Biochimica et Biophysica Acta, 1104 (1992) 215–225

BBAMEM 75534

The role of Mg^{2+} and K^+ in the phosphorylation of Na^+,K^+-ATPase by ATP in the presence of dimethylsulfoxide but in the absence of Na^+

Carlos F.L. Fontes [1], Hector Barrabin [1], Helena M. Scofano [1] and Jens G. Nørby [2]

[1] *Departamento de Bioquímica, ICB, CCS, Universidade Federal do Rio de Janeiro, Rio de Janeiro (Brasil) and* [2] *Institute of Biophysics, University of Aarhus, Aarhus (Denmark)*

(Received 8 August 1991)

Key words: ATPase, Na^+/K^+-; Phosphoenzyme; Potassium; Magnesium; Dimethylsulfoxide

We have previously demonstrated that Na^+,K^+-ATPase can be phosphorylated by 100 μM ATP and 5 mM Mg^{2+} and in the absence of Na^+, provided that 40% dimethylsulfoxide (Me_2SO) is present. Phosphorylation was stimulated by K^+ up to a steady-state level of about 50% of E_{tot} (Barrabin et al. (1990) Biochim. Biophys. Acta 1023, 266–273). Here we describe the time-course of phosphointermediate (EP) formation and of dephosphorylation of EP at concentrations of Mg^{2+} from 0.1 to 5000 μM and of K^+ from 0.01 to 100 mM. The results were simulated by a simplified version of the commonly accepted Albers-Post model, i.e. a 3-step reaction scheme with a phosphorylation, a dephosphorylation and an isomerization/deocclusion step. Furthermore it was necessary to include an *a priori*, Mg^{2+}- and K^+-independent, equilibration between two enzyme forms, only one of which (constituting 14% of E_{tot}) reacted directly with ATP. The role of Mg^{2+} was two-fold: At low Mg^{2+}, phosphorylation was stimulated by Mg^{2+} due to formation of the substrate MgATP, whereas at higher concentrations it acted as an inhibitor at all three steps. The affinity for the inhibitory Mg^{2+}-binding was increased several-fold, relative to that in aqueous media, by dimethylsulfoxide. K^+ stimulated dephosphorylation at all Mg^{2+}-concentrations, but at high, inhibitory [Mg^{2+}], K^+ also stimulated the phosphorylation reaction, increasing both the rate coefficient and the steady-state level of EP. Generally, the presence of Me_2SO seems to inhibit the dephosphorylation step, the isomerization/deocclusion step, and to a lesser extent (if at all) the phosphorylation reaction, and we discuss whether this reflects that Me_2SO stabilizes occluded conformations of the enzyme even in the absence of monovalent cations. The results confirm and elucidate the stimulating effect of K^+ on EP formation from ATP in the absence of Na^+, but they leave open the question of the molecular mechanism by which Me_2SO, inhibitory Mg^{2+} and stimulating K^+ interact with the Na^+,K^+-ATPase.

Introduction

The transport ATPases like H^+,K^+-ATPase (EC 3.6.1.36), Na^+,K^+-ATPase (EC 3.6.1.37) and Ca^{2+}-ATPase (EC 3.6.1.38) catalyze the transduction of chemical energy in ATP to mechanical and potential energy related to the formation of ion gradients across biological membranes. Coupled to the ion transport, ATP is hydrolyzed via formation and hydrolysis of phosphorylated intermediates, where phosphate is bound to an aspartyl residue at the enzyme's substrate site [1–3]. In this process water acts as solvent, a substrate and probably also as a structural component important for the microenvironment of the sites of catalysis, ion binding and transport. The role of water is unclear (for reviews containing observations, theories and hypotheses related to the transport ATPases, see Refs. 4–9) and one way to study this subject is to determine effects of changes in the water-activity in the medium, e.g. by substituting water with water-miscible organic solvents like glycerol, ethyleneglycol, poly(ethylene glycol) or Me_2SO.

Several laboratories have been engaged in characterizing the importance of the activity of water in the reaction mechanism of Na^+,K^+-ATPase and the associated K^+-pNPPase activity (for references see Refs. 10

Abbreviations: Me_2SO, dimethylsulfoxide; EP, phosphoenzyme; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; P_i, inorganic orthophosphate; FITC, fluorescein isothiocyanate.

Correspondence: J.G. Nørby, Institute of Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark.

and 11). In our previous paper [11] we have studied the effect of Me_2SO on the initial steps of ATP-hydrolysis and showed that formation of the phosphorylated intermediate(s), EP, of Na^+,K^+-ATPase by ATP – usually believed to be closely connected with binding and subsequent transport of Na^+ – could proceed in the absence of Na^+, provided that medium water was substituted with 40% Me_2SO. Not only did we thus observe an apparent uncoupling between the first steps of ATP-hydrolysis (EP formation) and transport, but we furthermore obtained evidence that K^+ might activate the phosphorylation process and increase the steady-state level of EP (e.g. Fig. 2 in Ref. 11). This is also contrary to the generally accepted reaction schemes where the principal action of K^+ during transport is in catalyzing the dephosphorylation, i.e. the hydrolysis of EP. In the present paper we attempt to characterize this unexpected effect of K^+ by performing phosphorylation and dephosphorylation experiments at different concentrations of Mg^{2+} and K^+. The interplay between Mg^{2+} and K^+ in determining the level of EP and their influence on phosphorylation and dephosphorylation was evaluated by model simulations of the data. The results show that K^+ really stimulates dephosphorylation at all concentrations of Mg^{2+}. The Mg^{2+} ion, however, seems to exert both an activating (due to formation of MgATP) and an inhibitory effect, and 5 mM $MgCl_2$ inhibits, relative to lower $MgCl_2$-concentrations, both the phosphorylation and the dephosphorylation process. K^+ activates phosphorylation only when it is *a priori* inhibited by Mg^{2+}, i.e. there is no K^+-stimulation of the phosphorylation at low Mg^{2+}-concentrations. Furthermore, to simulate the time course of phosphorylation, it is necessary to assume that there is a Mg^{2+} and K^+ independent equilibrium between two unphosphorylated enzyme forms, only one of which is readily phosphorylated (in the absence of Na^+) by ATP.

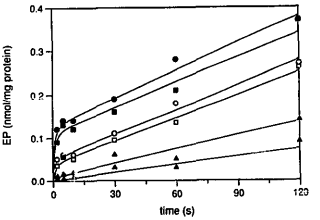

Fig. 1. Phosphorylation of Na^+,K^+-ATPase by 2 μM (open symbols) or 5 μM (closed symbols) inorganic [^{32}P]phosphate in the absence of Na^+, but in the presence of 40% Me_2SO, 100 μM unlabelled ATP, 5000 μM Mg^{2+} and K^+. The K^+-concentrations were 0.01 (△,▲), 0.5 (□,■) or 100 mM (○,●), and $E^{32}P$ was determined as described in Experimental procedures. All curves are drawn by eye. The experiments are used for constructing the 'P_i-contribution curves' shown in Figs. 8 and 9.

