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THE EFFECT OF CHELATORS ON Mg^{2+}, Na^+-DEPENDENT PHOSPHORYLATION OF (Na^+ + K^+)-ACTIVATED ATPase

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Summary

1. The effect of free Mg^{2+}, MgEDTA and MgCDTA on the phosphorylation of the (Na^+ + K^+)-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been studied.

2. 10 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) added simultaneously with [γ-^{32}P]ATP to a solution containing the enzyme, 1 mM Mg^{2+} and 150 mM Na^+ does not prevent formation of phospho-enzyme. When [γ-^{32}P]ATP is added after CDTA the level of phospho-enzyme obtained decreases with increase in the time interval between addition of CDTA and ATP. The inability of CDTA to prevent the formation of phospho-enzyme becomes more pronounced when the medium contains MgEDTA. In the presence of CDTA the maximum amount of phospho-enzyme formed increases with the MgEDTA concentration.

3. Without CDTA the steady-state level of phospho-enzyme is directly proportional to the logarithm of free Mg^{2+} concentration. Neither with suboptimal nor with optimal concentrations of free Mg^{2+} does MgEDTA have an effect on the level of phospho-enzyme formed.

4. Using the phospho-enzyme level as a measure of free Mg^{2+} the experiments show that CDTA reacts slower with Mg^{2+} than does EDTA, but the stability constant of MgCDTA complex is higher than of MgEDTA complex.

5. Due to the higher stability constant of MgCDTA, as compared to MgEDTA, addition of CDTA to a medium containing free Mg^{2+} and MgEDTA will not only chelate the free Mg^{2+}, but it will also shift the equilibrium from MgEDTA towards MgCDTA, i.e. MgEDTA acts as a source of free Mg^{2+} which is then chelated by CDTA. The experiments show that it takes minutes before Mg^{2+}, EDTA and CDTA come to equilibrium. Provided the dissociation of MgEDTA is faster than the formation of the MgCDTA complex, the medium will contain a concentration of free Mg^{2+} which at any given instant is near in equilibrium with a slowly decreasing concentration of MgEDTA; this free Mg^{2+}

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

can support phosphorylation. This can explain why the rate with which CDTA stops phosphorylation decreases with an increase in the MgEDTA concentration.

6. When phosphorylation is stopped by addition of unlabelled ATP, the rate of dephosphorylation is faster than when it is stopped by addition of CDTA both with and without EDTA in the medium. CDTA reacts too slowly with Mg^{2+} to be used as a chelator in studies where a fast removal of Mg^{2+} is required.

7. A previous finding has been verified, namely that the rate of spontaneous, of K^+-stimulated and of ADP-stimulated dephosphorylation is independent of the Mg^{2+} concentration during formation of phospho-enzyme.

Introduction

$(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is phosphorylated by ATP in the presence of Na^+ and Mg^{2+} (for references see ref. 1). When phosphorylation is stopped by chelation of the Mg^{2+} by excess CDTA, the rate of dephosphorylation is slow; it is increased by addition of K^+ [1,2]. As shown in a previous paper [1] EDTA, in concentrations which do not interfere with phosphorylation, decreases the rate of dephosphorylation when the phosphorylation is stopped by addition of CDTA in 10—5000-fold excess over free Mg^{2+}. EDTA decreases both the rate of dephosphorylation seen in the presence of Na^+ (which will be referred to as spontaneous dephosphorylation) and the rate of dephosphorylation seen after addition of potassium. CDTA present in the medium during phosphorylation does not have an EDTA effect, suggesting that the effect of EDTA is not due to chelation of a heavy metal ion, but due to EDTA as such.

There seem to be at least three possible explanations of the EDTA effect. 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 possibility is that EDTA in the medium decreases the rate by which CDTA stops phosphorylation so that the observed lower rate of dephosphorylation in the presence of EDTA would only be apparent. As will be seen from the present paper, this last possibility seems to be the explanation.

Materials and Methods

The $(Na^+ + K^+)$-activated ATPase (EC 3.6.13) from ox brain was prepared according to Klodos et al. [3] and the enzyme was washed free of EDTA. Activity of the enzyme measured with 3 mM ATP, 100 mM Na^+, 20 mM K^+, 30 mM histidine buffer, pH 7.4, at 37°C was about 2.6—3.5 μM ATP hydrolysed/mg protein per min and contained less than 1% of Mg^{2+}-activated ATPase (measured in the presence of $1 \cdot 10^{-3}$ M g-strophanthin). The enzyme (about 2 mg protein/ml) was stored at —20°C with loss of about 2% of activity per month.

Reagents. ATP labelled in the γ-position with ^{32}P was obtained from The Radiochemical Centre, Amersham, England and the sodium salts of ATP and ADP from Boehringer, Germany. [γ-^{32}P]ATP and ATP were purified and con-

verted to their Tris salts by chromatography on a DEAE-Sephadex G-25 (Pharmacia, Uppsala, Sweden) column [4]. EDTA was from Merck, Darmstadt, and CDTA from B.D.H. All other reagents used were of reagent grade.

Assay. The assay of phosphorylation and measurement of the amount of phosphorylated protein were carried out as described previously [1].

All results in the figures are mean values of 3—5 experiments with standard deviations shown in Fig. 3 and Table I. Concentrations of free Mg^{2+} were calculated according to stability constants given by Martell [5—7] taking into account the effect of temperature and pH ($1 \cdot 10^{5.37}$ M^{-1} for MgEDTA; $1 \cdot 10^{5.95}$ M^{-1} for MgCDTA; $1 \cdot 10^{4}$ M^{-1} for MgATP).

