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PHOSPHORYLATION OF THE ISOLATED HIGH-AFFINITY (Ca^{2+} + Mg^{2+})-ATPase OF THE HUMAN ERYTHROCYTE MEMBRANE

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Summary

Solubilized and purified high-affinity (Ca^{2+} + Mg^{2+})-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of the human erythrocyte membrane (Wolf, H.U., Dieckvoss, G. and Lichtner, R. (1977) Acta Biol. Med. Ger. 36, 847) has been phosphorylated and dephosphorylated under various conditions with respect to Ca^{2+} and Mg^{2+} concentrations. In the range, 0.001--100 mM, the rate of phosphorylation was dependent on Ca^{2+} concentration, showing a maximum at 10 mM. The phosphorylation rate was nearly independent of the Mg^{2+} concentration within the range 0.01—1 mM.

This enzyme has at least three Ca^{2+} binding sites with different affinities and regulatory functions: (1) binding to the high-affinity site yields phosphorylation of the enzyme; (2) binding to a low-affinity site (Ca^{2+} concentrations higher than 40 μM) inhibits dephosphorylation or the conformational change which is necessary for dephosphorylation; (3) by binding to an additional low-affinity site, Ca^{2+} at concentrations higher than 1 mM abolishes negative cooperative behaviour (shown below 1 mM Ca^{2+}) and causes weak positive cooperativity between at least two catalytic subunits in the phosphorylation reaction.

The phosphoprotein obtained at Ca^{2+} concentrations above 1 mM dephosphorylates spontaneously after removal of the divalent metal ions. Addition of Mg^{2+} accelerates the dephosphorylation rate.

Affinities of the inhibitory Ca^{2+} binding sites are reduced by the binding of substrate or K^+.

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Introduction

Erythrocyte membranes show two (Ca^{2+} + Mg^{2+})-ATPase activities which are responsible for different functions. The so-called low-affinity (Ca^{2+} + Mg^{2+})-ATPase seems to be involved in maintaining and changing the shape of erythrocytes. The second (Ca^{2+} + Mg^{2+})-dependent ATPase, designated as the high-affinity (Ca^{2+} + Mg^{2+})-ATPase because of its high affinities for Ca^{2+} and substrate, has been claimed to be responsible for active Ca^{2+} outward transport in human erythrocytes by Schatzmann [1]. Most recently, further experimental proof for this hypothesis could be demonstrated after reconstitution of a crude preparation of this enzyme [2].

By analogy with the known mechanisms of other metal ion transport ATPases, e.g., (Na^+ + K^+)-ATPase and Ca^{2+}-ATPase in the sarcoplasmic reticulum, it was expected that Ca^{2+} transport by the Ca^{2+} pump of erythrocytes may also involve intermediates. It has been shown by several authors that Ca^{2+} stimulates the phosphorylation of a membrane-bound protein [3–8] and in this paper we report experiments which demonstrate also that the solubilized and purified enzyme phosphorylates and dephosphorylates under appropriate conditions.

Materials and Methods

Materials. *trans*-1,2-Diaminocyclohexene-*N*,*N*,*N'*,*N'*-tetraacetate was obtained from Roth (Karlsruhe) and [γ-^{32}P]ATP (15—20 Ci/mM) from Amersham-Buchler. Diisopropyl phosphorofluoridate, morpholinopropanesulphonic acid, saccharose, *N*,*N*,*N'*,*N'*-tetramethylenediamine, Triton X-100 and Tween 20 were obtained from Serva, Heidelberg. All other chemicals were from Merck, Darmstadt. Metal ions were used as chlorides.

Water was twice redistilled in a quartz apparatus. Protein determinations were made according to the method of Lowry et al. [9]. Erythrocyte membranes were prepared essentially as described before [10,11]. (Ca^{2+} + Mg^{2+})-ATPase activity was measured as described before [10–12].

Mg^{2+} and Ca^{2+}-buffers. The Ca^{2+} concentration for measurement of the enzyme activity was adjusted by using 0.4 mM Mg^{2+}- and Ca^{2+}-EDTA buffers [13]. Ca^{2+} concentrations below 500 μM for the phosphorylation reaction were adjusted by using Mg^{2+}- and Ca^{2+}-EGTA buffers ([EGTA] = 1 mM).

Concentrations of free Ca^{2+} and Mg^{2+} after addition of EDTA or EGTA were calculated on the basis of the dissociation constants for the interaction of the chelators with the divalent metal ions and with H^+ [13]. The dissociation constants were corrected for their temperature dependence. ΔH values and dissociation constants at 20 or 25°C were taken from the data of Moeller and Chu [14], Bohigian and Martell [15], Boyd et al. [16] and Anderegg [17].

Solubilization of (Ca^{2+} + Mg^{2+})-ATPase. Solubilization of the membrane-bound high-affinity (Ca^{2+} + Mg^{2+})-ATPase was achieved by using 0.2% Triton X-100 [18]. For application to gel chromatography, this solution had to be concentrated from 20 down to 4 ml using 1 g Sephadex G-25 coarse for 2.3 ml solution [19].

