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THE EFFECT OF Mg^{2+} AND CHELATING AGENTS ON INTERMEDIARY STEPS OF THE REACTION OF Na^+, K^+-ACTIVATED ATPase

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Summary

(1) It has been investigated how varying concentrations of free magnesium with and without EDTA influence the properties of the phospho-enzyme formed in the presence of sodium by the ($Na^+ + K^+$)-activated enzyme system.

(2) The phospho-enzyme formed in the presence of sodium and a high concentration of free magnesium has the same rate of (a) spontaneous dephosphorylation, (b) dephosphorylation after addition of potassium, and (c) dephosphorylation after addition of ADP, as a phospho-enzyme formed in the presence of sodium and a low concentration of magnesium.

(3) With sodium and a given concentration of free magnesium, high or low, EDTA present during formation of the phospho-enzyme leads to a decrease in the rate of (a) spontaneous dephosphorylation, and (b) dephosphorylation after addition of potassium to the phospho-enzyme.

(4) The rate of dephosphorylation after addition of ADP to phospho-enzyme formed without and with EDTA is the same. But as the rate of spontaneous dephosphorylation is lower with EDTA than without, ADP gives a higher increase in the rate of dephosphorylation of phospho-enzyme formed with EDTA than without.

(5) The experiments thus show that the reported different sensitivity towards potassium and ADP of phospho-enzyme formed in the presence of a low and high concentration of free magnesium, respectively, is due to the EDTA used to decrease the free magnesium concentration and not to the decrease in the free magnesium as such.

Abbreviation: CDTA, *trans*-1,2-Diaminocyclohexane-*N,N,N',N'*-tetraacetic acid or its salt.

The term spontaneous dephosphorylation used in the paper refers to the decrease in amount of phospho-enzyme seen when phosphorylation, in the presence of sodium and magnesium, is stopped by complexing Mg^{2+} with excess CDTA.

Introduction

The magnesium requirement of the sodium-dependent ADP-ATP exchange catalyzed by Na^+,K^+-ATPase (ATP phosphohydrolase EC 3.6.1.3) is lower than the requirement for magnesium for the overall hydrolysis of ATP in the presence of sodium and potassium. From this, Fahn et al. [1,2] suggested that the hydrolysis of ATP proceeds via formation of two phospho-enzymes, $E_1 \sim P$ and E_2-P, with a requirement for a low concentration of Mg^{2+} for formation of $E_1 \sim P$ and for a higher Mg^{2+} concentrations for the conversion of $E_1 \sim P$ into E_2-P (see Scheme).

$$E_1 + ATP \rightleftharpoons EATP$$

$$EATP \overset{Mg^{2+},\, Na^+}{\rightleftharpoons} E_1 \sim P + ADP$$

$$E_1 \sim P \overset{Mg^{2+}}{\rightleftharpoons} E_2 - P$$

$$E_2 - P \overset{K^+}{\rightleftharpoons} E_2 + P_i$$

$$E_2 \rightleftharpoons E_1$$

$E_1 \sim P$ is dephosphorylated by ADP but not by K^+, while E_2-P is dephosphorylated by potassium but not by ADP.

Experiments by Post et al [3] apparently supported this proposal. They found that the phospho-enzyme formed in the presence of a high magnesium concentration (1 mM Mg^{2+}) was dephosphorylated at a high rate by potassium but not by ADP, while with phospho-enzyme formed in the presence of a low Mg^{2+} concentration (1 mM Mg^{2+}, 3 mM EDTA = 2 μM free Mg^{2+}) the opposite was the case as predicted by the scheme by Fahn et al. In disagreement with this, Fukushima and Tonomura [4] found no difference in the rate of dephosphorylation by ADP of phospho-enzyme formed in the presence of a high, 5 mM, and a low concentration of magnesium, 10 μM.

As will be seen from the following, this discrepancy is not due to differences in the concentrations of free magnesium used in the experiments. It is the EDTA used in the experiments by Post et al. to decrease the concentration of free Mg^{2+} which leads to the formation of a phospho-enzyme which apparently has properties distinct from that formed in the absence of EDTA.

The results to be presented raise some doubts about the concept of two phospho-enzymes as intermediates in the hydrolysis of ATP by Na^+,K^+-ATPase.