Results and Discussion

Effect of Mg^{2+} and EDTA

(a) Effect of EDTA on dephosphorylation after addition of CDTA or unlabelled ATP. The enzyme was phosphorylated for 10 s at 0° C in a medium containing 25 μM ATP, 150 mM Na^+, 30 mM Tris · HCl, pH 7.4 (at 0° C) and in one set of experiments with 1 mM Mg^{2+} and in another with 4 mM Mg^{2+} plus 3 mM EDTA, i.e. in both sets of experiments with a concentration of free Mg^{2+} of about 1 mM. The amounts of phospho-enzyme formed in these two media are the same and steady-state levels were obtained at times shorter than 10 s. After 10 s the phosphorylation of the enzyme was stopped, either by addition of 10 mM CDTA or of 1 mM unlabelled ATP and the rate of dephosphorylation (without further additions = spontaneous dephosphorylation) was followed; the results are presented in Fig. 1.

When phosphorylation of the enzyme is stopped by the addition of 10 mM CDTA, the rate of spontaneous dephosphorylation is highly dependent on the conditions under which the enzyme has been phosphorylated. With 4 mM Mg^{2+} plus 3 mM EDTA in the medium the rate of spontaneous dephosphorylation is much slower than when the enzyme has been phosphorylated in the presence of 1 mM Mg^{2+} without EDTA in the medium. This difference in rates of spontaneous dephosphorylation is not observed with the phosphorylation is stopped by unlabelled ATP instead of CDTA. Moreover, the spontaneous dephosphorylation after the addition of unlabelled ATP is much faster than the dephosphorylation after the addition of CDTA. This difference is most pronounced for the phospho-enzyme formed in the presence of EDTA, but also exists for the phospho-enzyme formed without EDTA.

There seem to be two possible explanations of this difference. One is that CDTA does not stop the phosphorylation as fast as unlabelled ATP, and that this lack of effect of CDTA is more pronounced in the presence of EDTA. The other that CDTA interferes with dephosphorylation. If the first explanation is correct, then unlabelled ATP added after CDTA should increase the rate of dephosphorylation, while if the second explanation is correct there should be no effect of addition of unlabelled ATP, provided that the unlabelled ATP does not contain ADP (ADP-dependent dephosphorylation).

Fig. 2 shows that the addition of unlabelled ATP after addition of CDTA leads to an increased rate of dephosphorylation. The ADP content of the ATP used was less than 1.7%, i.e. 17 μM in the experiment shown in Fig. 2. In con-

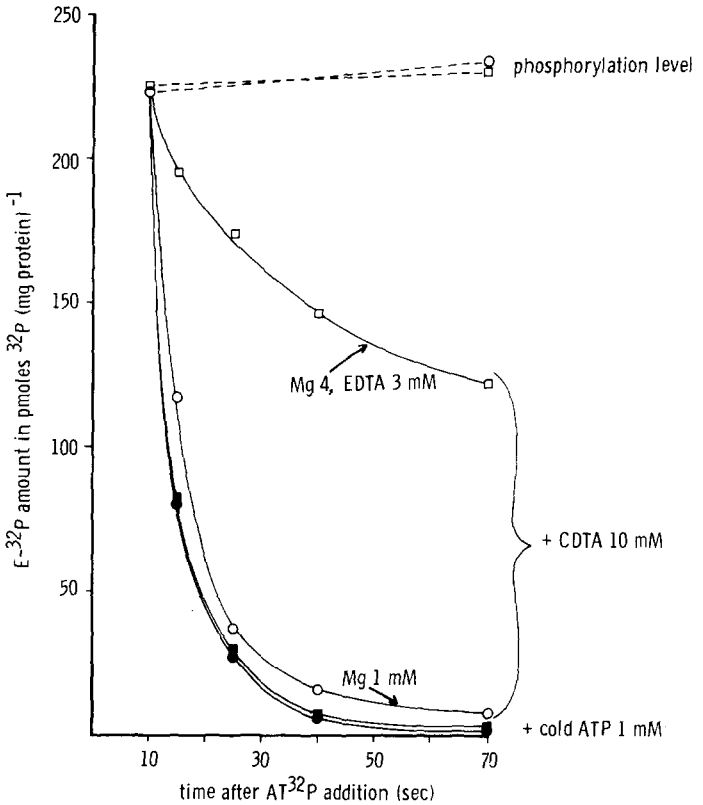

Fig. 1. The dephosphorylation of phospho-enzyme after addition of 10 mM CDTA or of 1 mM unlabelled ATP to phospho-enzyme formed in the presence of 1 mM Mg^{2+} or in the presence of 4 mM Mg^{2+} plus 3 mM EDTA. Medium contained: 25 μM [γ-^{32}P]ATP, 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C) and 1 mM $MgCl_2$ (○●) or 4 mM $MgCl_2$ plus 3 mM EDTA (□■). Phosphorylation time, 10 s; temperature, 0°C. Dashed line, phosphorylation control; unbroken line, phosphorylation stopped by 10 mM CDTA (empty symbols), or 1 mM cold ATP (filled symbols). Results are presented in pmol ^{32}P/mg protein after subtraction of appropriate blanks of unspecific phosphorylation. The following blanks were used for control of phosphorylation level: 150 mM KCl, 1 mM $MgCl_2$ (or 4 mM $MgCl_2$ plus 3 mM EDTA) (blank was about 22 pmol ^{32}P/mg protein); for the spontaneous dephosphorylation with CDTA: 10 mM CDTA, 150 mM choline chloride (blank was about 26 pmol ^{32}P/mg protein); for spontaneous dephosphorylation with unlabelled ATP: 150 mM NaCl, 1 mM $MgCl_2$ (or 4 mM $MgCl_2$ plus 3 mM EDTA) and 1 mM unlabelled ATP added prior to [γ-^{32}P] ATP (blank was about 13 pmol/mg protein).

trol experiments with phosphorylation under the same conditions as above (10 s of phosphorylation at 0°C followed by addition of CDTA) the same dephosphorylation rate as seen in Fig. 2 after addition of ATP required at least 100 μM ADP (not shown).