Purification of (Ca^{2+} + Mg^{2+})-ATPase. Purification was achieved by gel chro-

matography on Sepharose CL-6B columns in the presence of mixed micelles consisting of Triton X-100 or Tween 20 and phospholipids in a given molar ratio [11]. The column (40 × 2.6 cm) was equilibrated thoroughly with 200 mM Na^+ or K^+, 5 mM Ca^{2+}, 0.1 mM Mg^{2+}, 2 mM cysteine, 10 mM morpholinopropanesulphonic acid (pH 6.7), 0.1 mM Ca^{2+}-EGTA, 0.1 mM diisopropyl phosphorofluoridate, 1 mM *N*-α-*p*-tosyl-L-lysine chloromethylketonehydrochloride and mixed micelles, which had been made from 0.2 mg phosphatidylcholine/ml + 2.75 mg Tween 20/ml by ultrasonication for 30 min (Branson sonifier B 12) at 0°C under purified N_2. The medium for elution of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase was always identical with the equilibration solution. For further experiments, the eluate had to be concentrated, as described above, down to 3—5 ml.

Phosphorylation. 0.1 ml of the enzyme solution was incubated at 0°C in a final volume of 1 ml in a standard medium, which contained (unless otherwise stated) at final concentration, 10 mM Ca^{2+}, 0.01 mM Mg^{2+}, 50 mM Na^+ or K^+, 50 mM Tris-HCl (pH 7.5) and 0.1 mM CaEGTA. After 15 min the phosphorylation reaction was started under vigorous stirring, by the addition of 0.1 ml [γ-^{32}P]ATP (1.25 Ci/mM, final concn. 2 μM). After 2 min, 5 ml of an ice-cold solution of 10% trichloroacetic acid, 1 mM ATP and 20 mM H_3PO_4 were added and after 5 min, Ca^{2+} and Mg^{2+} were added to yield a final concentration of 10 mM. Then the trichloroacetic acid-insoluble fraction was separated by Millipore filtration (d = 2.5 cm, pore size = 0.22 μm) and washed four times with the same solution plus 10 mM Ca^{2+} and 10 mM Mg^{2+}. Addition of Ca^{2+} and Mg^{2+} to the reaction mixture and washing solutions was performed in order to decrease the blank phosphorylation (see below) value.

The radioactivities of the trichloroacetic acid-precipitated protein and of the Millipore filter were measured in a vial containing 20 ml Cerenkov solution [20].

Results

Phosphorylation experiments

Mg-ATPchase. In order to determine the yield of phosphoprotein of high-affinity (Ca^{2+} + Mg^{2+})-ATPase of human erythrocyte membranes, it was necessary to estimate the blank phosphorylation. This was performed by adding a 400-fold excess of unlabeled MgATP [21] after the phosphorylation reaction and by stopping the reaction as described after 30 s (Fig. 1). Radioactivity of the filter was also measured and the value subtracted from the blank phosphorylation. The resulting value, the so-called residual phosphorylation, shows a marked dependence on time (Fig. 2) and Ca^{2+} concentration (Fig. 3).

Effect of time. As shown in Fig. 2, the steady-state level has nearly been reached after 1—2 min in the presence of 1 μM Ca^{2+} and after 2 min in the presence of 10 mM Ca^{2+}. Therefore, the phosphorylation reaction was performed for 2 min in all further experiments. In contrast, Rega and Garrahan [6] reported that, in the presence of 0.1 mM Ca^{2+}, the steady-state level of phosphorylation of membrane-bound high-affinity (Ca^{2+} + Mg^{2+})-ATPase of the human erythrocyte membrane had been reached after 20—30 s.

Effect of Ca^{2+}. Fig. 3 illustrates the effect of varying Ca^{2+} concentrations

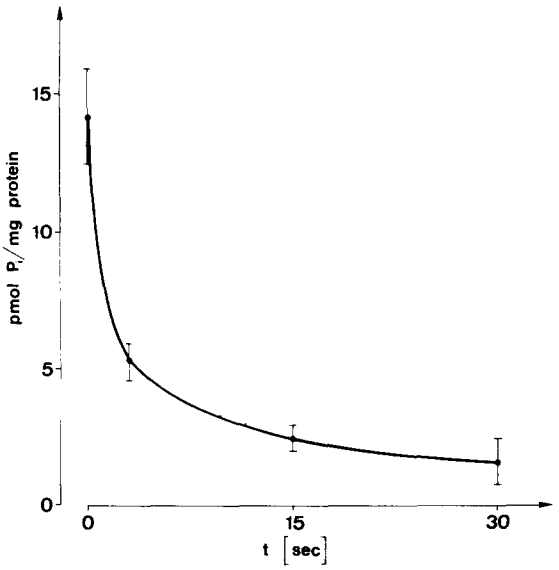


Fig. 1. MgATP-chase. Phosphorylation was carried out under standard conditions for 2 min. At the end of this period, 200 μl of 5 mM MgATP were added, the reaction continued and then terminated at the times indicated.