Material and Methods

The enzyme was prepared from ox brain by the method of Klodos et al. [5]. EDTA from the preparation procedure was removed by washing the enzyme at 0°C with 0.25 M sucrose, 30 mM histidine-HCl, pH 7.2 (pH measured at 20°C). The stock solution of the enzyme contained 2—2.2 mg protein · ml^{-1}. The specific activity was about 2.6—3.5 μM ATP hydrolyzed mg^{-1} protein · min^{-1} (3 mM ATP, 3 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 30 mM histidine buffer, pH

7.4 at 37°C). The Mg^{2+}-activated ATPase was about 1% of the total activity (3 mM ATP, 3 mM Mg^{2+}, 30 mM histidine buffer, pH 7.4 at 37°C, and in the presence of $1 \cdot 10^{-3}$ M *g*-strophanthin). The enzyme was stored at −20°C, and was relatively stable at this temperature; after 4 months it had lost about 10% of its original activity.

Reagents

ATP labelled in the γ position with ^{32}P was obtained from The Radiochemical Centre, Amersham, England, [U-^{14}C]ATP from New England Nuclear, and the Na^+ salts of ATP and ADP from Boehringer. [γ-^{32}P]ATP, [U-^{14}C]ATP and ATP were purified and converted to their Tris salt by chromatography on a DEAE Sephadex-25 (Pharmacia) column [6]. EDTA was from Merck, and CDTA from BDH. All reagents used were of reagent grade.

Assay

The phosphorylation and dephosphorylation experiments were run at 0°C. The test solution contained 30 mM Tris/HCl as buffer, pH 7.4 or 8.0 (measured at 0°C). Cations as chloride salts, EDTA and CDTA as Tris salts (neutralized with Tris base to the pH of the test medium) were added to the concentrations given in the figures. Enzyme (1.2—1.3 mg protein) was preincubated in the test solution for 10—15 min, and the reaction was then started by addition, under continuous stirring, of a mixture of ATP and [γ-^{32}P]ATP to a final concentration of 25 μM, and P_i to a concentration of 0.5 mM. This concentration of P_i did not affect the phosphorylation of the enzyme (result of preliminary experiment, not shown) but gave better reproducibility of results independent of the different batches of radioactive ATP. The reaction was stopped by addition of ice-cold $HClO_4$ (final concentration 4% w/v) and the precipitated protein was isolated and washed as described by Skou and Hilberg [7]. After the final wash, the protein was dissolved in 1 mM NaOH; an aliquot was used for counting and another for determination of protein.

For measurement of spontaneous dephosphorylation (see footnote, title page), the phosphorylation was carried out for 10 s, and then stopped by addition of CDTA (final concentration 10 mM). The reaction was then stopped at the times given in the figures by the addition of ice-cold $HClO_4$ and the phospho-protein isolated as described above. The CDTA solution was never more than two weeks old and stored at 4°C.

For measurement of K^+- or ADP-dependent dephosphorylation, the phosphorylation was stopped at 10 s by addition of CDTA and 5 s later K^+ or ADP was added. The reaction was then stopped as described above at the times given in the figures.

Protein was measured (in duplicates) by the method of Lowry et al. [8] with bovine serum albumin as standard.

A suitable portion of protein hydrolyzate was transferred to glass vials containing 10 ml of scintillation solution [6] and the ^{32}P was counted in a scintillation counter. Preliminary experiments showed that the efficiency of counting decreased with time, probably because of precipitation of the NaOH hydrolyzate. This was prevented by addition of 1 ml of a solution containing 5% trichloroacetic acid (w/w) and 75 mM H_2SO_4.

All results in the figures are mean values of 2—5 experiments with standard deviations shown in Fig. 1 and Table I.

Concentrations of free Mg^{2+} are calculated on the basis of stability constants given by Martell [9] taking into account the effects of pH and temperature.

Control of the level of non-specific phosphorylation

With Mg^{2+} and a high concentration of K^+ (but no Na^+) there is a certain level of phosphorylation which comes to about 30—40 pmol/mg protein. The same level is found after potassium dephosphorylation of phosphoenzyme formed in the presence of magnesium and sodium.

This "high K^+" control level of phosphorylation consists of two parts, one of about 20—30 pmol/mg protein (10—13% maximum phosphorylation which is removed by the addition of ADP or cold ATP; and another, 10—12 pmol/mg protein, which cannot be removed by ADP or ATP.

Experiments with [U-^{14}C]ATP show that the ADP-removable part is due to binding of ATP to the protein. The ATP-nonremovable phosphorylation found with [^{32}P]ATP as substrate, 10—12 pmol/mg protein, is also seen when $HClO_4$ was added to the protein before the addition of [^{32}P]ATP.