Experimental procedures

Enzyme preparations. Membrane-bound Na^+,K^+-ATPase from pig-kidney outer medulla was prepared, and contaminating Na^+ and K^+ determined, as described [11]. In the enzyme stock solution (2–5 mg protein/ml, 250 mM sucrose, 1 mM EDTA, 12.5 mM imidazole, pH = 7.4 at 20°C) the concentrations of Na^+ and K^+ were less than 20 and 15 μM, respectively. At 37°C and under standard conditions [12], the Na^+,K^+-ATPase activity was 15–18 U/mg protein, and the maximal phosphorylation level [13] was determined to 1.8–2.0 nmol/mg protein. Protein was measured by the method of Lowry et al. [14].

Chemicals. [γ-^{32}P]ATP was prepared according to Glynn and Chapell [15] with the modifications described [11]. The contaminant [^{32}P]P_i was 0.3–1 mol%. $MgCl_2$, EDTA (both analytical grade), Tris and unlabelled ATP were purchased from Sigma, and Me_2SO (spectroscopic grade) from Merck. [^{32}P]P_i was obtained from the Brazilian Institute of Atomic Energy and purified according to De Meis and Tume [16].

Determination of the time course of phosphorylation. The enzyme (50–120 μg/ml) was preincubated at 27°C in 5 mM Tris-HCl (pH = 7.0), 40% Me_2SO, 2 mM EDTA-Tris, $MgCl_2$ to give the desired free Mg^{2+}-concentration and KCl in the concentrations indicated. After adjustment of the pH to 7.0, the reactions were started by addition of 100 μM [γ-^{32}P]ATP. The volume was 0.5 ml. At each time-point, the amount of acid-stable phosphoenzyme, EP, was determined as described [11]. The P_i-concentration in the assay was estimated by extraction of an aliquot with phosphomolybdate according to De Meis and Carvalho [17].

Corrections for phosphorylation by P_i. Inorganic phosphate (P_i) is an inevitable contaminant of the [γ-^{32}P]ATP and it is also slowly produced during the phosphorylation experiments. In the presence of Me_2SO and Mg^{2+} the affinity of Na^+,K^+-ATPase for P_i is very high [18], and we therefore investigated to what extent P_i contributed to the EP formation. Fig. 1 gives the time course of $E^{32}P$ formation resulting from phosphorylation with 2 or 5 μM [^{32}P]P_i in the presence of 100 μM unlabelled ATP, 5 mM Mg^{2+} and 0.01, 0.5 or 100 mM K^+. From this, the 'P_i-contribution curves' to be used in the simulation of the phosphorylation data from ATP were constructed. These curves are shown as dotted lines in Figs. 8 and 9. It has been shown convincingly, that with μmolar P_i-concentrations, at least 1 mM Mg^{2+} is required to obtain significant phosphorylation from P_i [19], and we have there-

fore only applied the corrections at 5 mM Mg^{2+} in the present work.

Dephosphorylation reactions. The Na^+,K^+-ATPase (about 200 μg/ml) was phosphorylated at 27°C in an assay medium containing 5 mM Tris-HCl (pH = 7.0), 40% Me_2SO, 2 mM EDTA-Tris, $MgCl_2$ and KCl to give the desired concentrations as indicated in the figures, and 100 μM [γ-^{32}P]ATP. After one minute of reaction, the incorporation of radioactive phosphate was abolished by a twenty times dilution with an identical medium except for the presence of 100 μM unlabelled ATP. As indicated (Figs. 4 and 5) the K^+-concentration was also changed in some experiments. At different time-points thereafter, 0.8 ml aliquots were withdrawn and vigorously mixed with 3 ml ice-cold solution of 125 mM perchloric acid, 5 mM P_i and 5 mM Na-pyrophosphate. The $E^{32}P$ was then isolated and counted as described [11].

Calculation of the concentration of free Mg^{2+}. For each experiment $[Mg^{2+}]$ was calculated from the known concentrations of $MgCl_2$, EDTA, ATP and H^+, using the protonation and stability constants reported by Schwarzenbach et al. [20], Fabiato and Fabiato [21] and the procedure devised by the latter authors. The values for $[Mg^{2+}]$ thus obtained are probably only approximate, most of all because the effect of Me_2SO on the stability constants is unknown. We have therefore given not only the concentration of Mg^{2+}, but also that of total $MgCl_2$, EDTA and ATP in each experiment.

Results

Phosphoenzyme level from phosphorylation of Na^+,K^+-ATPase by ATP at various concentrations of Mg^{2+} and K^+

The effect of $[Mg^{2+}]$ and $[K^+]$ on the level of phosphoenzyme is shown in Figs. 2 and 3. The data are obtained after 60 s incubation with 100 μM ATP and the concentrations of Mg^{2+} and K^+ indicated. The highest values obtained for EP are about 60% of the maximal phosphorylation capacity. The results suggest both 'activating' and 'inhibitory' effects of both K^+ and Mg^{2+}, and they also point towards some kind of interaction between K^+ and Mg^{2+}: The lower the Mg^{2+}-concentration, the lower the $[K^+]$-values for 'half-maximal' activation or inhibition. Thus, at 0.1 μM Mg^{2+}, only the 'inhibitory' effect of K^+ was observed (Figs. 2A and 2B). The same relationships could be deduced from Fig. 3: Mg^{2+} apparently both activates and inhibits, and the half-maximal values are increased by increasing $[K^+]$.

It is important to note (see Figs. 6–9), that the 60-s EP-values are close to steady-state values for all K^+-concentrations equal to or larger than 0.5 mM irrespective of the Mg^{2+}-concentration. Likewise, steady

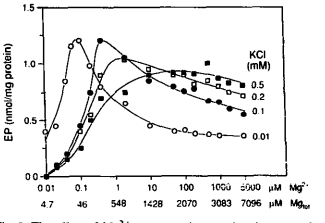

Fig. 3. The effect of Mg^{2+} concentration on phosphoenzyme level (EP) at four different K^+ concentrations. The enzyme was phosphorylated with 100 μM ATP for 60 s in 40% Me_2SO, and EP was determined as described under Experimental procedures. No Na^+ was added, and the concentrations of Mg^{2+} and K^+ were as indicated in the figure, together with the total concentration of $MgCl_2$ (Mg_{tot}) used in preparing the solutions (see Experimental procedures). The EP values are not corrected for the small contribution from phosphorylation by P_i (see Figs. 1, 8 and 9). The curves are drawn by eye.

