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Purification of the Ca^{2+}-dependent ATPase of human erythrocyte membranes

In 1961, DUNHAM AND GLYNN[1] described an ATPase from human erythrocytes, which is stimulated by low Ca^{2+} concentrations. Since then, this enzyme has been the object of various investigations, in which it was referred to the active Ca^{2+} transport across the red cell membrane (SCHATZMANN[2]). In recent studies ROSENTHAL *et al.*[3] showed that fibrils existing in red cell membranes (HAGGIS[4]), exhibit a very weak Ca^{2+}-dependent ATPase activity. Thus, efforts were made to purify the Ca^{2+}-ATPase with the aim to get information about the identity of the membrane part responsible for Ca^{2+}-ATPase activity, the part which forms actin-like fibrils[3-7], and the part responsible for the contraction of erythrocyte ghosts[8].

In this paper a method is described to obtain a Ca^{2+}-ATPase preparation, which is, in comparison with earlier preparations[1,3,9,10], characterized not only by an increased specific activity but also by increased affinities to the substrate MgATP and to Ca^{2+}. This method is based on the principle that enzymes can be stabilized when catalytic and regulatory sites are protected by the respective molecules (TANFORD[11]). Regarding the sensitive Ca^{2+}-ATPase of human erythrocytes this means that (1) in all steps of the preparation the presence of optimum concentrations of divalent cations (Mg^{2+}, Ca^{2+}) was provided by a buffer system[12], (2) in all preparation steps 0.2 mM substrate (MgATP) was present and (3) the detergent desoxycholate often applied for the purification of ATPases of various sources was omitted, because it decreases the affinity of the enzyme to both substrate and Ca^{2+} (ref. 12).

Human erythrocytes (group O/Rh^+), which were in contact with ACD-AG stabilizer (sodium citrate, citric acid, glucose, adenine, guanosine) for less than 50–60 h, were thoroughly washed with 0.155 M NaCl (centrifugation for 30 min at 5000 × *g*) several times to remove leucocytes and fat. The washed erythrocytes were incubated at 4° in a medium which contained Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, HPO_4^{2-} and glucose at plasma concentrations, and 10 mM Tris–maleate, pH 7.0, for several days. 120 ml of packed erythrocytes were hemolysed at 1° in a solution containing (final concentrations) 20 mM glycine buffer, 0.2 mM MgATP, 20 mM Na^+, 10 mM K^+, 2 mM Mg^{2+}, 0.025 mM Ca^{2+} (adjusted to this value by means of a metal ion buffer, which consisted of 0.33 mM Ca^{2+}, 0.67 mM Mg^{2+} and 1 mM *trans*-1,2-cyclohexanediaminetetraacetate[12]), and 0.167 % Tween 20. The final volume was 1200 ml, the final pH 9.2. After centrifugation at 22000 × *g* for 30 min (MSE High Speed 18) the hemoglobin was removed by suspending the sediment in a solution similar to that applied for hemolysis (except that the glycine buffer concentration was lowered to 2 mM and $[Na^+]$ was increased to 40 mM; Tween 20 was omitted) followed by centrifugation for 30 min at 40000 × *g*, 4–5 times. During the washing operations two apparently different sediments appeared, called "heavy sediment" and "light sediment". They could be separated easily on account of their different sedimentation behaviour: as the "heavy sediment" formed a compact button, the fluffy "light sediment" could be removed completely by suction. The last washing was performed at pH 7.3 (2 mM Tris buffer); subsequently the sediments were suspended in a 10-fold volume of 1 M sucrose + 1 mM Mg^{2+}. They were frozen at —80° and stored at —20°.