The control used in the following experiments has been enzyme incubated under the same conditions as the test, but with $HClO_4$ added before [^{32}P]ATP, and the results are presented after subtraction of this control value. It means that in the experiments with dephosphorylation with potassium there will remain a certain level of protein-bound ^{32}P which does not represent phosphorylated enzyme, but [^{32}P]ATP bound to the protein (see Figs 5 and 6), while in the experiments with dephosphorylation with ADP, the ^{32}P bound to the protein will go to zero (see Fig. 7).

TABLE I

THE EFFECT OF Mg^{2+} ON PHOSPHORYLATION OF Na^+, K^+-ATPase

Medium contained (total volume - 3 ml): 1.3 mg protein with specific activity of 209.4 μmol ATP hydrolyzed · mg^{-1} · h^{-1}; 25 μM ATP; 16 mM Na^+; 30 mM Tris-HCl; Mg^{2+} or Mg^{2+} + EDTA at concentrations shown. Phosporylation time, 10 s; temperature, 0°C. Figures in paranthesis show number of experiments.

Mg^{2+} (mM)	EDTA (mM)	E-^{32}P amount (pmol ^{32}P · mg protein^{-1}) ± s.d.	
		pH 7.4	pH 8.0
0	10	n.d.	n.d.
0	0	n.e.	185 ± 8 (2)
0.1	0	215 ± 20 (2)	235 ± 7 (4)
1	0	212 ± 17 (2)	261 ± 26 (8)
2	0	n.e.	289 ± 22 (2)
3	0	n.e.	283 ± 21 (2)
4	3	214 ± 16 (2)	246 ± 18 (2)
1	3	146 ± 12 (2)	92 ± 8 (5)

Abbreviations: n.d. not detectable: n.e. not estimated.

Results

Phosphorylation

Under the experimental conditions used (0°C, 25 μM ATP/[γ-^{32}P]ATP, Na^+,Mg^{2+}) the phosphorylation reaches a steady-state level in less than 5 s (the shortest time used), and does not decline before either the ATP is used up (several minutes) or the phosphorylation is stopped by the addition of CDTA, cold ATP or ADP. In the following, the ^{32}P phosphorylation was stopped by addition of CDTA after a 10 s incubation, i.e. after a time interval sufficient to give steady-state values.

In the presence of Na^+ without added Mg^{2+} there is a relatively high level of phosphorylation (Table I). It is increased by the addition of 0.1 mM Mg^{2+}, while a further increase in the Mg^{2+} concentration does not lead to further increase in the amount of phospho-enzyme formed. The formation of phospho-enzyme in the presence of Na^+ without added Mg^{2+} can be prevented by the addition of 10 mM EDTA, suggesting that the phosphorylation is due to traces of Mg^{2+} in the enzyme preparation. It is furthermore seen from the table that 4 mM Mg^{2+} with 3 mM EDTA gives the same amount of phospho-enzyme as 1 mM Mg^{2+} without EDTA, and that 3 mM EDTA decreases the amount of phospho-enzyme formed in the presence of 1 mM Mg^{2+}; this last effect is more pronounced at pH 8.0 where more Mg^{2+} is complexed by EDTA than at pH 7.4.

Sodium is necessary for the phosphorylation. With Mg^{2+} but without Na^+ no phospho-enzyme is formed in the 10 s period.

With 1 mM Mg^{2+}, with 4 mM Mg^{2+} + 3 mM EDTA, or with 1 mM Mg^{2+} + 3 mM EDTA, the steady-state level of phospho-enzyme increases with the Na^+ concentration up to about 30 mM (Fig. 1). The increase and decrease, respectively, seen in the figure with concentrations of Na^+ above 30 mM are statistically insignificant.