The effect of addition of unlabelled ATP after CDTA therefore suggests that CDTA does not stop the phosphorylation of the enzyme as fast as unlabelled ATP. Further evidence for this view will be found in the following experiments.

(b) Effect of MgEDTA on phosphorylation. Fig. 3 shows that CDTA added 5 s before ATP almost completely prevents formation of phospho-enzyme when the system is preincubated for 10—15 min in the presence of 150 mM Na^+ and 1 mM Mg^{2+} and no EDTA. If, however, the enzyme is preincubated with 150

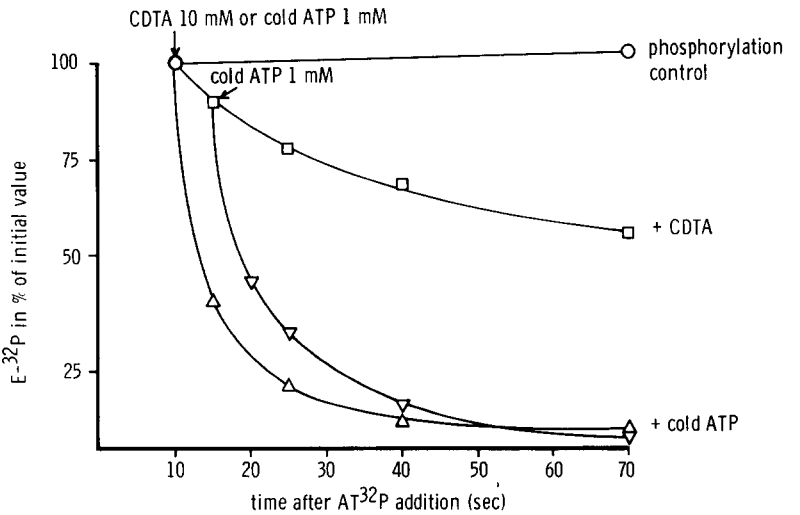

Fig. 2. The spontaneous dephosphorylation of phospho-enzyme after addition of CDTA, of unlabelled ATP or of CDTA followed by unlabelled ATP. Medium contained: 25 μM [γ-^{32}P]ATP, 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C), 4 mM $MgCl_2$ plus 3 mM EDTA. Phosphorylation time, 10 s; temperature 0°C. ○———○, phosphorylation control; □———□, 10 mM CDTA added after 10 s of phosphorylation; △———△, 1 mM unlabelled ATP added after 10 s of phosphorylation; ▽———▽, 10 mM CDTA added after 10 s of phosphorylation. 1 mM unlabelled ATP was added 5 s after the CDTA.

mM Na^+ and 4 mM Mg^{2+} in the presence of 3 mM EDTA, CDTA is not able to prevent phosphorylation.

This effect of EDTA is not only seen when the enzyme has been preincubated for 10—15 min with Mg^{2+} and EDTA before addition of CDTA, but also when Mg^{2+} and EDTA are added 5 s before CDTA, and even when EDTA and

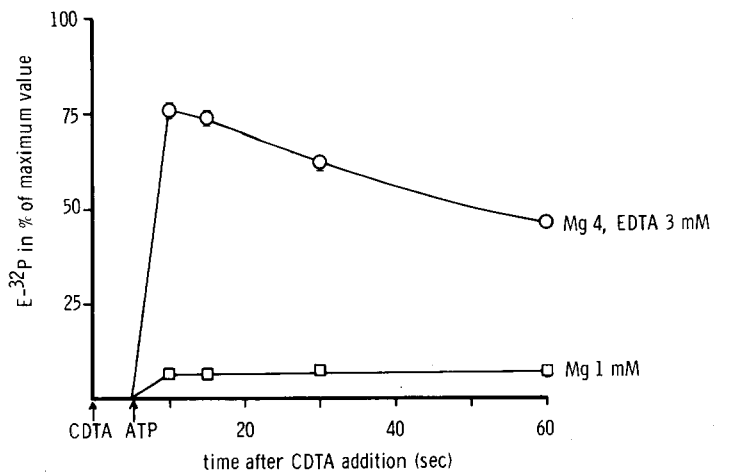

Fig. 3. The effect of CDTA on the level of phospho-enzyme phosphorylated in the presence of 1 mM $MgCl_2$ and of 4 mM $MgCl_2$ plus 3 mM EDTA. 10 mM CDTA and, 5 s later, 25 μM [γ-^{32}P] ATP were added to a medium containing enzyme, 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C), 1 mM $MgCl_2$ (□) or 4 mM $MgCl_2$ plus 3 mM EDTA (○). Values ± S.D. (n = 5). Control phosphorylation level without addition of CDTA (100%) was 254 ± 8 pmol ^{32}P/mg protein.