on phosphorylation of purified high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase in the presence of low and high Mg^{2+} concentrations. The phosphoprotein level reaches a maximum value at 10 mM Ca^{2+} independently of Mg^{2+} concentration. The Hill plot (Fig. 4) shows a negative co-operativity for Ca^{2+} concentrations up to 1 mM. At Ca^{2+} concentrations above 1 mM, Hill coefficients slightly

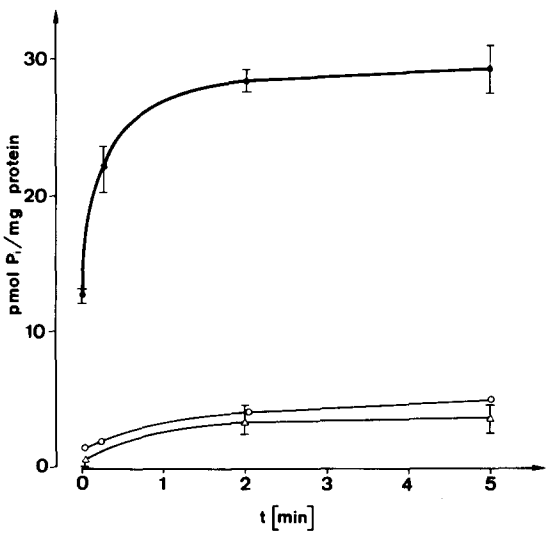


Fig. 2. Effect of time. Phosphorylation was carried out under standard conditions in the presence of 10 mM Ca^{2+} (●———●) and 1 μM Ca^{2+} (○———○). The residual phosphorylation in the presence of 10 mM Ca^{2+} is shown (△———△). In the presence of 1 μM Ca^{2+} the residual phosphorylation was 0 pmol P_i/mg protein.

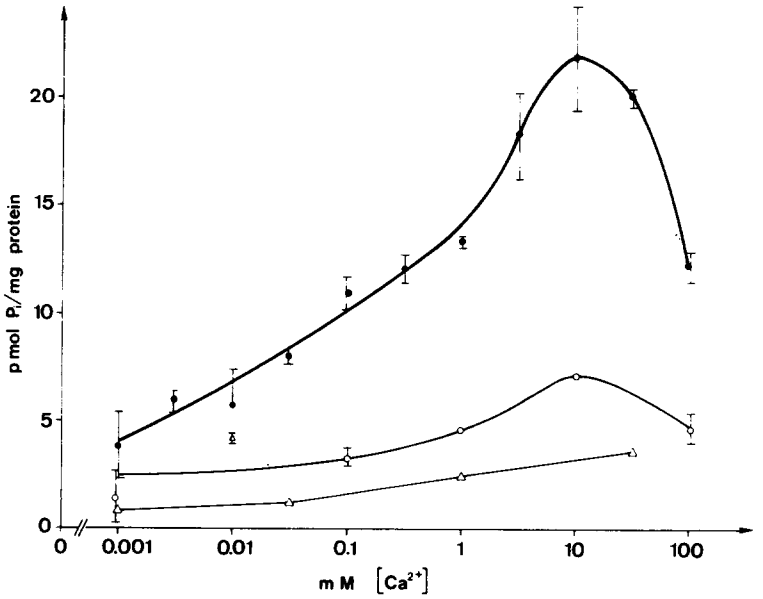


Fig. 3. Effect of Ca^{2+}. Phosphorylation was performed in the presence of 10 μM Mg^{2+} (●——●) and 10 mM Mg^{2+} (○——○) at varying Ca^{2+} concentrations. Up to 0.5 mM Ca^{2+}, EGTA at a final concentration of 1 mM was present. Free Ca^{2+} concentrations were calculated on the basis of the dissociation constants as described in the text. In the presence of 10 mM Mg^{2+}, the residual phosphorylation remained, throughout, at 0.6 pmol P_i/mg protein, whilst in the presence of 10 μM Mg^{2+} the residual phosphorylation rose with the Ca^{2+} concentration (△——△).

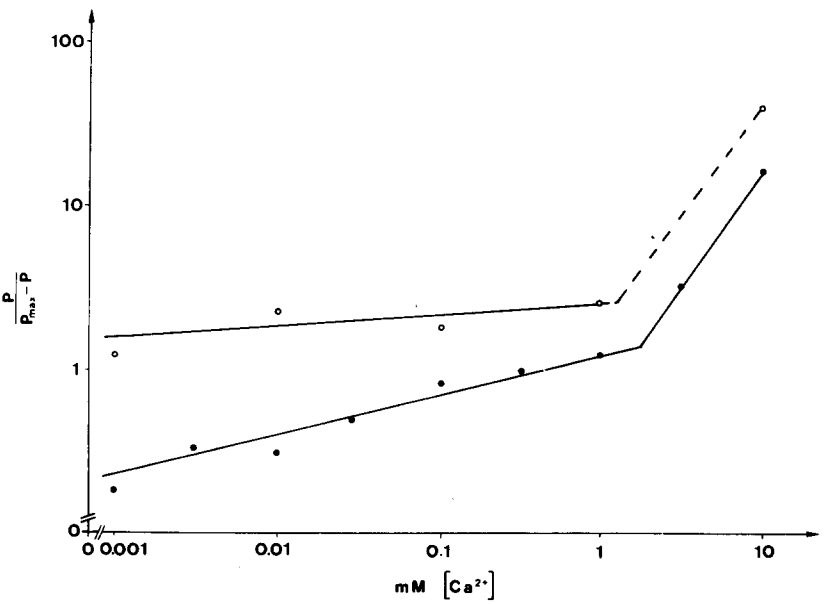


Fig. 4. Hill plot of Ca^{2+}-dependent phosphorylation in the presence of 10 μM (●——●) and 10 mM Mg^{2+} (○——○). $P \equiv$ pmol P_i/mg protein.

above 1 are obtained, possibly indicating a weak positive co-operativity. Mg^{2+} does not support or abolish these effects.