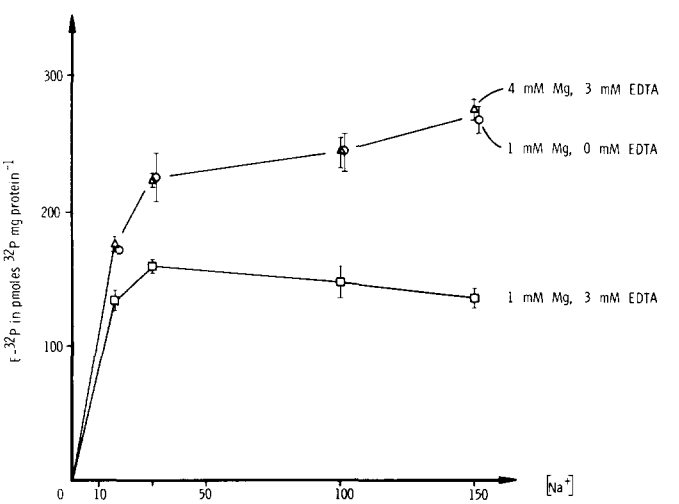

Fig. 1. The effect of Na^+ on phosphorylation of Na^+,K^+-ATPase. Phosphorylation time, 10 s; temp., 0°C. Medium contained: 1.2 mg of protein with the specific activity of 156.3 μM ATP hydrolyzed · mg protein^{-1} · h^{-1} (at 37°C), 25 μM ATP, 30 mM Tris/HCl, pH 7.4. Mean values ± S.D. (n = 3). ○———○, 1 mM Mg^{2+}; △———△, 4 mM Mg^{2+} + 3 mM EDTA; □———□, 1 mM Mg^{2+} + 3 mM EDTA.

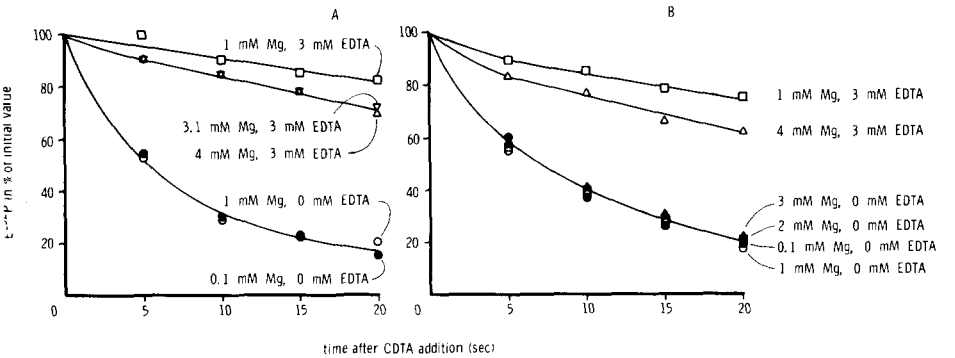

Fig. 2. The effect of Mg^{2+} and EDTA on spontaneous dephosphorylation at two pH values. Phosphorylation time, 10 s; temp., 0°C. Medium contained: 25 μM ATP, 16 mM Na^+, 30 mM Tris/HCl. (A) pH 7.4; (B) pH 8.0. ●——●, 0.1 mM Mg^{2+}; ○——○, 1 mM Mg^{2+}; ■——■, 2 mM Mg^{2+}; ▲——▲, 3 mM Mg^{2+}; □——□, 1 mM Mg^{2+} + 3 mM EDTA; △——△, 4 mM Mg^{2+} + 3 mM EDTA; △——△, 3.1 mM Mg^{2+} + 3 mM EDTA.

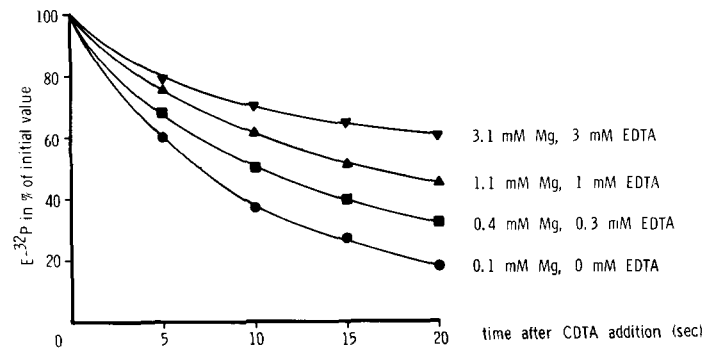

Fig. 3. The effect of EDTA on spontaneous dephosphorylation at constant free Mg^{2+} concentration. Phosphorylation time, 10 s; temp., 0°C. Medium contained: 25 μM ATP, 16 mM Na^+, 30 mM Tris/HCl, pH 8.0. Total Mg^{2+} and EDTA concentrations chosen gave free Mg^{2+} concentration of about 0.1 mM. ●——●, 0.1 mM Mg^{2+}; ■——■, 0.4 mM Mg^{2+} + 0.3 mM EDTA; ▲——▲, 1.1 mM Mg^{2+} + 1 mM EDTA; ▲——▲, 3.1 mM Mg^{2+} + 3 mM EDTA.

