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CHARACTERIZATION OF THE PHOSPHORYLATED INTERMEDIATE OF THE ISOLATED HIGH-AFFINITY (Ca^{2+} + Mg^{2+})-ATPase OF HUMAN ERYTHROCYTE MEMBRANES

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Summary

Phosphorylation of solubilized and purified high-affinity (Ca^{2+} + Mg^{2+})-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of human erythrocyte membranes shows no dependence on cyclic AMP concentration in the range 0.1—1000 μM.

Ca^{2+}-dependent phosphoprotein is sensitive to hydroxylamine and molybdate treatment. The phosphate linkage shows maximum stability at low pH values, which is progressively lost as the pH rises, with a shoulder around pH 6. SDS gel electrophoresis of the phosphorylated protein yields a peak which shows relative mobility corresponding to a molecular weight of 145 000 and sensitivity to MgATP-chase and hydroxylamine treatment. This indicates that the phosphoprotein represents the phosphorylated intermediate of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase of human erythrocyte membranes.

Introduction

Erythrocyte membranes contain several enzymes which are able to produce phosphorylated proteins by different mechanisms: metal ion ATPases show phosphorylated intermediates, whilst kinases are able to phosphorylate other membrane proteins [1—8].

For the distinction of these different enzyme systems it is necessary to characterize the phosphorylation reaction, the nature of the chemical linkage

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and the molecular weight of the phosphorylated proteins. Phosphorylation of the erythrocyte membrane, which is believed to be involved in the enzymatic reaction of the Ca^{2+} pump, has been reported by several authors [9—17]. The results of investigations on isolated high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase of erythrocyte membranes have been presented by us in a preceding paper [18]. Now we report experimental results which demonstrate that the Ca^{2+}-dependent phosphoprotein of solubilized and purified high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase of human erythrocyte membranes shows characteristics similar to those of the phosphorylated intermediates of other transport ATPases and which exclude the possibility that it is a product of a kinase reaction.

Materials and Methods

Chemicals. Except where noted, methods and sources of materials were as described in the preceding paper [18]. Acrylamide was obtained from Roth (Karlsruhe), ammonium persulphate, Coomassie brillant blue G 250, sodium dodecyl sulphate, 2-mercaptoethanol and *N,N'*-methylenebis-acrylamide were from Serva, Heidelberg: all other chemicals were from Merck, Darmstadt, F.R.G.

SDS gel electrophoresis. SDS gel electrophoresis at pH 7 was carried out according to the method of Weber and Osborn [19]. In order to prepare gels at pH 2.4 polymerization was performed as described before [20], but with the differences that lithium was substituted by sodium, the concentration of ascorbic acid was 0.0083%, and the SDS concentration was 0.2%. After SDS gel electrophoresis at pH 2.4 and 2°C the gels were cut below the tracking dye, cut into slices of 1 mm thickness and the radioactivity of two slices was measured in a vial after addition of 20 ml Cerenkov solution [21]. Some gels were stained according to the method of Fairbanks et al. [22].

Hydroxylamine and molybdate treatment. The trichloroacetic acid-insoluble phosphoprotein was washed at room temperature, once by Millipore filtration (d = 2.5 cm, pore size = 0.22 μm) with 10 ml H_2O (room temperature) and once with either 10 ml freshly dissolved 0.2 M NH_2OH (pH 5.5) or 0.2 M Na_2MoO_4 (pH 2). For the control, hydroxylamine was replaced by 0.2 M NaCl (pH 5.5). The precipitate on the Millipore filter was exposed to the described solutions for 10 min at 23°C. Afterwards, the solutions were removed by suction and then the precipitate was washed four times with trichloroacetic acid solution as described in the preceding paper [18].

Results and Discussion

Effect of cyclic AMP

Erythrocyte membranes contain at least two kinases which are able to phosphorylate membrane proteins in the presence of ATP. Some authors [1,3,4,6] described phosphorylation of proteins 3, 4 and 5 (nomenclature of Steck [23]) by Mg^{2+}-cyclic AMP-dependent kinase which is inhibited by Ca^{2+}. Mg^{2+}-dependent but cyclic AMP-independent kinase which phosphorylates protein 2 has been described by Guthrow et al. [1] and Avruch and Fairbanks [5]. As shown in Fig. 1, no cyclic AMP-dependent phosphorylation can be detected after