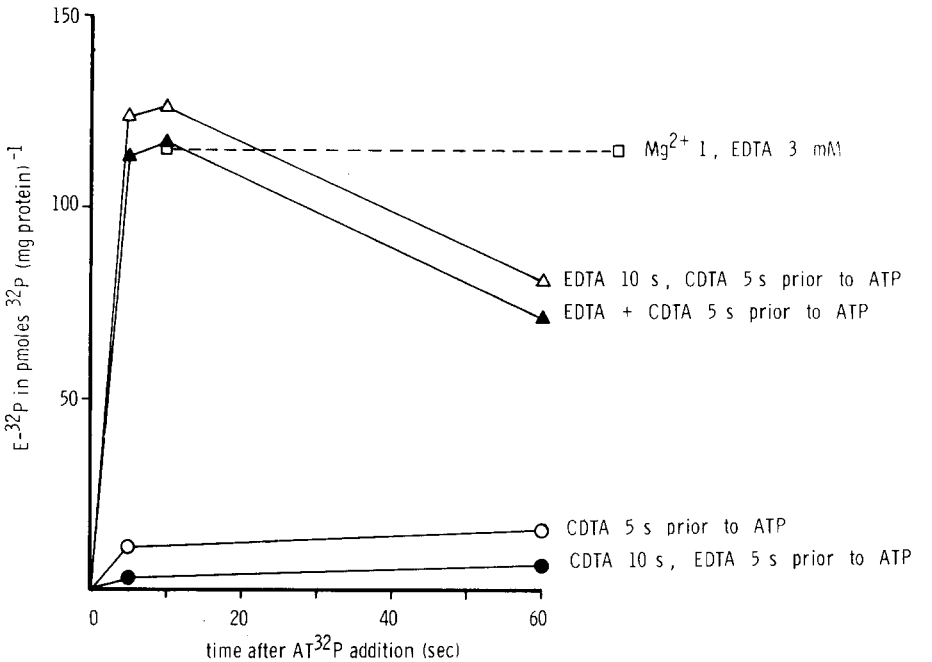

Fig. 4. The effect of CDTA on the phosphorylation of the enzyme with and without EDTA in the medium. Medium contained 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C) and 1 mM $MgCl_2$. Temperature, 0°C. The reaction was started by the addition of 25 μM [γ-^{32}P]ATP at zero time. □- - - - -□, phosphorylation control with 1 mM Mg^{2+} plus 3 mM EDTA but without CDTA; ○———○, 10 mM CDTA added 5 s prior to the addition of ATP; ●———●, 10 mM CDTA added 10 s and 3 mM EDTA 5 s prior to the addition of ATP; △———△, 3 mM EDTA added 10 s and 10 mM CDTA 5 s prior to the addition of ATP; ▲———▲, 3 mM EDTA and 10 mM CDTA added simultaneously 5 s prior to the addition of ATP.

CDTA are added simultaneously. This is shown in Fig. 4, where the concentrations of Mg^{2+}, EDTA and CDTA are 1, 3 and 10 mM, respectively (1 mM Mg^{2+} plus 3 mM EDTA give about 40% of the maximum amount of phospho-enzyme which can be obtained with saturating concentrations of Mg^{2+}). CDTA added 5 s prior to ATP, only seems to prevent phosphorylation when it is added before EDTA or without EDTA.

CDTA has a higher affinity for Mg^{2+} than EDTA, but is claimed to react more slowly with divalent cations than EDTA [8]. This means that the formation of a MgEDTA complex is still possible even when CDTA and EDTA are added simultaneously. The experiments shown in Fig. 4, where EDTA and CDTA are added simultaneously and in concentrations which chelate all free Mg^{2+} seem to suggest that phosphorylation can be obtained without free Mg^{2+}, but with only MgEDTA and MgCDTA in the medium.

In the absence of CDTA, at a given concentration of free Mg^{2+} the amount of phospho-enzyme formed is independent of the concentration of MgEDTA. This is shown in Fig. 5, where the concentration of free Mg^{2+} is 100 μM and the concentration of MgEDTA 1, 2, 3 and 4 mM. However, if under the same conditions, 10 mM CDTA (a concentration high enough to chelate all free Mg^{2+}) is added 5 s prior to ATP, phosphorylation is not prevented and the amount of phospho-enzyme formed increases with the MgEDTA concentration.

Fig. 5 (and Fig. 4) also shows that the level of phospho-enzyme stays constant for at least 60 s in the presence of free Mg^{2+} + MgEDTA while with MgEDTA + CDTA it decreases with time. The decrease is not caused by denaturation of the enzyme in the presence of CDTA. This possibility was tested in experiments in which the enzyme was preincubated with CDTA for 5—300 s followed by the addition of 4 mM Mg^{2+} plus 3 mM EDTA and 5 s later 25 μM ATP. The same amount of phospho-enzyme was formed independently of the duration of preincubation with CDTA.

10 mM CDTA should be more than enough to chelate the Mg^{2+} (1 mM or less) not chelated by EDTA. Therefore, the results of the experiments shown in