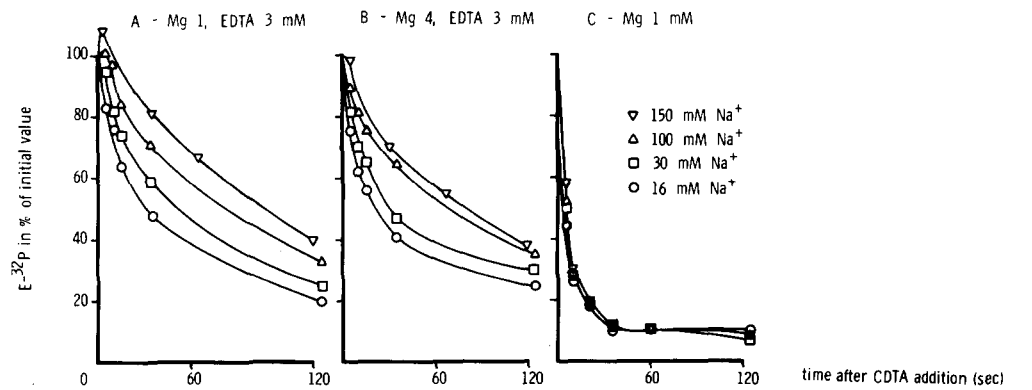

Fig. 4. The effect of Na^+ on spontaneous dephosphorylation. Phosphorylation time, 10 s; temp., 0°C. Medium contained: 25 μM ATP, 30 mM Tris/HCl, pH 8.0; (A) 1 mM Mg^{2+} + 3 mM EDTA; (B) 4 mM Mg^{2+} + 3 mM EDTA; (C) 1 mM Mg^{2+}. ○——○, 16 mM Na^+; □——□, 30 mM Na^+; △——△, 100 mM Na^+; ▽——▽, 150 mM Na^+.

Dephosphorylation

In the following experiments, the enzyme was phosphorylated in different media. After an incubation of 10 s, CDTA was added to a final concentration of 10 mM, to stop further phosphorylation. Dephosphorylation was then followed as a function of time: (a) without further additions i.e. spontaneous dephosphorylation; (b) after addition of potassium; (c) after addition of ADP.

The experimental results are described in relation to the conditions under which the phospho-enzyme is formed.

(a) Spontaneous dephosphorylation. As seen from Fig. 2A, the rate of spontaneous dephosphorylation is relatively fast and is the same for phospho-enzyme formed in the presence of 0.1 and 1 mM Mg^{2+}. However, if the phospho-enzyme is formed in the presence of 3 mM EDTA, but with about the same concentration of free Mg^{2+}, as above, i.e. 0.1 mM (3.1 mM Mg^{2+} + 3 mM EDTA) and 1 mM (4 mM Mg^{2+} + 3 mM EDTA) respectively, the rate of dephosphorylation is much slower. This effect of EDTA is also found at pH 8.0 (Fig. 2B).

It is also seen from Fig. 2 that in the presence of 3 mM EDTA the rate of spontaneous dephosphorylation is lower with 1 mM magnesium than with 3.1 or 4 mM magnesium.

The decrease in rate of dephosphorylation by EDTA increases with the EDTA concentration. This is seen from Fig. 3, where the concentration of free Mg^{2+} is kept constant at about 0.1 mM, while the concentration of EDTA is increased.

In the presence of EDTA, at a given Mg^{2+} and EDTA concentration, the rate of spontaneous dephosphorylation decreases with an increase in the Na^+ concentration, (Fig. 4A and B). This effect of Na^+ seems to be present also in the absence of EDTA in the medium (Fig. 4C): it is, however, not very pronounced and is only seen during the first 5 s of dephosphorylation.

(b) Effect of potassium on the dephosphorylation. In the following experiments the medium in which the enzyme was phosphorylated contained 150 mM Na^+, Mg^{2+}, EDTA and CDTA in the concentrations given in the figures. The phosphorylation was stopped after 10 s by addition of CDTA, and 5 s later K^+ was added. The reaction was stopped with $HClO_4$ after the times given in the figures. The amount of phospho-enzyme is given as a percentage of the amount at the time of K^+ addition.