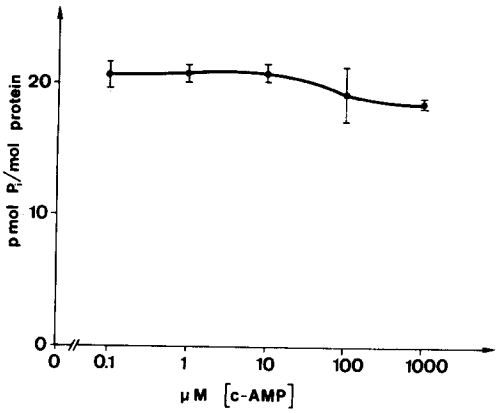

Fig. 1. Effect of cyclic AMP. Phosphorylation was carried out under standard conditions in the presence of cyclic AMP (c-AMP). The residual phosphorylation remained constant throughout at 5.0 pmol P_i/mg protein.

phosphorylation under standard conditions and in the presence of cyclic AMP in the range 0.1—1000 μM.

Effect of hydroxylamine and molybdate

Ca^{2+}-stimulated phosphorylation is sensitive to hydroxylamine and molybdate treatment. This type of sensitivity suggests that the terminal phosphate of ATP is bound by an acylphosphate linkage [24]. The phosphoenzyme level is reduced by 0.2 M hydroxylamine at pH 5.5 to approx. 20% of the control value. This value is nearly the same as that obtained with MgATP-chase. These results confirm those of others [11,14,15]. Molybdate treatment yields only 50% hydrolysis of the phosphate bound, in agreement with the results of Makinose [25] who also reported incomplete hydrolysis of the acylphosphate linkage of (Ca^{2+} + Mg^{2+})-ATPase of the vesicles of sarcoplasmic reticulum under treatment with molybdate.

Effect of pH

Recent studies of Lippmann and Tuttle [26] have shown that acylphosphate linkages can be characterized by hydroxylamine-, molybdate- and pH-sensitivity. Results shown in Fig. 2 indicate that the stability of the phosphorylated protein is maximal at low pH values, the stability being progressively reduced as the pH rises with a shoulder around pH 6. This again is consistent with an acylphosphate bond. Since the ions of the buffer system do influence hydrolysis of the phosphate bond [27], the same buffer system (citric acid/phosphoric acid/boric acid [28]) was employed covering the whole pH range tested (pH 2—11).

SDS gel electrophoresis

The molecular weight of the phosphorylated intermediate of membrane-bound high-affinity (Ca^{2+} + Mg^{2+})-ATPase of the human erythrocyte membrane has been estimated to be 145 000 by means of SDS gel electrophoresis [11,13, 17]. The conditions used (15°C; in some cases, pH 7) are not optimal for the stability of the phosphoprotein. Therefore, we developed a system where gel