Effect of Mg^{2+}. To exclude the possibility that lower phosphorylation at the optimal Ca^{2+} concentration in the presence of high Mg^{2+} concentrations (Fig. 3) may be due to an Mg^{2+} effect or to variations in the purity of the enzyme obtained from different preparations, phosphorylation was performed as follows: in experiment A, phosphorylation was performed in the presence of 10 mM Ca^{2+} and 10 μM Mg^{2+} for 2 min and then stopped; in experiment B, phosphorylation was achieved also in the presence of 10 mM Ca^{2+} and 10 μM Mg^{2+}, after 2 min 10 mM Mg^{2+} were added, the reaction continued for 2 min and then stopped; in experiment C, phosphorylation was performed in the presence of 10 mM Ca^{2+} and 10 mM Mg^{2+} for 2 min and then stopped. Phosphorylation in the presence of high Ca^{2+} and Mg^{2+} concentrations yields an only slightly decreased phosphoprotein level than in the presence of high Ca^{2+} and low Mg^{2+} concentrations (experiment B, 80%: experiment C, 84% of the control value A). Fig. 5 illustrates that the variation of Mg^{2+} concentration in the presence of low and high Ca^{2+} concentrations has no increasing effect on the phosphoprotein level.

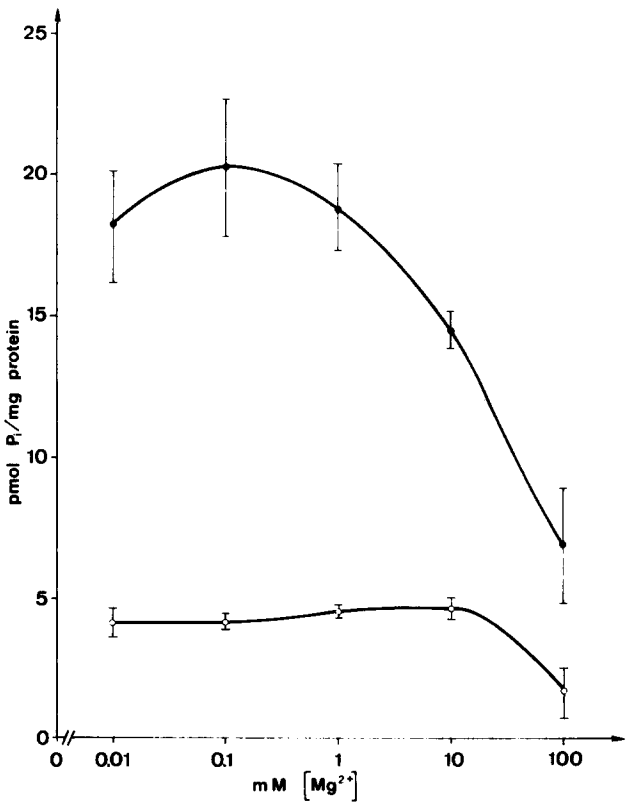


Fig. 5. Effect of Mg^{2+}. Phosphorylation was performed in the presence of 10 μM Ca^{2+} (○———○) and 10 mM Ca^{2+} (●———●) in the presence of varying Mg^{2+} concentrations. The residual phosphorylation remained constant, throughout, at 0.43 pmol P_i/mg protein at 10 μM Ca^{2+} and 5.9 pmol P_i/mg protein at 10 mM Ca^{2+}. In the former case, the concentration of free Ca^{2+} was adjusted by using 1 mM EGTA.

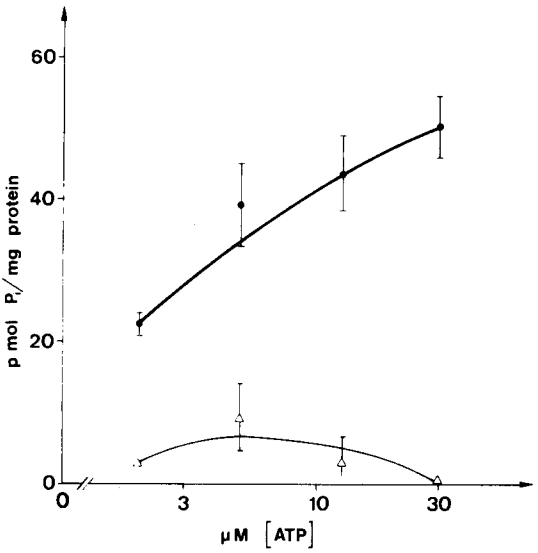


Fig. 6. Effect of ATP. Phosphorylation under standard conditions and varying ATP concentrations ($2.3 \cdot 10^6$ cpm—$11.2 \cdot 10^6$ cpm $^{32}P_i$/ml reaction medium). Phosphorylation (●———●), residual phosphorylation (△———△). For the estimation of residual phosphorylation in the presence of 30 μM ATP, 0.5 ml of 5 mM MgATP was added.