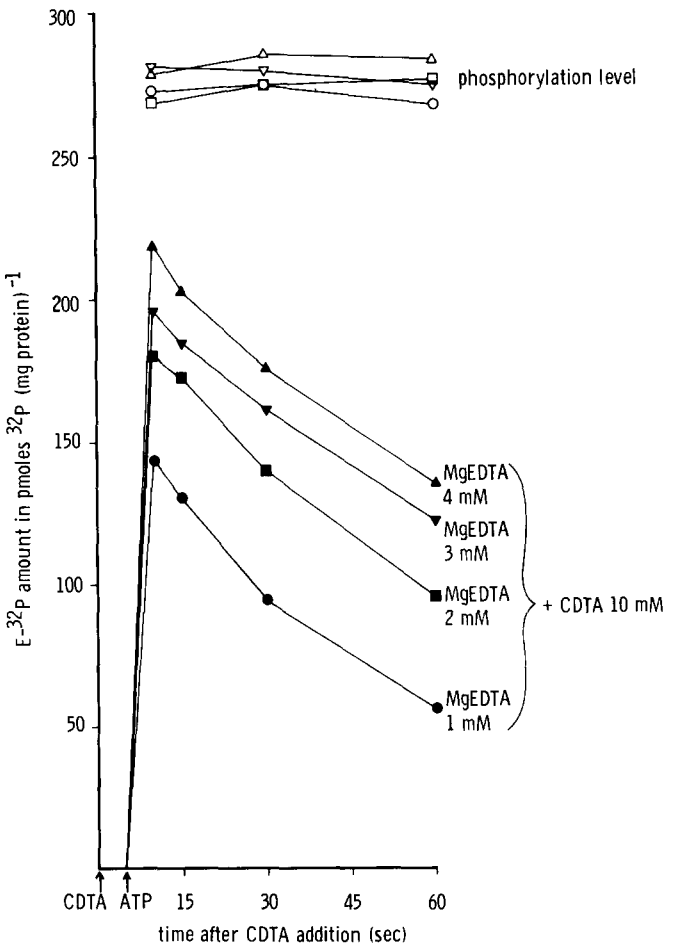

Fig. 5. The effect of 100 μM free Mg^{2+} and varying concentrations of MgEDTA on the formation of phospho-enzyme. In one set of experiments 10 mM CDTA was added 5 s prior to the addition of 25 μM [γ-^{32}P]ATP (filled symbols). In another set of experiments, the control, no CDTA was added (empty symbols). Temperature, 0°C. Medium contained: 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C), and 1.07 mM $MgCl_2$ plus 1 mM EDTA (= 99 μM free Mg^{2+} and 0.96 mM MgEDTA) (○); 2.03 mM $MgCl_2$ plus 2 mM EDTA (= 100 μM free Mg^{2+} and 1.92 mM MgEDTA) (□); 2.995 mM $MgCl_2$ plus 3 mM EDTA (= 102 μm free Mg^{2+} and 2.88 mM MgEDTA) (▽); 3.95 mM $MgCl_2$ plus 4 mM EDTA (= 100 μM free Mg^{2+} and 3.84 mM MgEDTA) (△).

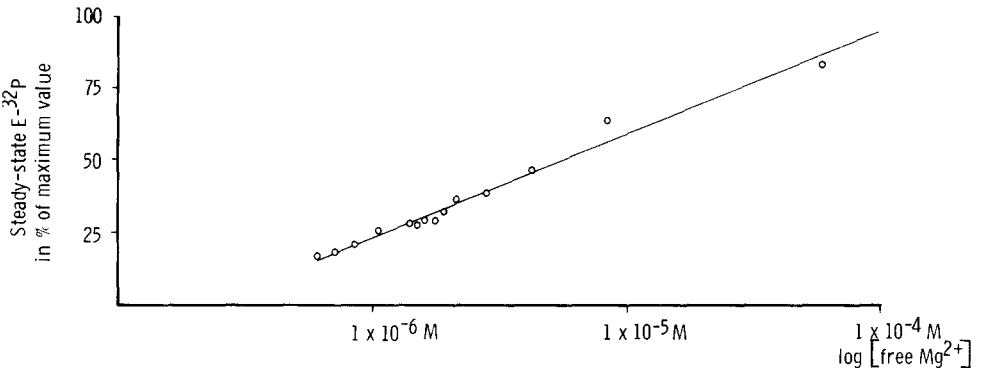

Fig. 6. The steady-state level of phosphorylation of the enzyme as a function of the concentration of free Mg^{2+}. Medium contained: 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C), 25 μM [γ-^{32}P]ATP, 1 mM $MgCl_2$ and 1—10 mM EDTA. Phosphorylation time, 60 s; temperature, 0°C. Control phosphorylation level with 1 mM free Mg^{2+} (100%) = 212 ± 6 pmol ^{32}P/mg protein (n = 3).

Fig. 3, 4 and 5 seem to suggest that MgEDTA in the presence of CDTA can support formation of phospho-enzyme. As CDTA has a higher affinity for Mg^{2+} than EDTA the addition of 10 mM CDTA must lead to a gradual decrease in MgEDTA concentration and increase in MgCDTA concentration. Assuming that MgEDTA can support formation of phospho-enzyme, this gradual decrease in MgEDTA after addition of CDTA could explain the decrease in the level of phospho-enzyme seen as a function of time after CDTA has been added.

(c) Effect of free Mg^{2+} on phosphorylation. If it is correct, that MgEDTA can support phosphorylation it should also support formation of phospho-enzyme in the absence of CDTA and with concentrations of free Mg^{2+} too low to give maximum phosphorylation. This was tested in experiments where the concentration of MgEDTA was varied 30-fold at two fixed concentrations of free Mg^{2+}. The concentrations of free Mg^{2+} chosen, 2.1 and 8.3 μM, give about 40 and 60% of maximal phosphorylation, respectively. The results in Table I show that in the absence of CDTA phosphorylation at a given concentration of free Mg^{2+} is completely unaffected by changes in the concentration of MgEDTA. This suggests that MgEDTA cannot support phosphorylation, but only free Mg^{2+} can. Further support for this is given in Fig. 6 which shows that the steady-state level of phospho-enzyme is proportional to the logarithm of free Mg^{2+} concentration (up to $1 \cdot 10^{-4}$ M).

(d) Effect of CDTA on dephosphorylation in the presence of EDTA. It can be concluded from the above results that neither MgEDTA nor MgCDTA support phosphorylation, only free Mg^{2+} does. But why is there phosphorylation under conditions where the medium contains MgEDTA and CDTA in such a high concentration that it should have chelated all the free Mg^{2+}?