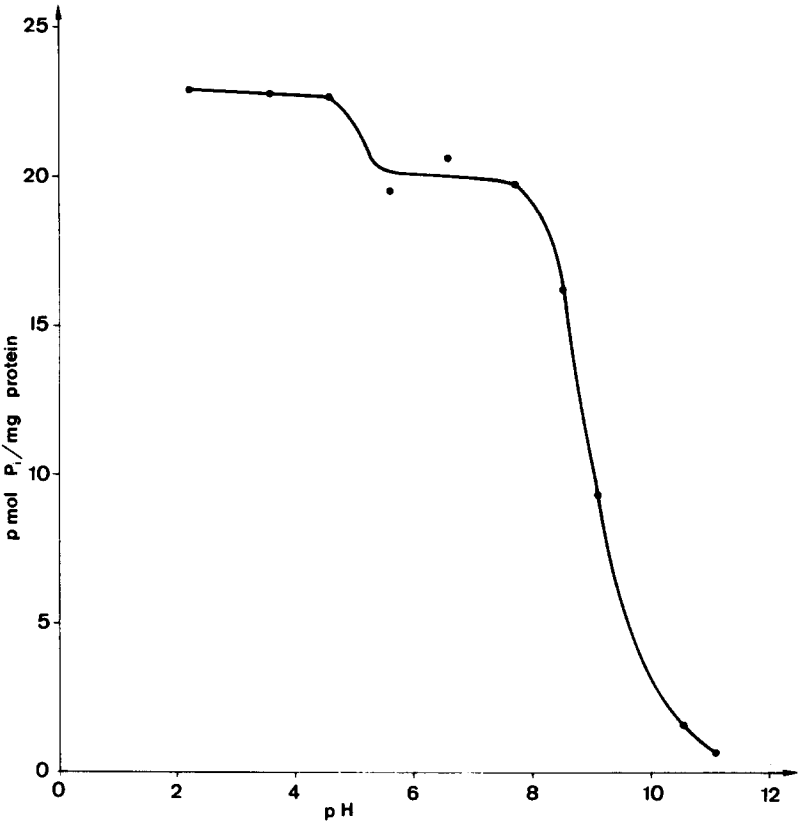

Fig. 2. Effect of pH. Phosphorylation was carried out under standard conditions. After 2 min phosphorylation, the reaction was stopped by adding 50 μl of 1 M HCl, the pH decreasing to 2. After addition of 9 ml buffer (40° C) with the desired pH (citric acid/phosphoric acid/boric acid) incubation was carried out for 2 min at 40° C. Then, 2 ml of ice-cold 50% trichloroacetic acid solution (plus 5 mM MgATP and 100 mM P_i) were added and the mixture was cooled immediately to 0° C. After addition of Ca^{2+} and Mg^{2+} (final concentrations 10 mM) Millipore filtration was performed as described in the text.

electrophoresis can be run at low temperatures and pH values [20]. Fig. 3 shows SDS gel electrophoresis of the purified enzyme at pH 2.4 (right-hand gel) and of human erythrocyte membranes at different pH values, the left-hand gel representing pH 7 and the middle gel pH 2.4. For estimation of the relative mobilities of [γ-^{32}P]ATP and $^{32}P_i$ at pH 2.4, a small quantity of [γ-^{32}P]ATP was warmed for 10 min to 40°C and then subjected to SDS gel electrophoresis (Fig. 4). The radioactivity is distributed into two peaks, the higher peak in front of the tracking dye is assumed to be [γ-^{32}P]ATP and the smaller peak behind the tracking dye seems to be $^{32}P_i$ [29]. SDS gel electrophoresis of the trichloroacetic acid-precipitated phosphoprotein (Fig. 5) yields a peak of which the relative mobility is in good agreement with that of the protein band with the highest molecular weight shown in the right-hand gel of Fig. 3. This band corresponds to the protein band of molecular weight 145 000 (α-unit [30]) on the left-hand gel run at pH 7 (Fig. 3). This confirms earlier findings of others for membrane-bound (Ca^{2+} + Mg^{2+})-ATPase [11,13—17]. The radioactivity shows rapid turnover after the addition of MgATP (Fig. 5, lowest line and