Effect of K^+. As compared to phosphorylation under standard conditions in the presence of 50 mM Na^+, the phosphoprotein level in the presence of 20 mM Na^+ and 30 mM K^+ is reduced to approx. 90% of the control value.

Effect of ATP. Fig. 6 shows the effect of different concentrations of [γ-^{32}P]-ATP on the phosphoprotein level of purified high-affinity (Ca^{2+} + Mg^{2+})-ATPase. The concentrations of free Ca^{2+} and Mg^{2+} remained constant throughout. Since the Ca^{2+} concentration is 1000 times higher than the Mg^{2+} concentration, and since their dissociation constants with ATP are almost equal, ATP will be present mainly as the CaATP complex [22].

Dephosphorylation

Dephosphorylation of the phosphoprotein can be achieved either by chelating the ion which is necessary for the stability of the phosphoprotein and/or by addition of the ion which is necessary for the dephosphorylation step. EDTA and EGTA form stable complexes with divalent metal ions: by this reaction, H^+ is liberated. Chelating of the high Ca^{2+} concentrations used for optimal phosphorylation needs high EDTA or EGTA concentrations and therefore, a decrease in pH was obtained (Table I). This effect could not be abolished completely, since it was not practicable to raise the pH of the chelating solution because the phosphoprotein is quite unstable at pH values higher than 7 (see following paper). In addition, the buffer concentration could not be raised, because Tris-HCl concentrations higher than 50 mM decrease markedly the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase (Wolf, H.U., unpublished results). However, in spite of the difficulties which were encountered using Tris-HCl, we decided to use this buffer thereafter in order to enable us to compare our results directly with those of other authors. The concentration ratios

TABLE I

TURNOVER OF THE PHOSPHOPROTEIN

Phosphorylation under standard conditions. After 2 min phosphorylation, addition of chelator (and Mg^{2+} in No. 2) to reach the indicated total concentrations. The resulting pH values were measured in control experiments and the free divalent metal ion concentrations were calculated as described. Concentrations are expressed in μM.

No.	$[Ca^{2+}]_t$	$[Mg^{2+}]_t$	$[L]_t$	Chelator	pH	$[Ca^{2+}]_{free}$	$[Mg^{2+}]_{free}$	Residual phosphorylation after 3 s	S.D. (%)	Residual phosphorylation after 30 s	S.D. (%)
1	10 000	10	20 000	EDTA	6.4	0.524	0.149	64.8	3.8	19.2	1.6
2	10 000	7883	18 840	EDTA	5.8	15.80	2471	35.0	2.7	11.0	0.2
3	10 000	10	9757	EGTA	4.5	8660	10	81.5	2.8	41.3	1.2

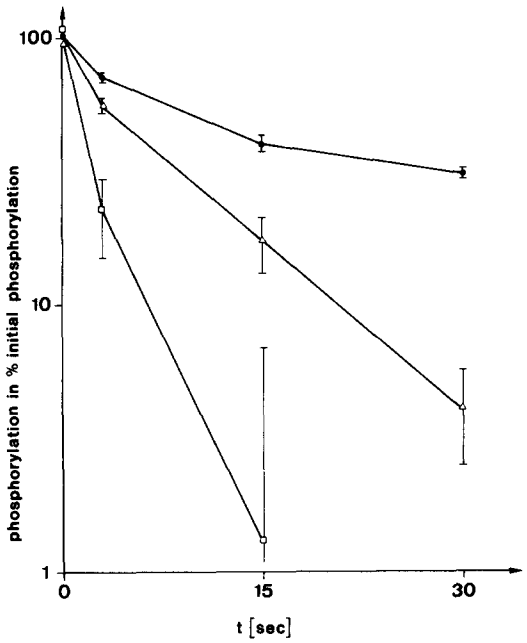


Fig. 7. Dephosphorylation. Phosphorylation under standard conditions. Upper curve (●———●): after 2 min, addition of 9.8 mM EGTA. The pH value decreased to 4.5. $[Ca^{2+}]_{free}$ was calculated to be 8.6 mM and $[Mg^{2+}]_{free}$ to be 10 μM. Middle curve (△———△): after 2 min, addition of 20 mM EDTA. The pH value decreased to 6.4. $[Ca^{2+}]_{free}$ was calculated to be 0.5 μM and $[Mg^{2+}]_{free}$ to be 0.1 μM. Lower curve (□———□): after 2 min, addition of 21.8 mM EDTA and 10.5 mM Mg^{2+}. The pH value decreased to 5.8. $[Ca^{2+}]_{free}$ was calculated to be 15.8 μM and $[Mg^{2+}]_{free}$ to be 2.4 mM. In all curves the blank phosphorylation was subtracted.

of Ca^{2+} and Mg^{2+} to be used in these experiments are very limited because of the individual pH values and dissociation constants of the ligands.