Although, as mentioned above, CDTA has a higher affinity for Mg^{2+} than EDTA has, it reacts relatively slowly with Mg^{2+}. This is seen from results presented in Fig. 7 in which phosphorylation by ATP was started at various times after addition of CDTA or EDTA to enzyme preincubated with Na^+ and Mg^{2+}.

Comparison of the effects of equimolar concentrations of CDTA (Fig. 7A) and EDTA (Fig. 7B) shows: (1) The final steady-state level of phospho-enzyme

TABLE I

THE EFFECT OF MgEDTA ON PHOSPHORYLATION OF THE ENZYME AT TWO CONCENTRATIONS OF FREE Mg^{2+}

Medium contained: 1.2 mg of protein, 25 μM ATP, 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (At 0°) and $MgCl_2$ and EDTA in concns. given in the table. Concentrations are given in μM.

Mg^{2+} total added	EDTA total	Mg^{2+} free	MgEDTA	EDTA free	MgATP	ATP free	E-^{32}P * in pmol ^{32}P/mg protein after phosphorylation for		
							5 s (n = 4)	10 s (n = 2)	60 s (n = 2)
36	100	2.13	33	67	0.52	24.48	68 ± 5	75 ± 5	81 ± 0.5
169	500	2.13	166	334	0.52	24.48	61 ± 6	72 ± 5	74 ± 3
335	1000	2.12	332	668	0.52	24.48	62 ± 3	72 ± 1	74 ± 0.5
500	1500	2.12	497	1003	0.52	24.48	58 ± 2 **	69 ± 3	74 ± 0.1
667	2000	2.12	664	1336	0.52	24.48	59 ± 4	67 ± 3	73 ± 3
1000	3000	2.12	997	2003	0.52	24.48	59 ± 2	68 ± 1	74 ± 3
76	100	8.23	66	34	1.90	23.10	125 ± 1	130 ± 4	131 ± 7
340	500	8.27	330	170	1.91	23.09	127 ± 6	134 ± 5	130 ± 1
670	1000	8.27	660	340	1.91	23.09	126 ± 2	131 ± 0.1	130 ± 1
1000	1500	8.28	990	510	1.91	23.09	123 ± 1	127 ± 3	128 ± 4
1330	2000	8.28	1320	680	1.91	23.09	123 ± 4	128 ± 5	129 ± 3
1990	3000	8.28	1980	1020	1.91	23.09	123 ± 2	131 ± 2	131 ± 1
1000	—	977	—	—	22.7	2.3	206 ± 0.2	208 ± 7	204 ± 13

* E-^{32}P, ^{32}P-labelled phospho-enzyme.

** n = 3.

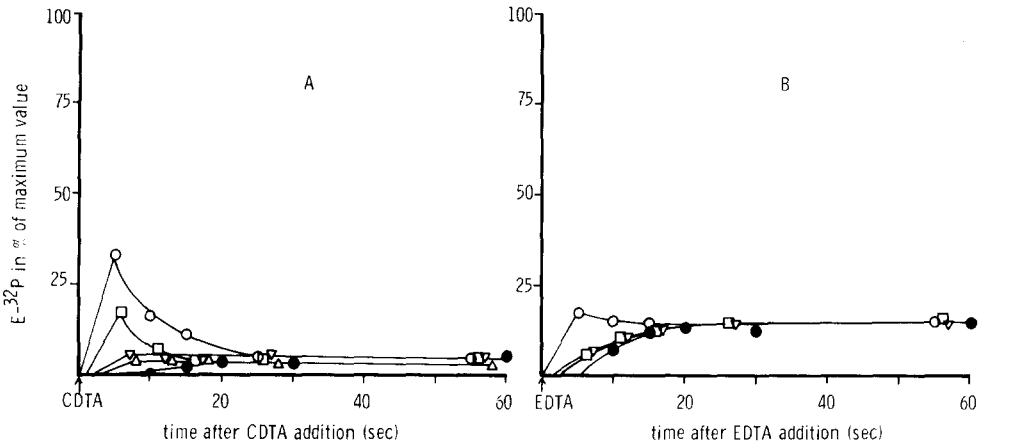

Fig. 7. The phosphorylation of the enzyme in the presence of chelons added either simultaneously with or prior to the addition of ATP. A, effect of 10 mM CDTA; B, effect of 10 mM EDTA. Medium contained: 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C), 1 mM $MgCl_2$. Temperature, 0°C. ○———○, chelon added together with 25 μM [γ-^{32}P]ATP; □———□, chelon added 1 s before addition of 25 μM [γ-^{32}P]-ATP; ▽———▽, chelon added 2 s before addition of 25 μM [γ-^{32}P]ATP; △———△, chelon added 3 s before addition of 25 μM [γ-^{32}P]ATP; ●———●, chelon added 5 s before addition of 25 μM [γ-^{32}P]-ATP. Control level of phosphorylation without addition of chelons (100%) = 286 ± 15 pmol ^{32}P/mg protein (n = 2).

with CDTA is lower than with EDTA. According to Fig. 6 this shows that the concentration of free Mg^{2+} is lower with CDTA than with EDTA; moreover, even 10 mM EDTA does not chelate enough of the added 1 mM Mg^{2+} to prevent formation of phospho-enzyme. (2) The final level of phospho-enzyme is attained faster with EDTA which shows that the rate of removal of free Mg^{2+} is higher in the presence of this chelon. (3) CDTA added simultaneously with ATP does not prevent phosphorylation, even in the absence of EDTA. The maximum amount of phospho-enzyme obtained decreases as the time interval between the addition of CDTA and ATP increases. There is no phosphorylation when the interval is 5 s or more (compare with Fig. 3).