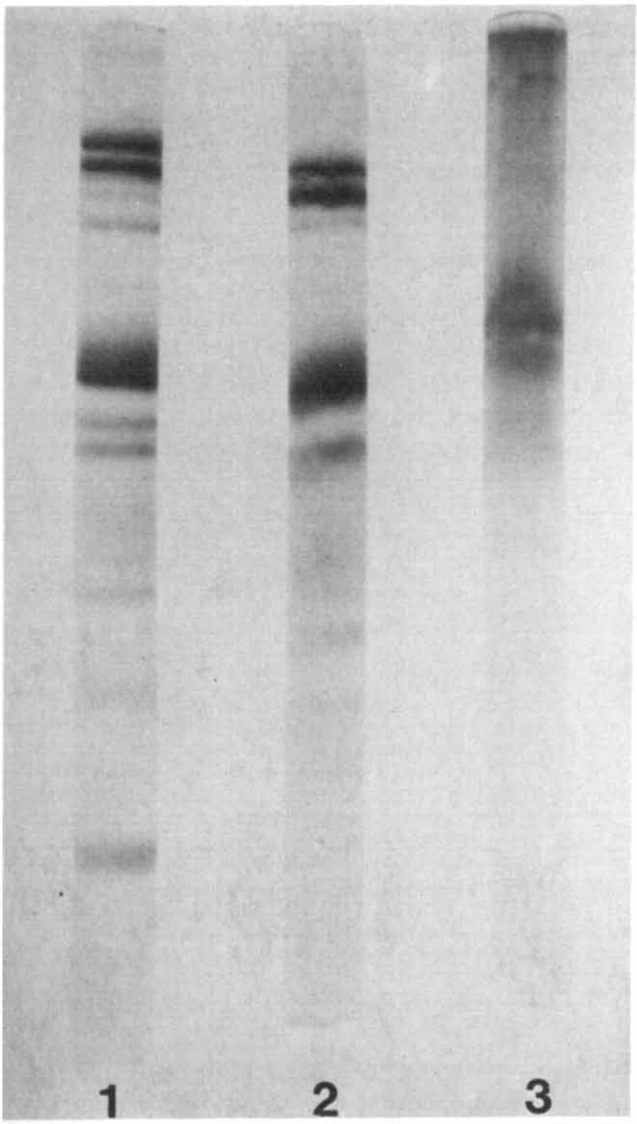

Fig. 3. SDS gel electrophoresis of the human erythrocyte membrane (left-hand and middle gels) and the purified enzyme (right-hand gel). Gel 1 was prepared according to the method of Weber and Osborn [19] at pH 7. Gels 2 and 3 were run at pH 2.4 under the conditions as described in the text. Gel 1 was run for 3.5 h at 9 mA/tube at room temperature and gels 2 and 3 for 5.5 h at 3 mA/tube at 2°C.

Fig. 6, middle line) and the phosphoenzyme linkage is sensitive to hydroxylamine (Fig. 5, lowest line).

The smaller peak near the tracking dye, which does not stain with Coomassie blue, shows the same characteristics in the washed preparation (Fig. 6) as the phosphoenzyme, e.g., sensitivity to MgATP-chase and hydroxylamine treatment. The possibility can be excluded that this peak represents inorganic phosphate which runs faster than the tracking dye (Fig. 4). On the other hand, it is unlikely that phosphorylation of phospholipids [29] or of the small polypep-

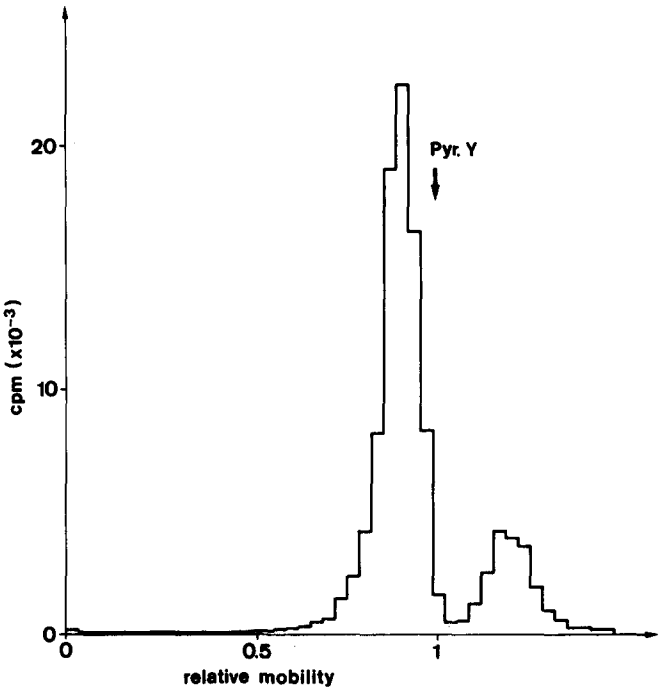

Fig. 4. SDS gel electrophoresis of [γ-^{32}P]ATP and $^{32}P_i$. SDS gel electrophoresis was performed under the conditions as described in the text. The gel was run for 3.5 h and 9 mA/tube, then cut into slices of 1 mm thickness and the radioactivity of two slices was measured in a vial with 20 ml Cerenkov solution [21].

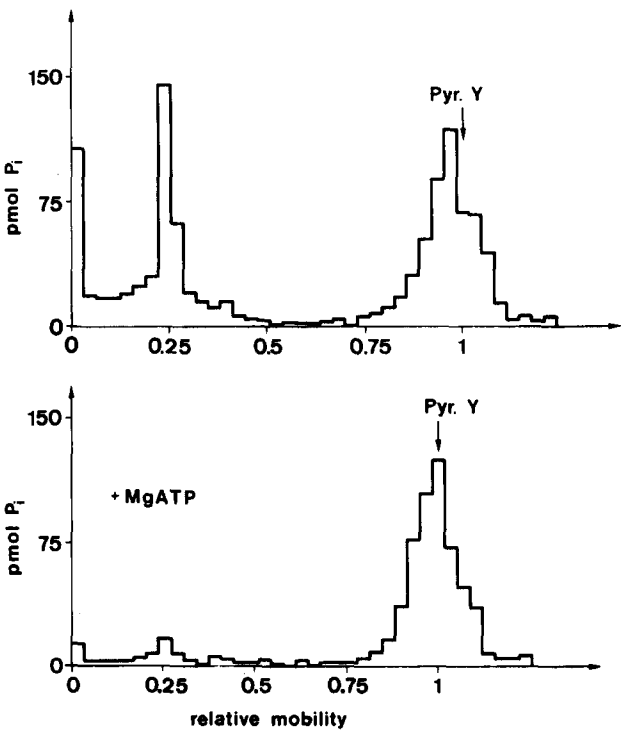

Fig. 5. SDS gel electrophoresis of the phosphorylated enzyme. Phosphorylation was performed under standard conditions, the volume of the reaction solution was 3 ml. The trichloroacetic acid-precipitated protein was solubilized, subjected to gel electrophoresis at pH 2.4 and 2°C and the radioactivity measured, as described in Materials and Methods. MgATP-chase in the lower line was performed by adding 200 μl of 5 mM MgATP per ml and terminated as described.