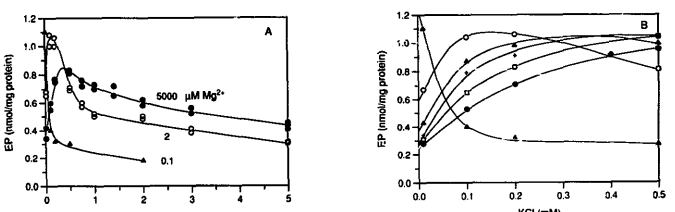

Fig. 2. The effect of K^+ concentration on phosphoenzyme level (EP) at different concentrations of Mg^{2+}. The enzyme was phosphorylated with 100 μM ATP for 60 s in 40% Me_2SO, and EP determined as described under Experimental procedures. No Na^+ was added, $[K^+]$ was as indicated on the x-axis, and the following Mg^{2+} concentrations (μM) were used: 0.1 (▲), 2 (○), 10 (△), 50 (+), 500 (□) or 5000 (●). The corresponding values for $[Mg_{tot}]$ are shown in Fig. 3. Panel B corresponds to the left part of panel A, i.e. for $[K^+] \leq 0.5$ mM. The EP values are not corrected for the small contribution from phosphorylation by P_i (see Figs. 1, 8 and 9). All curves are drawn by eye.

state is achieved with 0.01 and 0.1 mM K^+ when Mg^{2+} is kept lower than about 10 μM. However, at high [Mg^{2+}], like 5000 μM, and low [K^+], e.g. 0.01–0.1 mM, the phosphorylation reaction is slow and there is a steady contribution to EP-formation from P_i, so that the EP-level is not at steady state but still increasing at the 60-s time point (Fig. 8).

Whether or not the 60-s values of EP represent steady state or not, the apparent activating and inhibitory effects of K^+ and Mg^{2+} and their interdependence can only be elucidated by studying the two reactions of which the measured EP level is the resultant. Consequently we have measured dephosphorylation and phosphorylation rates under a series of different ionic conditions.

Dephosphorylation of the phosphointermediates of Na^+,K^+-ATPase at low (0.1 μM) and high (5000 μM) [Mg^{2+}], as a function of the K^+-concentration

To characterize the dephosphorylation, the enzyme was first phosphorylated for 60 s under conditions where a reasonable level of EP was obtained. The concentration of Mg^{2+} was always the same during phosphorylation and dephosphorylation.

With 0.1 μM Mg^{2+}, phosphorylation was performed with 0.01 or 0.1 mM K^+ yielding about 1 or 0.35 nmol EP/mg protein (see also Fig. 3). The time course for dephosphorylation was then determined as described under Experimental procedures, and the data are given in Fig. 4, from which the following conclusions can be drawn: (a) There is about 0.1 nmol EP/mg protein which is not (or only very slowly) dephosphorylated in the 5-min duration of the experiment. This corresponds to about 5% of the maximal capacity; (b) The rate of dephosphorylation increases with increasing [K^+] in the dephosphorylation medium and at 0.5 mM K^+ it is about as fast as we can measure accurately; (c) The dephosphorylation time course seems to be independent of [K^+] in the phosphorylation medium, as exemplified by the dephosphorylation curves with 0.1 mM K^+. The curves in Fig. 4 can be semi-quantitatively characterized by 'dephosphorylation rate constants' for the disappearance of EP, k_{dephow}, estimated as ln2 divided by $t_{\frac{1}{2}}$ for the decay of EP. With 0.01 mM K^+ there is a biphasic disappearance of EP: 50% with $k = 0.2\ s^{-1}$, 50% with k about $0.01\ s^{-1}$. With 0.1 mM K^+, k_{dephow} is about $0.2\ s^{-1}$ and with 0.5 mM K^+ k_{dephow} is $0.5\ s^{-1}$.

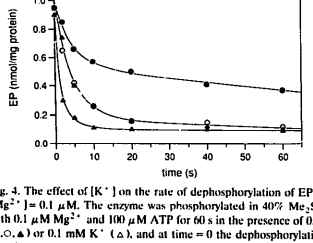

Fig. 4. The effect of [K^+] on the rate of dephosphorylation of EP at [Mg^{2+}] = 0.1 μM. The enzyme was phosphorylated in 40% Me_2SO with 0.1 μM Mg^{2+} and 100 μM ATP for 60 s in the presence of 0.01 (●,○,▲) or 0.1 mM K^+ (△), and at time = 0 the dephosphorylation reaction was started as described under Experimental procedures. During dephosphorylation [K^+] was 0.01 (●), 0.1 (△,○) or 0.5 mM (▲). The y-axis values for (△) must be divided by 4 to represent real EP-concentrations. At 300 s EP was 0.18 nmol/mg protein for the upper curve (●), whereas the two lower curves did not change from 60 to 300 s. All curves are drawn by eye.

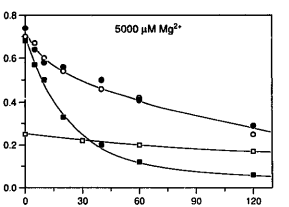

Fig. 5. The effect of [K^+] on the rate of dephosphorylation of EP at [Mg^{2+}] = 5000 μM. The enzyme was phosphorylated in 40% Me_2SO with 5000 μM Mg^{2+} and 100 μM ATP for 60 s in the presence of 0.01 (□) or 0.5 mM K^+ (○,●,■), and at time = 0 the dephosphorylation reaction was started as described under Experimental procedures. During dephosphorylation [K^+] was 0.01 (□,○), 0.5 (●) or 100 mM (■). At 300 s, EP was 0.13 (□,●), 0.08 (○) and 0.05 nmol/mg protein (■), respectively. The curves are drawn by eye, and the EP values are not corrected for the contribution to phosphorylation from contaminating P_i (see Figs. 1, 8 and 9).

When phosphorylation and dephosphorylation is carried out with 5000 μM Mg^{2+}, the dephosphorylation rate is slower than with 0.1 μM Mg^{2+}. In three examples of experiments about 0.7 nmol EP/mg protein was formed when the enzyme was phosphorylated for 60 s in the presence of 0.5 mM K^+. The time course of the subsequent dephosphorylation in the presence of 0.01, 0.5 or 100 mM K^+ is given in Fig. 5, and the approximate values for k_{dephow} are 0.015, 0.015 and $0.04\ s^{-1}$, respectively. In one experiment, the phosphorylation and dephosphorylation were both carried out in 0.01 mM K^+. As expected from Fig. 3, the yield of EP under these conditions is low (around 0.3 nmol/mg protein), and considering that there also here seems to be some 'stable' EP it is difficult to quantify the dephosphorylation curve. The values for k_{dephow}, see Table I, will serve as a guideline in the model-simulation of the phosphorylation time courses described in the following sections.