Chelating of Ca^{2+} and Mg^{2+}. Ca^{2+}-stimulated phosphorylation undergoes dephosphorylation after chelating the divalent metal ions. When phosphorylation is achieved in the presence of high Ca^{2+} concentrations, dephosphorylation occurs completely within 30 s after addition of 20 mM EDTA (Fig. 7, middle curve). The pH value decreases from 7.5 to 6.4. $[Ca^{2+}]_{free}$ was calculated to be equal to 0.524 μM and $[Mg^{2+}]_{free}$ to be 0.149 μM.

Chelating of Ca^{2+} and addition of Mg^{2+}. Phosphorylation in the presence of 10 mM Ca^{2+} and 10 μM Mg^{2+} and after 2 min, addition of 21.8 mM EDTA and 10.5 mM Mg^{2+} yields complete dephosphorylation within 15 s (Fig. 7, lower curve). The pH value decreases from 7.5 to 5.8. $[Ca^{2+}]_{free}$ was calculated to be equal to 13.5 μM and $[Mg^{2+}]_{free}$ to be 2.8 mM. These concentrations are within the range for optimum activity for high-affinity (Ca^{2+} + Mg^{2+})-ATPase of human erythrocyte membranes (Wolf, H.U., unpublished results).

Chelating of Ca^{2+}. Selective chelating of Ca^{2+} can be achieved by using EGTA (Fig. 7, upper curve). Addition of 9.7 mM EGTA to 10 mM Ca^{2+} yielded a decrease in pH from 7.5 to 4.5 and resulted in free metal ion concentrations of 8.6 mM Ca^{2+} and 10 μM Mg^{2+}.

Discussion

Influence of the Ca^{2+} binding sites on phosphorylation

The Ca^{2+} pump of human erythrocyte membranes needs Ca^{2+} and Mg^{2+} [23,24]. Ca^{2+}-stimulated phosphorylation of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase of the human erythrocyte membrane has been reported from several authors [3—6,8], but these results were obtained using a membrane-bound enzyme.

Ca^{2+} concentrations higher than 0.04 mM have an inhibitory effect on the activity of this enzyme system (Wolf, H.U., unpublished results). This may be due to the blocking of the dephosphorylation step, since the phosphoprotein level increases with increasing Ca^{2+} concentrations (Fig. 3). From these results, it may be deduced that the enzyme has at least two Ca^{2+} binding sites, one activating with high affinity for Ca^{2+} and the other inhibiting with low affinity for Ca^{2+}. These reactions and all further results are combined to form a scheme representing phosphorylation and dephosphorylation reactions of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase. In this scheme, the activating influence of Ca^{2+} is designated as CaE (step II) and the inhibitory influence as $\underset{\downarrow}{Ca}$ (step III).

