calmodulin and displays a biphasic activation pattern for the substrate ATP. Furthermore, three tested inhibitors (vanadate, *N*-ethylmaleimide and *p*-nitrophenyl phosphate) of the (Ca^{2+} + Mg^{2+})-ATPase show the same affinities as in membranes prepared according to the method of Wolf [14] (not shown). All these similarities prove that the isolation procedure used by Gietzen et al. [8] yields a functional (Ca^{2+} + Mg^{2+})-ATPase.

When the purification was carried out in a medium containing phosphatidylserine instead of phosphatidylcholine, the calmodulin requirement was dispensed with, yet the affinity for Ca^{2+} was precisely the same and the affinity

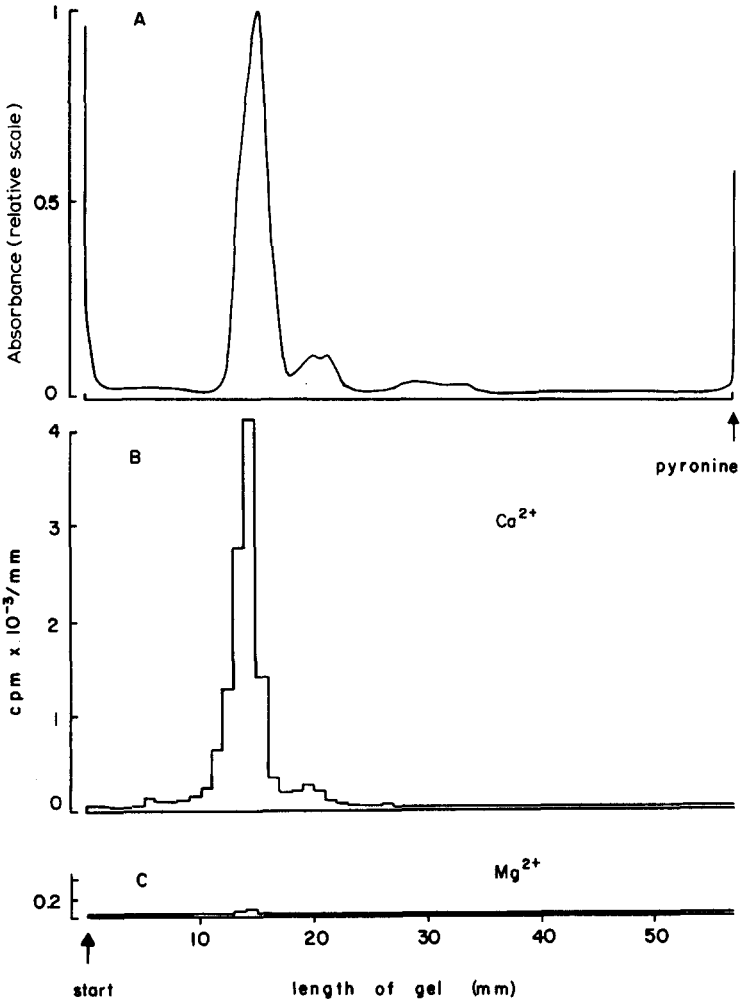

Fig. 3. (A) SDS-polyacrylamide gel pattern of the purified (Ca^{2+} + Mg^{2+})-ATPase stained with Coomassie brillant blue R 250 (for method see below). (B,C) Phosphorylated intermediate of the enzyme after incubation with [γ-^{32}P]ATP for 30 s at 0—2°C. The phosphorylation was carried out in 0.5 ml of a medium containing (mM): KCl, 120; Mops, 30 (pH 7.0 at 0°C); bovine brain calmodulin, 1 μg; 0.2 ml enzyme solution (approx. 5 μg protein); and (B) $CaCl_2$, 0.62; Tris-EGTA, 0.57; or (C) $MgCl_2$, 2; and Tris-EGTA, 2. The reaction was started by adding 50 μl of 5 μM [γ-^{32}P]ATP and stopped after 30 s by adding 2 ml of cold 6% trichloroacetic acid, 50 mM H_3PO_4 and 0.5 mM Na_2ATP. Finally, 200 μg bovine serum albumin were added as carrier. The precipitate was washed four times with 3 ml of the stopping solution. All procedures were carried out at 0—2°C. SDS-polyacrylamide gel electrophoresis was carried out on 6% polyacrylamide gels according to the method of Fairbanks et al. [16] with 10—15 μg enzyme protein at 15°C. Gels were cut in 1 mm slices, digested at 60°C in 0.2 ml of 60% $HClO_4$ + 0.4 ml of 30% H_2SO_4 in plastic vials and counted in a Packard Tricarb 460 C scintillation counter.

for Mg^{2+} nearly the same as in a phosphatidylcholine medium plus calmodulin. Abolition of the calmodulin requirement after isolation in phosphatidylserine was also observed by Carafoli et al. [10]. With regard to Ca^{2+}, Niggli et al. [17] recently arrived at the same result for the purified protein reconstituted in phosphatidylcholine or phosphatidylserine liposomes (i.e., after removal of

detergents): In phosphatidylserine without calmodulin, the Ca^{2+} dependence of the ATPase activation was the same as in phosphatidylcholine with calmodulin. Thus, phosphatidylserine imitates the effect of calmodulin on the isolated (Ca^{2+} + Mg^{2+})-ATPase. Since the enzyme in situ is calmodulin sensitive, this means that phosphatidylserine is not the prevalent phospholipid in the membrane near the enzyme, provided that the present finding reflects the natural situation.

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