TABLE I

Steady-state level of EP (EP_{ss}) and rate coefficients characterizing phosphorylation and dephosphorylation of Na^+,K^+-ATPase in 40% Me_2SO with different Mg^{2+}- and K^+-concentrations

The values in this table are obtained from the dephosphorylation curves (k_{dephos}; Figs. 4 and 5) and the phosphorylation experiments (EP_{ss}, k_{phos}, k_{obs}; Figs. 6–9) as described in the text and in legends. k_{phos} is r_0/EP_{max} and k_{obs} is $\ln 2/t_{\frac{1}{2}}$.

$[K^+]$ (mM)	EP_{ss} (% of EP_{max})	k_{phos} (s^{-1})	k_{dephos} (s^{-1})	k_{obs} (s^{-1})
	$[Mg^{2+}] = 0.1$ μM			
0.01	50	0.2	0.2–0.01	0.1
0.1	27	0.2	0.2	0.17
0.5	15	0.1	0.5	0.2
	$[Mg^{2+}] = 5000$ μM			
0.01	18 [a]	0.006	0.015	0.02
0.1	30 [a]	0.01		0.02
0.5	35	0.05	0.015	0.04
2	25	0.05		0.07
100	7	0.01	0.04	0.07

[a] Not steady state, see Fig. 8.

Time course of Na^+,K^+-ATPase phosphoenzyme formation (phosphorylation) from ATP at various concentrations of Mg^{2+} and K^+

The phosphorylation experiments are illustrated for 0.1 (Fig. 6), 0.5 (Fig. 7) and 5000 μM Mg^{2+} (Figs. 8 and 9) and with the K^+ concentrations given in the figures. The points are experimental data, and the curves are simulated using model C in Fig. 10 as outlined below. The 60-s values of these curves correspond to the EP concentrations given in Figs. 2 and 3 under the same ionic conditions. The characterization of the curves that follows, serve as the basis for the choice of the minimal model in Fig. 10 to describe the time-courses of EP formation.

At 0.1 μM Mg^{2+} (Fig. 6) the steady-state level of EP (EP_{ss}) decreases with increasing $[K^+]$, and if one were to characterize the time course (data points) with a single constant, k_{obs} values of about 0.1 s^{-1}, 0.17 s^{-1} and 0.2 s^{-1} would be obtained for $[K^+] = 0.01$, 0.1 and 0.5 mM, respectively (k_{obs} is calculated as $\ln 2/t_{\frac{1}{2}}$, where $t_{\frac{1}{2}}$ is the time it takes to reach $\frac{1}{2} \times EP_{ss}$). The same effect of K^+ on EP_{ss} is observed at $[Mg^{2+}] = 0.5$ μM (Fig. 7), where k_{obs} is around 0.15 s^{-1} for both 0.1 and 0.5 mM K^+.

With 5000 μM Mg^{2+} (Figs. 8 and 9), the trend for the K^+-effect is reversed, in the sense that 'initial' rate and 'final' level *increase* with increasing $[K^+]$ up to about 0.5–2 mM. The values for k_{obs} are smaller than 0.02 s^{-1} ($[K^+] = 0.01$ and 0.1 mM) and equal to about 0.04 s^{-1} at $[K^+] = 0.5$ mM (Fig. 8). With the higher K^+-concentrations (Fig. 9), k_{obs} is increased to 0.07 s^{-1}.

The phosphorylation curves in Figs. 6, 8 and 9 can also yield a rough estimate of k_{phos} which is determined as the initial rate, r_0 nmol/mg protein per s, divided by the total concentration of $E = E_{tot} = EP_{max} = 2$ nmol/mg protein (Experimental procedures). Note that this method estimates a *minimal* value for k_{phos}, since the concentration of enzyme readily available for phosphorylation may be less than 2 nmol/mg protein as shown below. Values for k_{phos}, k_{dephos} and k_{obs} are presented in Table I.

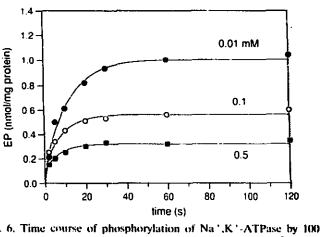

Fig. 6. Time course of phosphorylation of Na^+,K^+-ATPase by 100 μM ATP in the presence of 40% Me_2SO and 0.1 μM Mg^{2+} with the K^+-concentrations shown in the figure. The data were obtained as described under Experimental procedures, the total $MgCl_2$-concentration being 46 μM. The curves are calculated from the model in Fig. 10 as described in the text and with the constants given in Table II. The apparent k_{obs} for phosphorylation, determined from the approximate $t_{\frac{1}{2}}$-values, are 0.1 (●), 0.17 (○) and 0.2 s^{-1} (■).

Strategy for, and results of, model simulations of phosphorylation curves

We have now demonstrated that Na^+,K^+-ATPase can be phosphorylated by ATP in the absence of Na^+ when Me_2SO is present, and that K^+ and Mg^{2+} have profound influence on this process. In order to evalu-

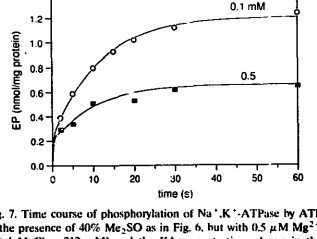

Fig. 7. Time course of phosphorylation of Na^+,K^+-ATPase by ATP in the presence of 40% Me_2SO as in Fig. 6, but with 0.5 μM Mg^{2+} (total $MgCl_2 = 212$ μM) and the K^+-concentrations shown in the figure. The curves are calculated from the model in Fig. 10 with the constants given in Table II. The apparent k_{obs} for phosphorylation obtained from this figure is 0.14 (○) and 0.17 s^{-1} (■).