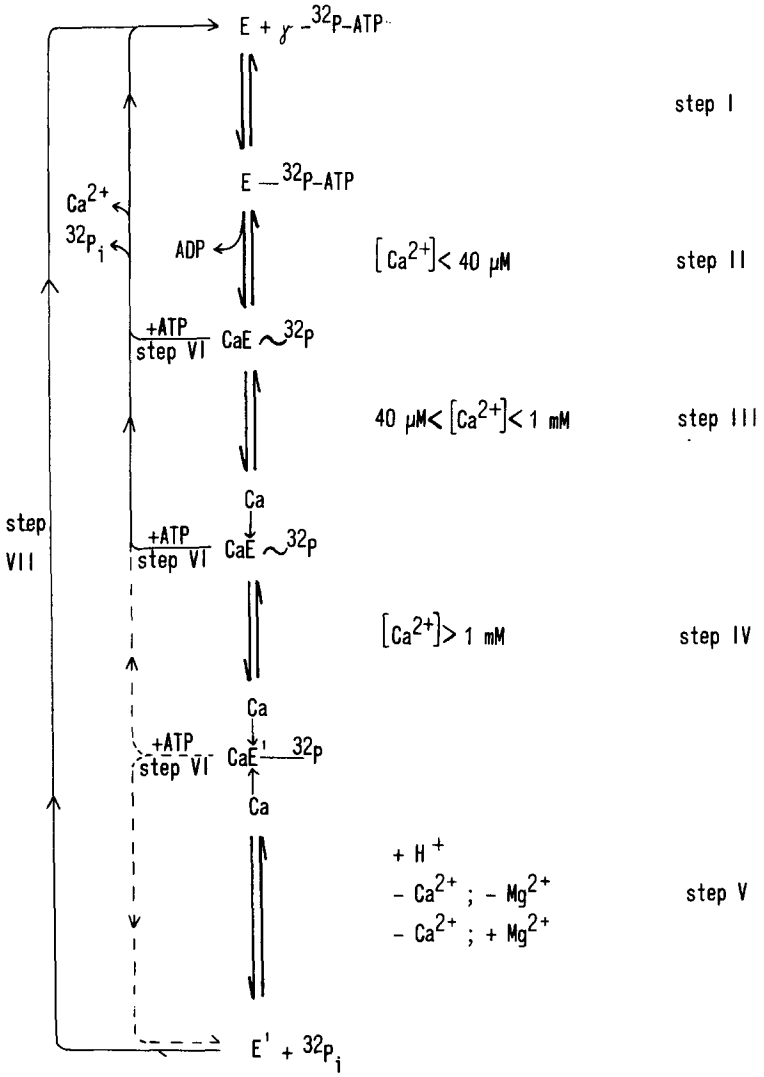
E

The existence of at least two Ca^{2+} binding sites of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase of human erythrocyte membranes has been reported by Scharff and Foder [25]. Ferreira and Lew [26] demonstrated the involvement of two Ca^{2+} binding sites in the translocation step of the Ca^{2+} pump of the human erythrocyte membrane. This is also known from the ($Ca^{2+} + Mg^{2+}$)-ATPase of the vesicles of sarcoplasmic reticulum [27,28]. The Hill plot (Fig. 4) shows a positively co-operative behaviour of at least two subunits at Ca^{2+} concentrations above 1 mM. The conformational change of the catalytic subunit induced by Ca^{2+} concentrations higher than 1 mM is designated as E′ in the scheme (step IV).

The phosphorylation step seems to be regulated not only by Ca^{2+} binding sites involved in activation and inhibition, but also by co-operative effects as known from enzymes consisting of more than one catalytic subunit. In fact, Wolf [19] found that the enzyme exists at least in a dimeric structure.

The existence of low-affinity Ca^{2+} binding sites which are involved in the inhibition of ATPase can also be seen in Fig. 2: the fastest phosphorylation rate and highest phosphoprotein level can be achieved in the presence of high Ca^{2+} concentrations. This confirms earlier findings of Katz and Blostein [5], who reported that high Ca^{2+} concentrations block the turnover of the phosphoprotein.

As seen in Fig. 1, dephosphorylation occurs after addition of 1 mM unlabelled MgATP though the Ca^{2+} concentration is 10 mM. This may be explained by assumption that binding of the substrate lowers the affinity of inhibitory Ca^{2+} binding sites. Bond [29] reported that conformational changes of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase of the human erythrocyte membrane induced by Ca^{2+} can be influenced by further addition of MgATP. This is also known in the case of ($Ca^{2+} + Mg^{2+}$)-ATPase of sarcoplasmic reticulum vesicles [30—33]. Binding of ATP by ($Na^+ + K^+$)-ATPase can also influence the affinities of Na^+ and K^+ binding sites [34]. It may be assumed that the conforma-



Scheme I. Scheme of the reactions involved in the reaction cycle of phosphorylation and dephosphorylation. Step I represents formation of the enzyme-substrate complex. The subsequent steps, II, III and IV, show the effects of increasing Ca^{2+} concentrations. Step V demonstrates spontaneous dephosphorylation and step VI substrate-induced dephosphorylation. The product of spontaneous dephosphorylation is an enzyme species, E', fixed in a 'frozen' state. Step VII indicates that the increase in temperature abolishes the 'frozen' state of this enzyme species, according to one of the possible explanations mentioned in Discussion.

tational changes of high-affinity (Ca^{2+} + Mg^{2+})-ATPase induced by binding of the substrate will occur at all phosphoenzyme states proposed in the scheme (step VI).

High-affinity (Ca^{2+} + Mg^{2+})-ATPase is activated by monovalent cations, K^+ having the highest potency [19,25]. On the other hand, in the presence of 30 mM K^+ + 20 mM Na^+, the phosphoprotein level is reduced to 90% as compared to the phosphorylation in the presence of 50 mM Na^+, in good agreement

with the results of Knauf et al. [3]. It is tempting to assume that there may exist a competitive mechanism between K^+ and Ca^{2+} at the inhibitory Ca^{2+} binding sites, reducing the phosphoprotein level and thus activating the ATPase.

Substrate of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase

It has still not been clarified completely, whether the substrate of high-affinity (Ca^{2+} + Mg^{2+})-ATPase is ATP, MgATP, CaATP or any combination of these. Wolf [10,19] reported that the enzyme forms a complex with MgATP which is inactive in the absence of Ca^{2+}. On the other hand, Rega and Garrahan [6] found phosphorylation of the enzyme in the absence of Mg^{2+} and considered free ATP to be the substrate, as has been shown for the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum. Schatzmann [35] also discussed the possibility that free ATP may act as the substrate of high-affinity (Ca^{2+} + Mg^{2+})-ATPase rather than MgATP.

The results of this paper indicate that CaATP can also act as a substrate of this enzyme: the Ca^{2+} concentration is 1000 times higher than the Mg^{2+} concentration; since both complexes with ATP have nearly the same value of the dissociation constant [22], ATP will be present mainly as CaATP. $[ATP]_{free}$ is decreased to $1 \cdot 10^{-8}$ M. Assuming that free ATP is the substrate (K_M = 1—2 μM, [35]), the concentration of this substrate would be too small to obtain a detectable enzymatic reaction. On the basis of these experiments, it is not possible to decide whether free ATP, MgATP, CaATP or any combination of these act as a substrate for high-affinity (Ca^{2+} + Mg^{2+})-ATPase.