Due to the higher affinity for Mg^{2+} of CDTA than of EDTA, CDTA added to a medium containing free Mg^{2+} and MgEDTA will not only chelate the free Mg^{2+}, but also shift the equilibrium from MgEDTA towards MgCDTA and free EDTA, i.e. MgEDTA acts as a source of free Mg^{2+} which is then chelated by CDTA. But the reaction of CDTA with free Mg^{2+} is slow and as seen from Fig. 8, it takes minutes before equilibrium is reached. The final equilibrium concentration of free Mg^{2+} should be about 2 nM and, according to Fig. 6, phosphorylation should not be observed with this concentration of free Mg^{2+}. However, in the experiments shown in Fig. 8, even 6 min after the addition of CDTA there is a phosphorylation of about 16% of maximum. According to Fig. 6 this corresponds to a concentration of free Mg^{2+} of 600 nM.

To explain the phosphorylation seen after addition of CDTA in the presence of MgEDTA, because of the results of Fig. 6, it is necessary to assume that there is free Mg^{2+} in the medium, at least a certain time after the addition of CDTA. Since the effectiveness of CDTA in preventing phosphorylation decreases with increasing MgEDTA concentration, the source of free Mg^{2+} must

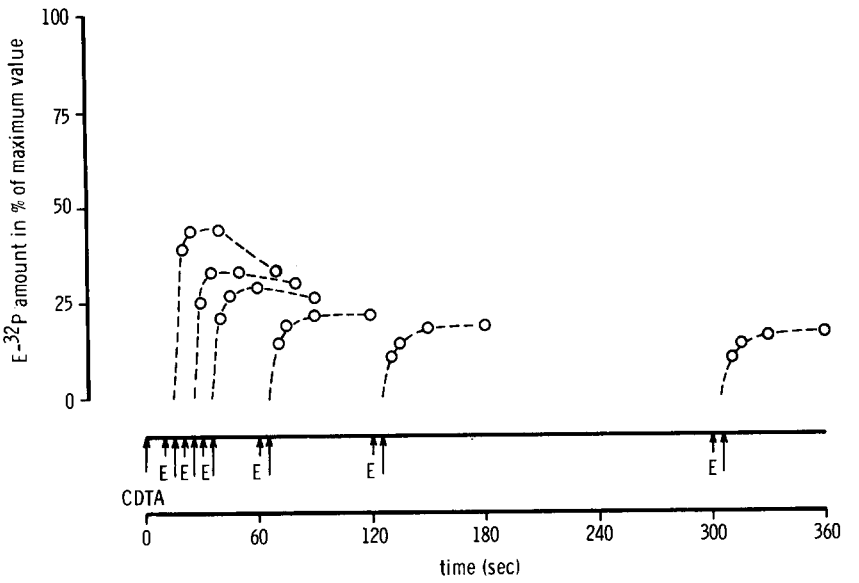

Fig. 8. The effect of preincubation of Mg^{2+}, EDTA and CDTA on the phosphorylation. CDTA was added at zero time to a mixture of 4 mM $MgCl_2$, 3 mM EDTA, 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C). Enzyme was added (↓) from 10 to 300 s later and 25 μM [γ-^{32}P]ATP was added 5 s after the enzyme (↑). The reaction was stopped 5, 10, 25 or 55 s after addition of ATP. Temperature, 0°C. The amount of phospho-enzyme is given in percent of control which was measured in the presence of 4 mM $MgCl_2$ plus 3 mM EDTA and which was 280 ± 19 pmol ^{32}P/mg protein (n = 3).

be the MgEDTA. Provided the dissociation of Mg^{2+} from EDTA is faster than the rate of complexing of Mg^{2+} with CDTA the medium will contain a concentration of free Mg^{2+} which at any instant is nearly in equilibrium with the remaining concentration of MgEDTA and which, according to Fig. 8, will decrease slowly with time; this free Mg^{2+} will support phosphorylation.

There are thus two problems related to the use of CDTA as a magnesium chelator in investigations on the rate of dephosphorylation of the phospho-enzyme, at least at 0°C and pH 7.4. One is that CDTA by itself reacts too slowly with free magnesium to give an immediate stop of the phosphorylation; the other is that in the presence of MgEDTA the rate by which CDTA can remove free Mg^{2+} is further delayed because MgEDTA acts as a buffer for free Mg^{2+}.

The experiments show that it is the Mg^{2+} not complexed by EDTA and CDTA that is necessary for phosphorylation. This does not necessarily mean that it is free Mg^{2+} which supports phosphorylation. Variation in the concentration of Mg^{2+} not complexed by EDTA and CDTA is dependent upon variation of other chelator, such as ATP, and is reflected in variations in the concentrations of free Mg^{2+}, MgATP and free ATP. And it is unknown which of these three components is/are necessary for phosphorylation.

Effect of Mg^{2+} on K^+-dependent and on ADP-dependent dephosphorylation

That CDTA is unable to stop phosphorylation immediately means that the rate of disappearance of phospho-enzyme measured after addition of CDTA

(to chelate Mg^{2+}) is not a real expression of the rate of dephosphorylation [1,2]. The observed fall in the amount of phospho-enzyme is the resultant of a dephosphorylation rate and a phosphorylation rate that decreases with time. MgEDTA will further decrease the rate by which CDTA stops the phosphorylation.

Phosphorylation in the sense of ^{32}P incorporation from [γ-^{32}]ATP can be stopped not only by removal of Mg^{2+}, but also by dilution of radioactive ATP; this can be done by adding unlabelled ATP.