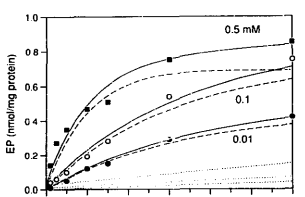

Fig. 8. Effect of K^+ (concentrations indicated on the figure) on the time course of phosphorylation of Na^+,K^+-ATPase by 100 μM ATP in the presence of 40% Me_2SO and high $[Mg^{2+}]$, i.e. $[MgCl_2] = 7100$ μM corresponding to $[Mg^{2+}] = 5000$ μM (see Experimental procedures for further details). The dotted curves represent the contribution to EP-formation by contaminating P_i, assuming that $[P_i]$ is about 1 μM, see Fig. 8 in Ref. 11. This contribution increases with K^+-concentration and the curves are calculated on the basis of the experiments shown in Fig. 1. The apparent 'non-zero' intercept of some of the 'P_i-contribution curves' signifies the rapid initial phosphorylation by $[^{32}P]P_i$ (contaminating the $AT^{32}P$) when K^+ is higher than 0.1 mM (see Fig. 1, circles and squares). The broken lines are simulations of phosphorylation by ATP, using the model in Fig. 10 with the rate constants of Table II, as described in the text. The full line, describing the total EP-concentration as a function of time, is then the sum of the other two curves for each experimental condition. Apparent k_{obs} for phosphorylation is smaller than about 0.02 (●,○) and 0.04 s^{-1} (■).

ate the effect of K^+ and Mg^{2+}, it is preferable to have a (simple) model, by which the data can be analyzed and simulated. In Table I some coefficients charac-

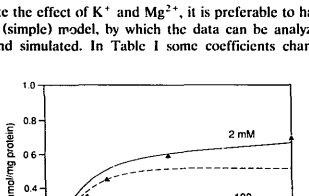

Fig. 9. Time course of phosphorylation of Na^+,K^+-ATPase from ATP under experimental conditions like those described for Fig. 8, i.e. with $[Mg^{2+}] = 5000$ μM, but with 2 or 100 mM K^+ as indicated. As in Fig. 8, the dotted curve (here common for the two K^+-concentrations) is the calculated contribution to EP-formation from contaminating P_i (the 'non-zero' intercept relates to the rapid initial phosphorylation by preformed $[^{32}P]P_i$ in $AT^{32}P$, see Fig. 8 and Fig. 1), the broken curves are calculated from the model in Fig. 10 and the rate constants in Table II, and the full curves are the sum of the dotted and the broken, thus simulating total EP-formation. Apparent k_{obs} for phosphorylation is around 0.07 s^{-1} in both experiments.

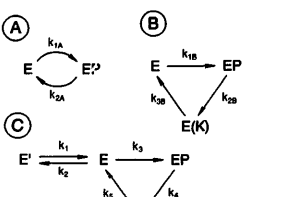

Fig. 10. Three simple, minimal models for the mechanism of formation of EP (the phosphointermediates of Na^+,K^+-ATPase) in the presence of Me_2SO but in the absence of Na^+. E(K) represents a dephosphoform that may have K^+ occluded. The models can be regarded as considerably reduced versions of the Albers-Post scheme in the sense that they contain a phosphorylation and a dephosphorylation sequence and, in the case of B and C, also an intermediate with a possibly occluded cation. The models were evaluated quantitatively, as described in the text, for their ability to simulate the phosphorylation data shown in Figs. 6–9, and only model C was found suitable. The rate coefficients corresponding to model C at different K^+- and Mg^{2+}-concentrations are listed in Table II.

terizing the experimental data of the phosphorylation experiments in Figs. 6–9 are collected, and in Fig. 10 we show three simple reaction schemes of slightly increasing complexity, which we will briefly investigate in order to choose the minimal model for simulation of the results.

When Model A (Fig. 10) is correlated with Table I, $k_{1A} = k_{phos}$, $k_{2A} = k_{dephos}$ and, as is well known, k_{obs} must then be equal to $k_{phos} + k_{dephos}$ $(= k_{1A} + k_{2A})$. This condition seems to be fairly well fulfilled by the rate coefficients obtained at $[Mg^{2+}] = 5000$ μM, but not at all at $[Mg^{2+}] = 0.1$ μM, where e.g. at $[K^+] = 0.1$ and 0.5 mM, k_{obs} is smaller than k_{dephos}.

Model B (Fig. 10) poses similar problems. Simulations of the time course for EP formation, using different combinations of k_{1B}, k_{2B} and k_{3B} (not shown) reveals a general, and not unexpected pattern: When k_{3B} is (considerably) larger than k_{2B}, a monotonic rise in EP towards steady state is observed. Model B is then equivalent to Model A, and $k_{obs} = k_{1B} + k_{2B}$. But when k_{3B} is smaller than or equal to k_{2B}, an overshoot in the EP curve appears. Consequently, the experimental results cannot be simulated with this model.

By introducing an a priori equilibrium between enzyme species as shown in Model C, Fig. 10, it becomes possible to simulate the results, as illustrated in Figs. 6–9. The ratio between [E'] and [E] (determined by the k_2/k_1 ratio), that gave the best results, was about 6.25. The presence of a suitable proportion of the readily phosphorylating species E, accounts for the initial rapid phase as seen in Fig. 6 and especially in Fig. 7. The

TABLE II

Rate coefficients for model C, Fig. 10, used in the simulation of phosphorylation curves (Figs. 6–9).

All coefficients have units of s^{-1}.

$[K^+]$ (mM)	k_1	k_2	k_3	k_4	k_5
$[Mg^{2+}] = 0.1\ \mu M$					
0.01	0.08	0.5	0.8	0.09	0.5
0.1	0.08	0.5	0.75	0.22	0.5
0.5	0.08	0.5	0.8	0.43	0.5
$[Mg^{2+}] = 0.5\ \mu M$					
0.1	0.08	0.5	4.5	0.17	0.45
0.5	0.08	0.5	4.0	0.5	0.45
$[Mg^{2+}] = 5000\ \mu M$					
0.01	0.08	0.5	0.025	0.0	0.05
0.1	0.08	0.5	0.047	0.01	0.05
0.5	0.08	0.5	0.15	0.028	0.05
2	0.08	0.5	0.14	0.04	0.05
100	0.08	0.5	0.033	0.055	0.05

rate of this rise is profoundly influenced by k_3. The subsequent slower rate of phosphorylation, that primarily determines the $t_{\frac{1}{2}}$ and thus k_{obs} (Table I), is mainly accounted for by the rate coefficient (k_1) for the E′ to E conversion. (At $[Mg^{2+}] = 5000\ \mu M$ (Figs. 6 and 9), the rapid and slow phase of phosphorylation are no longer observed, since k_3 is equal to or smaller than k_1). In the simulations, the rate coefficient for dephosphorylation, k_4, was kept close to those experimentally obtained (compare k_{dephos}, Table I and k_4 Table II), and finally, k_3, k_4 and k_5 together determines the simulated steady-state level of EP. It should be noted that the necessity of having the intermediate E(K) in the model is especially apparent at $[K^+] \geq 0.5$ mM: Here the value of k_5 is important for the adjustment of EP_{ss}.