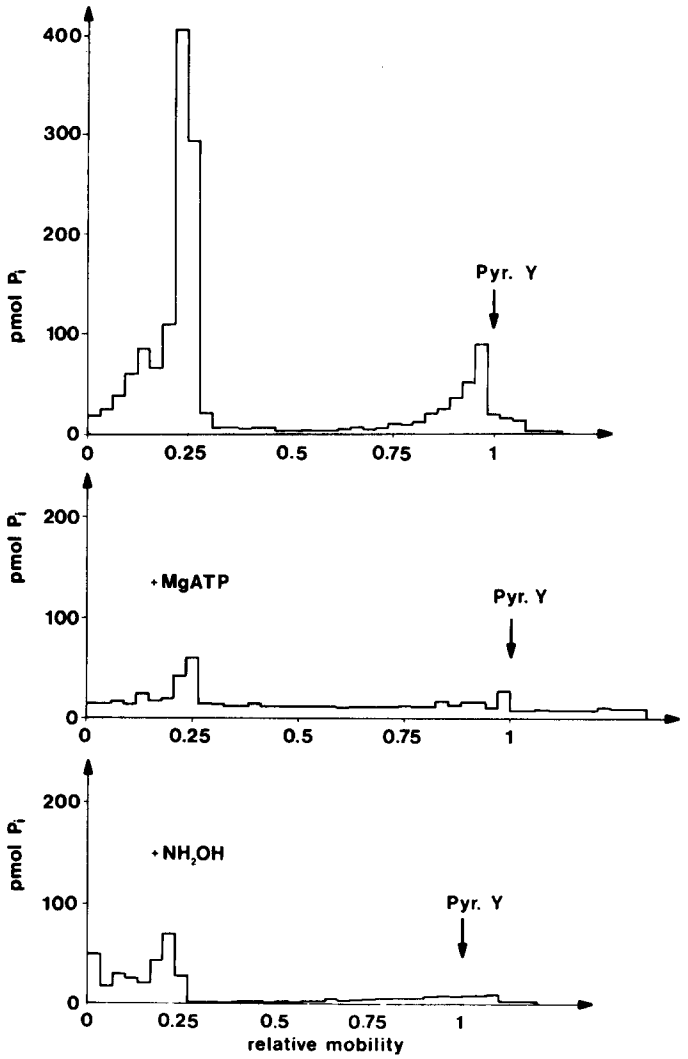

Fig. 6. SDS gel electrophoresis of the phosphorylated and dephosphorylated enzyme. The upper line shows SDS gel electrophoresis of the phosphoprotein, the middle line indicates the sensitivity to MgATP-chase and the lower line the sensitivity to hydroxylamine. Phosphorylation was performed under standard conditions, the volume of the reaction solution was 3 ml. The trichloroacetic acid-precipitated phosphoprotein was washed once with 10% trichloroacetic acid (plus 1 mM ATP, 20 mM P_i, 10 mM Ca^{2+} and 10 mM Mg^{2+}) before solubilization and gel electrophoresis. After sedimentation of the trichloroacetic acid-precipitated phosphoprotein, it was incubated for 10 min in 20 ml of 0.2 M hydroxylamine (pH 5.5) at room temperature, and after addition of 20 ml ice-cold 10% trichloroacetic acid (plus 1 mM ATP, 20 mM P_i, 10 mM Ca^{2+} and 10 mM Mg^{2+}) it was sedimented and then solubilized as described above.

tide which forms hydroxylamine-stable phosphoserine linkages [31] has taken place. Apparently, the concentrations of diisopropyl phosphofluoridate and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride used were not sufficient to inhibit completely the proteases of the erythrocyte (Wolf, H.U., unpublished data) which might have yielded a fragment of the catalytic subunit, with a molecular weight of 20 000—30 000, being still enzymatically

active. Similar results are known from $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum vesicles [32].

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