Potassium added to the prephosphorylated enzyme increases the rate of dephosphorylation, the more so the higher the K^+ concentration (Fig. 5). There is no difference in the effect of K^+ with 1 mM Mg^{2+} (Fig. 5A), or with 1 mM Mg^{2+} and 1.1 mM CDTA (Fig. 5B), i.e. with concentrations of free Mg^{2+} of about 1 mM and 2 μM, respectively.

However, if the medium in which the enzyme is phosphorylated contains EDTA, the rate of dephosphorylation at a given K^+ concentration is lower than without EDTA (Fig. 6, compare with Fig. 5). The effect of EDTA on the K^+ effect of EDTA can apparently be overcome by an increase in the K^+ concenhigh concentration of free Mg^{2+} (4 mM Mg^{2+} + 3 mM EDTA, Fig. 6A) and with a low concentration of free Mg^{2+} (1 mM Mg^{2+} + 3 mM EDTA, Fig. 6B). The effect of EDTA can apparently be overcome by an increase in the K^+ concentration (compare Fig. 5 and Fig. 6).

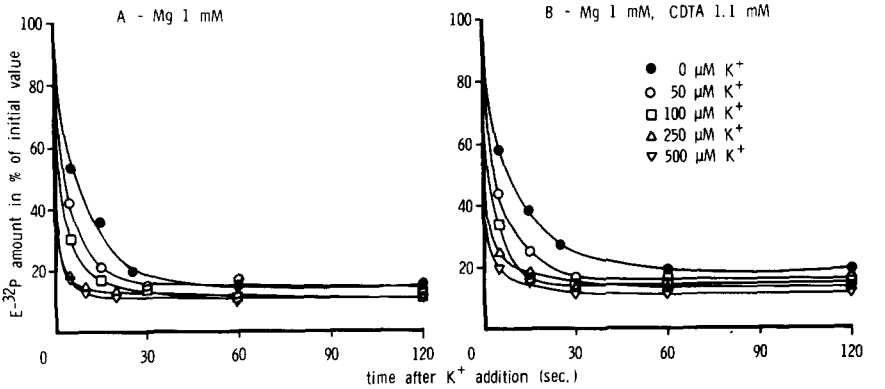

Fig. 5. The effect of Mg^{2+} on K^+-dependent dephosphorylation. Phosphorylation time 10 s; temp., 0°C. Medium contained: 25 μM ATP, 150 mM Na^+, 30 mM Tris/HCl, pH 7.4; (A) 1 mM Mg^{2+}; (B) 1 mM Mg^{2+} + 1.1 mM CDTA. Original amounts of E-^{32}P: (A) 232 ± 8 pmol ^{32}P · mg protein^{-1} and (B) 117 ± 13 pmol ^{32}P · mg protein^{-1}. Initial amounts of E-^{32}P at the moment of K^+ addition 122 ± 7 and 61 ± 4, respectively. ●———●, no K^+ added (spontaneous dephosphorylation); ○———○, 50 μM K^+; □———□, 100 μM K^+; △———△, 250 μM K^+; ▽———▽, 500 μM K^+.

The experiments shown in Fig. 5 and Fig. 6 have been repeated with a lower Na^+ concentration, 16 mM, (not shown). With this low Na^+ concentration a given potassium concentration gives a higher rate of dephosphorylation. For example with 1 mM Mg^{2+}, 40 μM of K^+ dephosphorylates 75% of the phosphoenzyme during the first 5 s, compared to 58% dephosphorylated by 50 μM K^+ during the same time, in a medium containing 150 mM Na^+.

EDTA shows the same effect on the dephosphorylation by K^+ with 16 mM Na^+ as with 150 mM Na^+, and the effect is independent of the Mg^{2+} concentration.