The final results of the simulations with Model C, Fig. 10, are shown as curves in Figs. 6–9 and the coefficients are given in Table II. The following features appear: The ratio $[E']/[E] = k_2/k_1$, and also the absolute values of k_1 and k_2, can be assumed to be independent of $[K^+]$ and $[Mg^{2+}]$. The rate coefficients for the phosphorylation and dephosphorylation reactions are all lower at $[Mg^{2+}] = 5000\ \mu M$ than at $[Mg^{2+}] = 0.1$ or $0.5\ \mu M$, indicating that these reactions are inhibited by high $[Mg^{2+}]$. It should also be noted, that the rate coefficient for phosphorylation, k_3, can be set to be proportional to $[Mg^{2+}]$ at low $[Mg^{2+}]$. This is an expected relationship: Under these conditions and with $[ATP] = 100\ \mu M$, [MgATP] is proportional to $[Mg^{2+}]$ and the MgATP concentration is much lower than its $K_{0.5}$ (see Discussion).

It appears from Table II that k_4 increases with $[K^+]$ at all Mg^{2+} concentrations, i.e. K^+ stimulates dephosphorylation. The 'phosphorylation rate coefficient' k_3 is independent of $[K^+]$ at low $[Mg^{2+}]$, but at $[Mg^{2+}] = 5000\ \mu M$, an increase in the K^+-concentration from 0.01 to 0.5 -2 mM leads to a 6 times larger k_3. Further increases in $[K^+]$ up to 100 mM may reduce k_3 to the level in the absence of K^+, but due to the low level of EP_{ss} and the relatively large contribution by 'P_i-phosphorylation', the simulation is probably equivocal. The behaviour of k_3 at $[Mg^{2+}] = 5000\ \mu M$ is also reflected in the change in EP_{ss} level with K^+-concentration, see Fig. 2A and Table I.

Discussion

The results of the present study are evaluated using a simplified reaction scheme – model C in Fig. 10 – that adequately can describe the data by mathematical simulation. The scheme consists, as an essential and necessary feature, of an *a priori* equilibrium between two enzyme forms, E′ and E, of which the form E is phosphorylated by ATP in an irreversible step (no ADP present) with rate coefficient k_3, followed by an irreversible dephosphorylation (P_i absent), rate coefficient k_4, and a deocclusion/isomerization reaction likewise considered irreversible (see below) with the coefficient k_5. The reaction scheme can be considered as a simplified version of the currently accepted more detailed model for the Na^+,K^+-ATPase reaction [2], in the sense that it contains both dephospho- and phosphoenzyme and also features a transport intermediate with a possibly occluded cation, E(K). The discussion below is aimed at: (1) comparing these reactions in Me_2SO with their properties in water, and (2) elucidating the roles of Mg^{2+} and K^+ in the different processes.

The a priori equilibrium between E and E′

This equilibrium is characterized by the Mg^{2+}- and K^+-independent rate constants $k_1 = 0.08\ s^{-1}$ and $k_2 = 0.5\ s^{-1}$, meaning that before addition of ATP and irrespective of the concentration of Mg^{2+} and K^+, E constitutes about 14% of E_{tot}. This model feature has important implications for the $E(K) \rightarrow E$ reaction sequence since this sequence as a logical consequence then must be irreversible. The operational basis for introducing the *a priori* equilibrium is given in the last section of Results, and although it is tempting to draw a parallel between the two forms in the present model and the well established major classes of conformation in aqueous media, the K^+-form E_2 and the Na^+-form E_1 [22], such an analogy is probably not justified. Especially one might argue that the E′/E-ratio should be K^+-dependent, which it is not, and further difficulties in this context are discussed earlier by us [11]. It is of interest to note, that the existence of a similar? equilibrium has been inferred by Fritzsch [23,24] who analyzed two sets of data for the inactivation of

Na^+,K^+-ATPase with ATP-analogs and one for the inactivation of Ca^{2+}-ATPase with FITC. In all three cases, by far the best model-fit was obtained if the model contained two slowly interconvertible enzyme states, only one of which was active in binding the inhibitor. The active state constituted 7, 13 and 17% and it was "speculated that the two states reflects different interactions of enzyme units".

Activation of phosphorylation by low (μM) concentrations of Mg^{2+}

With 100 μM ATP present, increasing $[Mg^{2+}]$ in the range 0.01–1 μM activates the phosphorylation step (k_3 in Table II) and increases the steady-state level of EP (EP_{ss}, somewhat dependent on $[K^+]$, see Fig.3). At higher Mg^{2+}-concentrations, EP_{ss} decreases again (Fig. 3) in connection with an inhibitory action of Mg^{2+} on the phosphorylation as well as the dephosphorylation process (Tables I and II). In aqueous media similar phenomena are observed: Increasing $[Mg^{2+}]$ activates because the substrate is MgATP – or ATP with Mg^{2+} as an obligatory cofactor – [25–27]. ATP binds without Mg^{2+} [28] but EP formation and hydrolysis only begins when Mg^{2+} is added [29,30]. When $[Mg^{2+}]$ becomes very high, hydrolysis and various partial reactions are inhibited (see Refs. 31–33 which also have references to earlier work, and below).

If we assume that MgATP is the substrate for Na^+,K^+-ATPase phosphorylation also in 40% Me_2SO, the activating effect of increasing $[Mg^{2+}]$ can very well be explained by an increase in [MgATP]. Using a stability constant for MgATP (admittedly determined in aqueous media) equal to $2 \cdot 10^4\ M^{-1}$ [21,34], we can calculate that when $[ATP_{tot}] = 100\ \mu M$ and $[Mg^{2+}]$ is raised from 0.1 to 0.5 μM, the MgATP-concentration increases with a factor of 5, from 0.2 to 1 μM. The concentration of free, uncomplexed ATP stays constant at approximately 100 μM. If the $K_{0.5}$ value for MgATP for phosphorylation is 2–4 μM as determined by us previously (Fig. 6, Ref. 11) we would expect the 'phosphorylation rate coefficient' k_3 to increase 3–5 times for this [MgATP] change. In the simulations it is increased from $0.8\ s^{-1}$ to $4\ s^{-1}$, Table II.