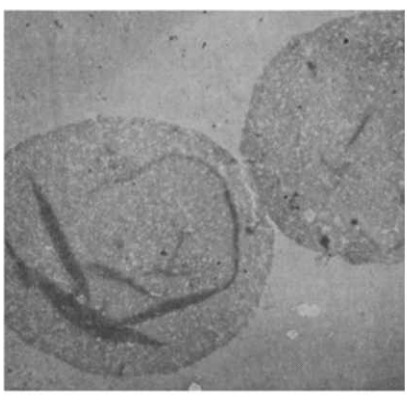
From 120 ml packed erythrocytes 2.0–2.4 ml of a faintly yellow "heavy sedi-

ment" was obtained, which showed strong green blueish fluorescence which disappeared after suspension in sucrose. The specific ATPase activity of the best preparation was 0.146 I.U./mg protein under optimum conditions (100 mM Na^+, 2 mM Mg^{2+}, 0.025 mM Ca^{2+}, 1 mM MgATP, pH 7.0) and 30°, while the activity of the "light sediment" was only about 0.02 I.U./mg protein. Under the following conditions for the "heavy sediment", alkaline phosphatase (EC 3.1.3.1) activity (10 mM glycine buffer, pH 9.5, 100 mM Na^+, 10 mM K^+, 1 mM Mg^{2+}, at 30°; substrate, 1 mM β-glycerophosphate) was not detectable and (Na^+, K^+, Mg^{2+})-ATPase activity (10 mM Tris–maleate buffer, pH 7.5, 100 mM Na^+, 10 mM K^+, 2 mM Mg^{2+}, at 30°; substrate, 1 mM MgATP) amounted to 0–5 % of the total ATPase activity.

Inorganic phosphate was determined in a Technicon Autoanalyzer by the method of FISKE AND SUBBAROW[13] as modified by LACY[14], using ascorbic acid as reducing agent. Protein determinations were carried out using a micro-Kjeldahl method after the removal of the membrane-bound phospholipids by methanol–chloroform (1:1, v/v)[15]. Ca^{2+} concentrations were adjusted by means of Ca^{2+} buffers consisting of varying concentrations of Ca^{2+} and Mg^{2+}, and EDTA or EGTA (ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid), as described elsewhere[12]. All experiments were performed in the presence of 10 mM Tris–maleate buffer, pH as indicated, and of 4 mM GSH + 0.2 mM GSSG.

Electron micrographs were made from the unfrozen "heavy sediment" after $1:10^2$ and $1:10^4$ dilutions in redistilled water. As shown in Fig. 1, the preparation consisted of ghosts, which exhibit a large number of holes, presumably as a result of extensive degradation of the membrane. In the $1:10^4$ dilution, an unexpected process was observed: the membranes decayed to form filament-shaped structures (Fig. 2). In a $1:10^5$ dilution this process of decay was completed: membrane fragments were no longer present. The reason for this decay may be the decrease of the ionic strength.

The preparation showed an ATPase activity, which was strongly stimulated by 0.1–50 μM Ca^{2+} at pH 5.8–8.4 (Fig. 3). The apparent dissociation constant of the



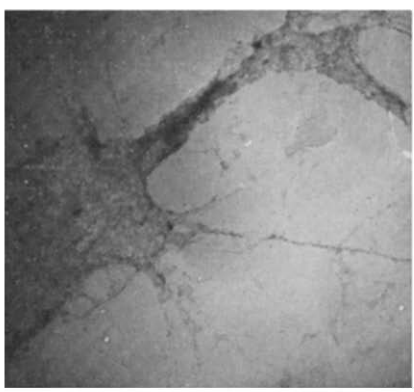
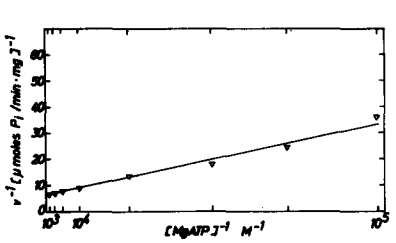


Fig. 1. Electron micrograph of the "heavy sediment" diluted $1:10^2$; unfixed preparation stained with uranyl acetate and lead citrate; magnification, × 6000. The ghosts appear well preserved.