It has to be noted that the measured phosphoprotein levels in Fig. 6 are probably diminished due to the reduced affinities of inhibitory Ca^{2+} binding sites induced by binding of the substrate.

Influence of Mg^{2+} on the phosphorylation

Mg^{2+} is necessary for ATPase activity and the translocation step and can induce conformational changes of the enzyme [29]. It does not accelerate the phosphorylation or inhibit the dephosphorylation step, either in the presence of high or low Ca^{2+} concentrations (Fig. 5). This result confirms also that Ca^{2+}-dependent phosphorylation of high-affinity (Ca^{2+} + Mg^{2+})-ATPase does not require Mg^{2+} [3,5,6,8]. It has been found by some authors that Mg^{2+} accelerates the turnover of the phosphoprotein: Rega and Garrahan [6] and Schatzmann and Bürgin [8] assumed that Mg^{2+} does not accelerate hydrolysis of the phosphoprotein but transforms it into a state which is easily hydrolysed. The latter reported that phosphorylation in the presence of 50 μM Ca^{2+} and 2 mM Mg^{2+} yields a phosphoprotein level which is higher than that in the absence of Mg^{2+}. We cannot confirm this finding since Fig. 5 illustrates that, in the presence of 10 μM Ca^{2+}, phosphorylation of the solubilized enzyme shows no dependence on Mg^{2+} concentration in the range 0.01—10 mM. As seen in experiments B and C, phosphorylation in the presence of high Ca^{2+} and Mg^{2+} concentrations yields a decrease in the phosphoprotein level of approx. 20% as compared to phosphorylation in the presence of high Ca^{2+} and low Mg^{2+} concentrations. This may be explained by competition of high Ca^{2+} and Mg^{2+} concentrations at the inhibitory Ca^{2+} binding sites. Thus, the different phosphoryl-

ation levels shown in Fig. 3 are mainly due to variations in the purity of the enzyme obtained from different preparations rather than to the different Mg^{2+} concentrations.

Influence of Ca^{2+} and Mg^{2+} on dephosphorylation

The influence of the divalent metal ions on the turnover and on the dephosphorylation, respectively, can be studied by chelating the ions after phosphorylation by addition of EDTA or EGTA (see Table I). Selective chelating of 10 mM Ca^{2+} by addition of EGTA (Fig. 7, upper curve) only yields a decrease in the pH value to 4.5 and no essential change in the free divalent metal ion concentration. The observed dephosphorylation of approx. 40%, thus, can be explained by a pH effect (scheme, step V). It is unlikely that the phosphate bond is split non-enzymatically at pH of 4.5, because the linkage is quite stable at low pH values. As seen in Fig. 7, lower curve, addition of EDTA and Mg^{2+} yields complete dephosphorylation within 15 s, though under the resulting concentrations of divalent metal ions (13.5 μM Ca^{2+}, 2.8 mM Mg^{2+}) a phosphoprotein level of approx. 2—3-fold of the residual phosphorylation would be expected (Fig. 5). The possibility that the enzyme is denatured at pH 5.8 can be excluded since, at this pH and 30°C, the enzyme still shows 50% of the optimum activity [19].

One possible explanation is that the dephosphorylated enzyme with the conformation, E′ (see scheme), cannot rephosphorylate at 0°C. The enzyme apparently is in a 'frozen' state, which can be abolished by raising the temperature (scheme, step VII), because no change in the kinetic data could be observed (measured at 30°C) after treatment at 0°C in the presence of 10 mM Ca^{2+} (Wolf, H.U., unpublished data). Another explanation is that EDTA concentrations of approx. 20 mM are 'toxic' to enzyme activity. However, this effect obviously would be directed mainly versus the phosphorylation step rather than versus the dephosphorylation step, since the dephosphorylation rate can be modulated by Mg^{2+}.

Chelating of Ca^{2+} and Mg^{2+} yields a diminished dephosphorylation rate but also complete dephosphorylation after 30 s (Fig. 7, middle curve).

Assuming that the differences in pH values can be neglected, we conclude that the dephosphorylation of the complex

$$\begin{array}{c} Ca \\ \downarrow \\ CaE'\text{—}P \\ \uparrow \\ Ca \end{array}$$

occurs spontaneously after chelation of the divalent metal ions and the addition of Mg^{2+} accelerates the breakdown of the phosphoprotein.

Rega and Garrahan [5] and Schatzmann and Bürgin [8] also studied the dephosphorylation of the phosphoprotein which was formed in the presence of low Ca^{2+} and Mg^{2+} concentrations. In contrast to our conditions, their chelating solution always contained high concentrations of substrate. On account of substrate-induced conformational changes of the enzyme, their results cannot be compared with ours. Cha and Lee [7] found that Mg^{2+}

stimulates phosphorylation of the membrane-bound Ca^{2+} pump and Ca^{2+} accelerates dephosphorylation. This result is in contrast to ours and to those of other authors [3,5—8]. At least their explanation that the Ca^{2+} pump cannot dephosphorylate at low temperatures is disproved by us and other authors [3,5—8].

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