Based on these considerations we can discuss two simple mechanisms that could explain the relationship between [MgATP] and k_3. Firstly, the binding of MgATP could be rate-limiting for phosphorylation and diffusion-controlled, i.e. with $k_{diff} = 10^7\ M^{-1}s^{-1}$ [30], but with $[MgATP] = 1\ \mu M\ (= 10^{-6}\ M)$ one should then find $k_3 = 10\ s^{-1}$. This is twice the 'model k_3' (for $[Mg^{2+}] = 0.5\ \mu M$ in Table II), and a more likely mechanism is therefore the second possibility: E and MgATP equilibrates in a diffusion-controlled binding process and with 1 μM MgATP, about 1/4 should be in EMgATP ($K_{0.5}$ is 2–4 μM, see above). The rate coefficient for the subsequent rate-limiting phosphorylation step EMgATP → EP might then be calculated as $4 \times (k_3$ of Table II$) = 4 \times 4\ s^{-1} = 16\ s^{-1}$, and this could be considered as the phosphorylation rate coefficient at optimal substrate concentration (and no Mg^{2+}-inhibition). We have compared this rate coefficient with estimates of the same parameter in aqueous media at room temperature under what may be considered optimal conditions for EP-formation, i.e. 25–150 mM NaCl, more than 25–100 μM ATP, 1–5 mM $MgCl_2$ and no K^+. Mårdh and co-workers found a value of 150–200 s^{-1} for ox brain [35] and guinea pig kidney [29], and Hobbs et al. [36] determined the phosphorylation rate coefficient for enzyme from electroplax to be 55 s^{-1}. These values are 3–10 times the value of 16 s^{-1} calculated above and one possible reason for this discrepancy could be that the 100 μM free ATP in our experiments act as a competitive inhibitor with MgATP. In recent (unpublished) experiments with the pig kidney enzyme used in the present investigation, R.C.Rossi and J.G.Nørby found a 'phosphorylation rate coefficient' of about 20 s^{-1} using the same type of rapid mixing apparatus as Mårdh. Thus it seems that phosphorylation in 40% Me_2SO without Na^+ proceeds with an efficiency at least comparable to the Na^+-stimulated phosphorylation in aqueous media.

With these very low concentrations of Mg^{2+}, we found no effect of K^+ on the phosphorylation rate, and although there are no other published studies concerning this matter, it is of interest that Kaniike et al. [37] observed that binding of ADP in 30% Me_2SO-medium was not affected by K^+ as it is in aqueous medium [28]. So when increasing $[K^+]$ leads to a decrease in EP_{ss} (Figs. 2, 3, 6 and 7) it is exclusively due to the K^+-activation of dephosphorylation (Fig. 4, k_{dephos} in Table I, k_4 in Table II).

The inhibition by Mg^{2+}, and the K^+-Mg^{2+} antagonism at the phosphorylation step

From the model simulations summarized in Table II it appears, that raising $[Mg^{2+}]$ from 0.5 to 5000 μM decreases both the rate coefficient for dephosphorylation (k_4) and that for isomerization/deocclusion (k_5) by a factor of 10, irrespective of the K^+-concentration (it has been suggested that inhibition by Mg^{2+} in aqueous media requires K^+ [38], but see Ref. 33 for a discussion of this subject). The phosphorylation rate coefficient (k_3) falls 30 to 150 times depending upon the K^+-concentration. This inhibitory effect of Mg^{2+} is probably also reflected in the relationship between EP_{ss} and $[Mg^{2+}]$ – from 2 to 5000 μM – at $[K^+] \leq 0.2$ mM shown in Fig. 2B. Due to the simplicity of the model employed in this study (C in Fig. 10) is it not possible to decide whether the differential inhibition is an expression of one, two or more inhibitor-binding sites for Mg^{2+}, but it seems safe to conclude that there are at least 2 Mg^{2+} sites: one (high affinity) at which Mg^{2+} is

an activator related to ATP on the substrate site, and a different site with lower affinity where Mg^{2+} inhibits. This would be in accord with the general view upon the reaction of Mg^{2+} with Na^+,K^+-ATPase [33,39-42] although the hypothesis, that both actions of Mg^{2+} may be elicited via one site also has been put forward [43]. We have not found any other published studies on the inhibition of Na^+,K^+-ATPase by Mg^{2+} in Me_2SO, but a comparison with the inhibitory effects of Mg^{2+} in aqueous media reveals a much higher affinity for inhibitory Mg^{2+} in Me_2SO (this paper) than in aqueous buffers. Here we find that in 40% Me_2SO the rate constants are reduced by at least a factor of 10 by 5 mM Mg^{2+}, whereas inhibition in aqueous media is characterized by $K_{0.5}$ (or K_i) values of 10–20 mM [41,42,44,45] or higher [27,31,46–48], probably depending on assay conditions. Maybe the increased inhibition in Me_2SO reflects the observation that 40% Me_2SO dramatically decreases K_m(Mg) for the pNPPase reaction [49] as well as the Mg^{2+} requirement for phosphorylation by P_i [19].

From the model analysis in Table II we can deduce that the unexpected, stimulating effect of K^+ on EP formation from ATP, that we observed in our first paper on this subject [11], is an effect of K^+ on the phosphorylation sequence (k_3), meaning that K^+ reacts with E or other intermediates in the $E \rightarrow EP$ sequence. The K^+-activation is seen at least for $[K^+] < 2$ mM but apparently only when the reaction(s) inhibited by Mg^{2+}. This activation is not necessarily reflected in the EP_{ss}-level as an increase, since K^+ also increases the dephosphorylation rate constant k_4. It seems unlikely from the present evidence that K^+ acts as a competitive ligand on the Mg^{2+} binding site(s), because K^+ by no means relieve the Na^+,K^+-ATPase system of the Mg^{2+}-inhibition. The activation could be described as an attenuation of the Mg^{2+}-inhibition by binding of K^+ to separate site(s). The reason that activation by K^+ is only observed at inhibitory $[Mg^{2+}]$ could be either that the K^+-binding step at high (but not at low) $[Mg^{2+}]$ is rate-limiting, or that the affinity of the K^+-site at low $[Mg^{2+}]$ is such that it is already saturated at 10 μM K^+.

Mg^{2+}-K^+ antagonism has also been observed in aqueous media. Tashima et al. [50] and Robinson [48] report that K^+ can counteract the inhibition of pNPPase by Mg^{2+}, and Mg^{2+} inhibition of Na^+,K^+-ATPase activity has a lower K_i, the lower the concentration of monovalent cations [46]. These authors describe the antagonism as competitive, whereas others find that K^+ acts as a partial inhibitor towards Mg^{2+} [51] or characterize the interaction between K^+ and Mg^{2+} as uncompetitive [31,32]. Partial inhibition requires that Mg^{2+} and K^+ be bound simultaneously, and there are indeed several indications that that can happen [31,32,40,41,51–54].

Comparison between rate constants in 40% Me_2SO and in aqueous media

In a previous section we noted that the phosphorylation coefficient k_3 in Me_2SO seemed to be comparable to that in water; at most it was a factor of 10 lower.