Fig. 2. Electron micrograph of the "heavy sediment" diluted $1:10^4$; unfixed preparation stained with uranyl acetate and lead citrate; magnification, × 6000. The ghosts decay to form filament-shaped structures.

enzyme–Ca^{2+} complex was dependent on the pH value, yielding a value of 2.3 μM at pH 7.0. Optimum activity was reached at 25 μM Ca^{2+}, the activation ratio $v_{[Ca^{2+}]opt.}/v_{[Ca^{2+}]=0} = 13.5$. In order to estimate the K_m value the substrate concentration was varied from 0.01–1.0 mM MgATP at pH 7.0, while Ca^{2+} was added at



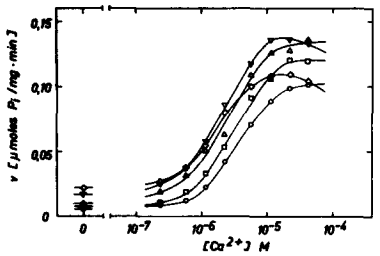


Fig. 3. Dependence of the Ca^{2+}-ATPase reaction rate on Ca^{2+} concentration at different pH values. Medium contained 100 mM Na^+, 2 mM Mg^{2+}, 1 mM MgATP. ○, pH 6.3; □, pH 6.7; △, pH 7.0; ▽, pH 7.5; ◇, pH 7.9: The Ca^{2+} concentration was adjusted by 0.4 mM Ca^{2+} buffer containing EDTA or EGTA (ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid) as chelating agent[12].

Fig. 4. $1/v - 1/[MgATP]$ plot for the estimation of K_m. Medium contained 100 mM Na^+, 2 mM Mg^{2+}, 25 μM Ca^{2+} (0.4 mM Ca^{2+} buffer[12]), pH 7.0; MgATP as indicated.

the concentration for optimum activity, *i.e.* 25 μM; under these conditions the K_m was 40 μM (Fig. 4). A second Ca^{2+}-dependent ATPase activity, different from the first with respect to kinetic constants (*e.g.* optimum pH > 8), is apparently present in the membrane preparation: this enzyme shows optimum activity at substrate concentrations of 0.04–0.1 mM at pH 8 (ref. 12).

The comparison between kinetic constants of the "heavy sediment" and those of other preparations of the present investigation, which were obtained at pH 7.5 without the addition of the nonionic detergent Tween 20 (but in the presence of Mg^{2+}, Ca^{2+} and substrate), showed that, in spite of a marked increase of the specific ATPase activity, neither the value of the enzyme–Ca^{2+} dissociation constant nor the K_m value was changed as a result of the increased pH value and the application of Tween 20 during hemolysis[12].

In contrast to this, a considerable difference can be noticed between preparations of other investigators[1,3,9,10] and the preparation described here with respect to kinetic constants and specific activity: The K_m value was as low as 40 μM (contrast ref. 1: 500 μM). Optimum activation occurred at 25 μM Ca^{2+} (contrast refs. 1 and 10: 300 μM; ref. 9: 100 μM and ref. 3: 6 mM). The activation ratio $v_{[Ca^{2+}]opt.}/v_{[Ca^{2+}]=0}$ had the high value of 13.5 (contrast ref. 10: 3 and ref. 1: 3.3). Calculated on the basis of the cell volume the increase of activity is 30–130 fold[1,10], on the basis of protein weight the increase is 280 fold[3].

Although at present there is no evidence that the membrane preparation of this investigation contains the fibrous protein which has been isolated by various investigators[3–7], this is suggested, for the fibrous protein prepared by ROSENTHAL *et al.*[3] exhibits Ca^{2+}-dependent ATPase activity also. The difference in degree of ATPase activity between the preparation of ROSENTHAL *et al.*[3] and the one described here, could be due to the use of different preparations: fibrils in the first case, entire membranes in the second, so that in the second case the membranes could contain

an ATPase not connected with fibrils. Nevertheless it should be noted that the preparation method employed here is characterized by the fact that in no step of the purification procedure the enzyme preparation was devoid of divalent cations such as Mg^{2+} and Ca^{2+} in contrast to the application of 0.5 mM Na_2EDTA[3]. The aim of this provision was to avoid "partial denaturation" of the enzyme, which possibly occurs when the binding sites for substrate and effectors are not protected by the respective molecules or ions during the course of enzyme purification and isolation[11].

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