If the above explanation of the effect of EDTA on dephosphorylation in the presence of CDTA is corect then the rate of dephosphorylation after addition of unlabelled ATP should be independent of EDTA. As seen from Fig. 9 (and Fig. 1), this seems to be the case both for the rate of spontaneous and of K^+-dependent dephosphorylation. It can also be seen from a comparison of Figs. 9A and 9B that the rates of spontaneous and K^+-dependent dephosphorylation after addition of unlabelled ATP are independent of the concentration of free Mg^{2+}.

Since ADP competes with ATP for binding sites on the enzyme [9.10] it can also stop phosphorylation from [γ-^{32}P]ATP. As seen from Fig. 10, the rate of dephosphorylation after addition of ADP is also independent of the concentrations of EDTA and free Mg^{2+}. Moreover, the rate of dephosphorylation in the presence of ADP is greater than in the presence of unlabelled ATP, showing that there is an effect of ADP which goes beyond that of unlabelled ATP. This effect presumably includes the reaction of ADP with phospho-enzyme leading to the formation of ATP and dephospho-enzyme.

The results suggest that the rates of spontaneous, of K^+-dependent, and of ADP-dependent dephosphorylation are independent of the Mg^{2+} concentration

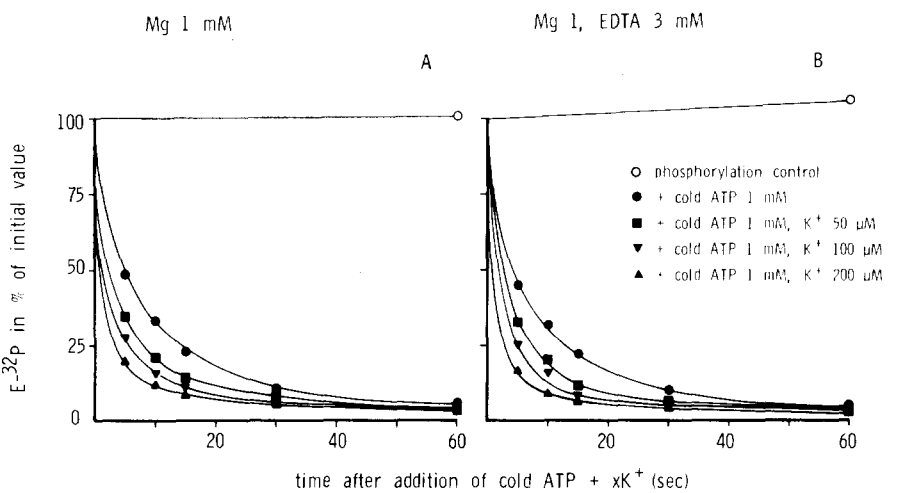

Fig. 9. The effect of Mg^{2+} on the K^+-dependent dephosphorylation. Medium contained: 25 μM [γ-^{32}P]-ATP, 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C) and 1 mM $MgCl_2$ (A) and 1 mM $MgCl_2$ plus 3 mM EDTA (B). Initial amount of phosphoenzyme: 284 ± 11 pmol ^{32}P/mg protein (A) and 98 ± 12 pmol ^{32}P/mg protein (B) (n = 2). ○———○, control of phosphorylation level; ●———●, + 1 mM unlabelled ATP (spontaneous dephosphorylation); ■———■, + 1 mM unlabelled ATP plus 50 μM KCl; ▼———▼, + 1 mM unlabelled ATP plus 100 μM KCl; ▲———▲, + 1 mM unlabelled ATP plus 200 μM KCl.

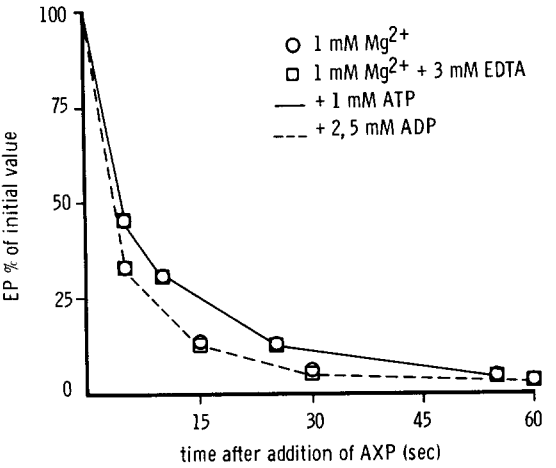

Fig. 10. The effect of Mg^{2+} on the ADP-dependent dephosphorylation. Medium contained: 25 μM [γ-^{32}P]ATP, 150 mM NaCl, 30 mM Tris · HCl, pH 7.4 (at 0°C) and 1 mM $MgCl_2$ (○) or 1 mM $MgCl_2$ plus 3 mM EDTA (□). Unbroken line, dephosphorylation after addition of 1 mM unlabelled ATP; dashed line, dephosphorylation after addition of 2.5 mM ADP. Initial amounts of phospho-enzyme: 295 ± 15 pmol ^{32}P/mg protein with 1 mM $MgCl_2$ and 100 ± 8 pmol ^{32}P/mg protein with 1 mM $MgCl_2$ plus 3 mM EDTA (n = 2).

present during the formation of phospho-enzyme. The observed effect of variation in the free Mg^{2+} concentration [2], varied by addition of EDTA, on rate of dephosphorylation of the phospho-enzyme, when phosphorylation has been stopped by addition of CDTA is apparent and due to inability of CDTA to give an immediate stop of phosphorylation, an effect which becomes more pronounced in the presence of EDTA.

CDTA does not react fast enough with Mg^{2+} to be useful as a chelator in investigations of processes where a fast removal of divalent cations is essential.

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