The experiments thus show that the dephosphorylating effect of potas-

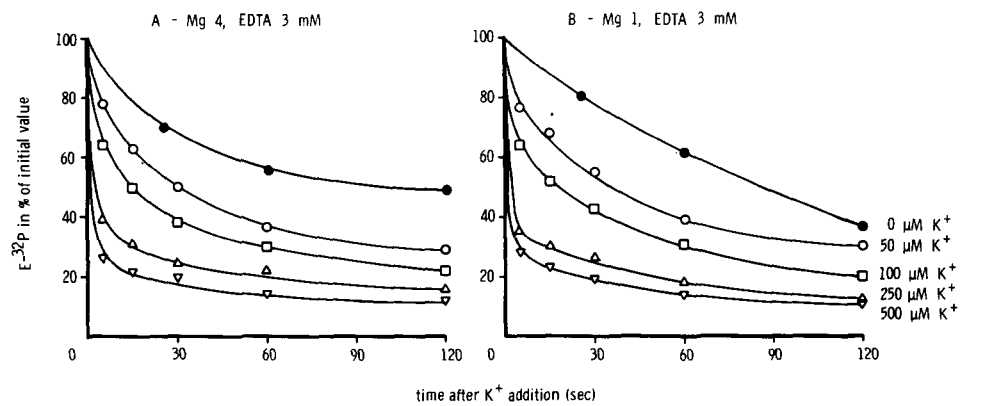

Fig. 6. The effect of Mg^{2+} on K^+-dependent dephosphorylation in the presence of EDTA. Phosphorylation time, 10 s; temp., 0°C. Medium contained: 25 μM ATP, 150 mM Na^+, 30 mM Tris/HCl, pH 7.4; (A) 4 mM Mg^{2+} + 3 mM EDTA; (B) 1 mM Mg^{2+} + 3 mM EDTA. Original amounts of E-^{32}P: (A) 219 ± 4 pmol ^{32}P · mg protein^{-1} and (B) 100 ± 16 pmol · mg protein^{-1}. Initial amounts of E-^{32}P at the moment of K^+ addition: 216 ± 2 and 111 ± 14 in A and B, respectively. ●———●, no K^+ added (spontaneous dephosphorylation); ○———○, 50 μM K^+; □———□, 100 μM K^+; △———△, 250 μM K^+; ▽———▽, 500 μM K^+.

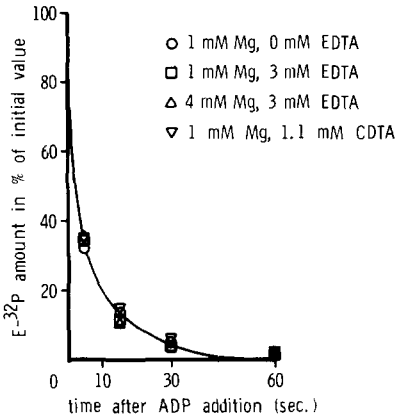

Fig. 7. The effect of Mg^{2+} on ADP-dependent dephosphorylation. Phosphorylation time, 10 s; temp., 0°C. Medium contained 25 μM ATP, 150 mM Na^+, 30 mM Tris/HCl, pH 7.4. 2.5 mM ADP added. ○———○, 1 mM Mg^{2+}; □———□, 1 mM Mg^{2+} + 3 mM EDTA; △———△, 4 mM Mg^{2+} + 3 mM EDTA; ▽———▽, 1 mM Mg^{2+} + 1.1 mM CDTA. For spontaneous dephosphorylation, see Figs 5 and 6.

sium is independent of the free magnesium concentration in the medium during phosphorylation, and that EDTA decreases the rate of dephosphorylation by a given potassium concentration independent of the free magnesium concentration in the medium.

(c) Effect of ADP on the dephosphorylation. The enzyme was phosphorylated for 10 s in the presence of 1 mM Mg^{2+}; 4 mM Mg^{2+} + 3 mM EDTA; 1 mM Mg^{2+} + 3 mM EDTA; 1 mM Mg^{2+} + 1.1 mM CDTA. The experiments were carried out in the presence of 150 mM Na^+ and in the presence of 16 mM Na^+, respectively. CDTA was added after 10 s and 5 s later ADP. In the figures the amount of phospho-enzyme at any given time is given as a percentage of the amount at the time of ADP addition.

Fig. 7 shows the results from experiments with 150 mM Na^+ and with addition of ADP in a concentration which is 100 times higher than the ATP/[γ-^{32}P]ATP concentration. It is seen that the rate of dephosphorylation in the presence of ADP is the same whether the enzyme is phosphorylated in a medium with a high or low concentration of free Mg^{2+}, in the presence or absence of EDTA or CDTA.

The same is found in experiments with ADP in concentrations 10, 25 and 50 times higher than that of ATP/[γ-^{32}P]ATP (not shown); the Mg^{2+} concentration in the medium does not affect the dephosphorylation by ADP.

In experiments with 16 mM Na^+ in the medium, the rate of dephosphorylation after ADP addition is the same as with 150 mM of Na^+ (not shown).

Discussion

In agreement with previous observations the enzyme requires magnesium and sodium for phosphorylation [10—27], and potassium increases the rate of dephosphorylation of the prephosphorylated enzyme [3,7,11,15—17,20,22, 28—31], and so does ADP [3,20,30—32].

The rate of the spontaneous and of the K^+-dependent dephosphorylation

is decreased by increase in the sodium concentration or by the presence of EDTA. The effect of Na^+ on the K^+-dependent dephosphorylation can be explained on the basis of a competition between sodium and potassium. The concentration of Na^+ required for this effect is high relative to the K^+ concentration, showing that it must be a competition at the site with a high affinity for K^+ relative to Na^+ [33]. That high concentrations of Na^+ are required to inhibit the effect of K^+ and the similarity between the effects of sodium and EDTA on both spontaneous and K^+-dependent dephosphorylation suggests that the spontaneous dephosphorylation is due to K^+ present in the enzyme preparation and reagents used (2—10 μM).

The experiments show that the phospho-enzyme formed in the presence of sodium and a high concentration of magnesium does not differ in its sensitivity towards K^+ and ADP from the phospho-enzyme formed in the presence of sodium and a low concentration of magnesium. This is in agreement with the observations of Fukushima and Tonomura [4]. It is, however, in disagreement with the observations of Post et al. [3], who found that the phospho-enzyme formed with a low concentration of Mg^{2+} was less sensitive to dephosphorylation by K^+ than the phospho-enzyme formed in the presence of a high Mg^{2+} concentration. The explanation of the discrepancy seems to be that Post et al. used EDTA to decrease the Mg^{2+} concentration. As seen from the present experiments, EDTA decreases the sensitivity towards K^+, but this is not due to the decrease in the concentration of free Mg^{2+}, but to an effect of EDTA per se.

Post et al. also found that the phospho-enzyme formed in the presence of 1 mM Mg^{2+} + 3 mM EDTA (low free Mg^{2+}) was more sensitive to dephosphorylation by ADP than the phospho-enzyme formed in the presence of 1 mM Mg^{2+} without EDTA (high free Mg^{2+}). As seen from the present experiments the rate of dephosphorylation by ADP is the same with and without EDTA suggesting no difference in sensitivity.

The explanation of the discrepancy seems to be that the EDTA-phospho-enzyme has a lower rate of spontaneous dephosphorylation than the non-EDTA-phospho-enzyme (see above). It means that ADP gives a higher increase in rate of dephosphorylation of EDTA- than of non-EDTA-phospho-enzyme and that therefore the EDTA-phospho-enzyme apparently has a higher sensitivity towards ADP.

The experiments thus show that the effect of potassium and of ADP on the dephosphorylation is independent of the magnesium concentration used for formation of the phospho-enzyme. Furthermore, that EDTA, independent of the Mg^{2+} concentration, decreases the rate at which K^+ dephosphorylates the phospho-enzyme, while EDTA has no effect on the rate of dephosphorylation by ADP.

But why is it that EDTA decreases the rate of spontaneous and of K^+-dependent dephosphorylation? There seems to be at least three possibilities. One is that the phospho-enzyme formed in the presence of EDTA differs from that formed without EDTA. Another is that EDTA in the medium interferes with the dephosphorylation reaction. A third is that EDTA decreases the rate by which CDTA stops the phosphorylation. And as will be seen from a subsequent paper, this last seems to be the explanation.

A comparison between Figs 5, 6 and 7 shows that there is a certain amount (10—13%) of the phospho-enzyme which is not dephosphorylated by the addition of K^+, but is dephosphorylated by ADP. This amount is the same independent of the Mg^{2+} concentration. As discussed in Methods, experiments with [U-^{14}C]ATP show that the enzyme preparation contains an amount of bound [U-^{14}C]ATP which corresponds to about 10—13% of the amount of ^{32}P-binding sites, and which can be displaced by ADP but not by potassium. It suggests that the 10—13% of ^{32}P binding, which cannot be displaced by potassium, is [γ-^{32}P]ATP bound to the protein and does not represent phospho-enzyme.

The experiments thus give no support for a magnesium concentration-dependent formation of two phospho-enzymes with different ADP and potassium sensitivities. How then to explain the lower requirement for magnesium for the Na^+-dependent ADP-ATP exchange reaction than for the Na^+, K^+-dependent hydrolysis? For a discussion of this problem, see e.g. Fukushima and Tonomura [4] and Skou [33].

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