If we turn to the dephosphorylation coefficient k_4, we can estimate a value of about 1 s^{-1} under maximal stimulation by K^+ (Table I and II). Without K^+ it is 0.01–0.2 s^{-1} (Table I). These values are much lower that those determined for dephosphorylation in aqueous media at room temperature: For the unstimulated (no K^+) dephosphorylation Mårdh [55] finds about 4 s^{-1} and this value has been confirmed by Forbush and Klodos as well as Rossi and Nørby (unpublished) on the kidney enzyme. In the presence of K^+, K-sensitive EP dephosphorylates very rapidly, i.e. k is about 200 s^{-1} or larger [29,35,55]. Thus it seems clear that Me_2SO inhibits the dephosphorylation. This is in accordance with the suggestions by Robinson [10] and with earlier observations at 0°C for both Na^+,K^+-ATPase [37] and Ca^{2+}-ATPase [56], but in contrast to the pronounced increase in the rate constant for non-enzymatic hydrolysis of acetyl phosphate when water is substituted with Me_2SO [57]. We should also note, that the inhibition by Mg^{2+} that we see in 40% Me_2SO may have its parallel in the inhibition of E_2P-hydrolysis (with a $K_{0.5} > 6$ mM for Mg^{2+}) described by Post et al. [58].

We have recently shown [59] that K^+-stimulated (we used Tl^+ as a K^+-congener) dephosphorylation and K^+-occlusion occur simultaneously. In model C, Fig. 10, E(K) can be considered as enzyme having occluded K^+ and the sequence $E(K) \rightarrow E$ (rate coefficient k_5) then contains the deocclusion step. In setting this sequence to be irreversible, we imply that either the presumed deocclusion reaction or the following isomerization step (or both) are in fact irreversible in 40% Me_2SO (see also above under The *a priori* equilibrium between E and E'). This will be the subject of further studies. In our simulations we have assigned a rate coefficient of 0.5 s^{-1} to the reaction $E(K) \rightarrow E$ in the uninhibited state, i.e. at low $[Mg^{2+}]$. This is similar to the rate constant for Rb^+ release from E(Rb): 0.2 s^{-1} in water, 0.5 s^{-1} in 20% Me_2SO [60], and to rate constants for K^+-release or Rb^+-release in flux or occlusion/deocclusion studies, $k = 0.2–0.3$ s^{-1} (see discussion by Karlish and Stein [61]), but it must be emphasized that these values are for the unstimulated deocclusion. In the presence of ATP or P_i, the rate constant is 10–100 times higher [41,54], and since we have 100 μM ATP, and hence would have a deocclusion constant in water much larger than 0.5 s^{-1} (Table II), we conclude that Me_2SO also inhibits the sequence $E(K) \rightarrow E$ in our model.

As regards the Mg^{2+}-inhibition of this step, there are observations with aqueous media, that Mg^{2+} acts as a 'product inhibitor' by binding to E_2K [31,40]. We

furthermore note, that Forbush [41] reported almost complete inhibition by Mg^{2+} ($K_{0.5} = 10$ mM) of the MgATP-stimulated deocclusion, i.e. an inhibition perhaps similar to the one we observe here (Table II), but with a different affinity.

Concluding remarks

Above we have reported and discussed certain features of the reaction mechanism of Na^+,K^+-ATPase which all seem to be effected by the presence of 40% Me_2SO. According to the simplified reaction scheme used in the analysis of the results, a cation independent equilibrium between two enzyme forms E′ and E exists in the Me_2SO-medium. In the absence of Na^+, E is readily phosphorylated by ATP, but the subsequent reactions, the K^+-activated dephosphorylation and a presumed deocclusion/isomerization sequence, are inhibited by Me_2SO. One interesting hypothesis to explain this is that the lowered water activity (or the reaction of the enzyme with Me_2SO) uncouples the occlusion of Na^+ and K^+ by stabilizing 'empty, occluded' conformations in the absence of Na^+ or K^+. According to this, the presence of 40% Me_2SO could increase the rate of $E \rightarrow EP$ in the absence of Na^+ by stabilizing an 'occluded Na^+-like' conformation. This would be analogous to the phosphorylation by ATP of the related enzyme, Ca^{2+}-ATPase from sarcoplasmic reticulum, seen in the absence of Ca^{2+} provided Me_2SO is present in the medium [62]. Likewise, addition of Me_2SO to the medium would decrease the rate of $E(K) \rightarrow E$ by stabilizing an 'occluded K^+-like' conformation. As in aqueous buffers, all the reactions are inhibited by Mg^{2+}, but the affinity for Mg^{2+} is increased by Me_2SO. Considering that K^+ in these experiments with 40% Me_2SO and high, inhibitory [Mg^{2+}] stimulates the phosphorylation reaction, one might speculate whether K^+ has taken the function that Na^+ has in aqueous media. In this connection it is of interest, that the Mg^{2+}/K^+ antagonism observed here in Me_2SO, has its parallel in a Mg^{2+}/Na^+ antagonism in aqueous media as described by both Pedemonte and Beaugé [40], Sachs [32] and Robinson and Pratap [33]. However, the nature of the Mg^{2+}-inhibitory site is not known at the moment and therefore the molecular mechanism by which Me_2SO increases the affinity for Mg^{2+}, and by which K^+ accelerates the phosphorylation reaction at high Mg^{2+}, remains to be elucidated. In any case, the present study has underlined the fact that the properties of this flexible, mischievous enzyme depend both on the specific ligands and on the other substances of the reaction medium like buffers, water and organic solvents.

Acknowledgements

We thank Dr. Bliss Forbush III, Yale University, for permitting us to use the data processing program developed by him. This work was supported by the Financiadora de Estudos e Projetos, by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro, by grant 12-8174 from The Danish Medical Research Council and by The Biomembrane Research Center, University of Aarhus.

References

1 Pedersen, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–150.
2 Nørby, J.G. (1987) Chem. Scr. 27B, 119–129.
3 Nørby, J.G. and Klodos, I. (1988) in The Na^+,K^+-Pump, Part A: Molecular Aspects (Skou, J.C., Nørby, J.G., Maunsbach, A.B. and Esmann, M., eds.), pp. 249–270, Alan R. Liss, New York.
4 Ahmed, K. and Foster, D. (1974) Ann. N.Y. Acad. Sci. 242, 280–292.
5 De Meis, L. (1985) Biochem. Soc. Symp. 50, 97–125.
6 Wiggins, P.M. and MacClement, B.A.E. (1987) Int. Rev. Cyt. 108, 249–303.
7 Watterson, J.G. (1987) Biochem. J. 248, 615–617.
8 De Meis, L. (1989) Biochim. Biophys. Acta 973, 333–349.
9 Wiggins, P.M. (1990) Microbiol. Rev. 54, 432–449.
10 Robinson, J.D. (1989) Biochim. Biophys. Acta 994, 95–103.
11 Barrabin, H., Fontes C.F.L., Scofano, H.M. and Nørby, J.G. (1990) Biochim. Biophys. Acta 1023, 266–273.
12 Ottolenghi, P. (1975) Biochem. J. 151, 57–66.
13 Nørby, J.G., Klodos, I. and Christiansen, N.O. (1983) J. Gen. Physiol. 82, 725–759.
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
15 Glynn, I.M. and Chappel, J.B. (1964) Biochem. J. 90, 140–149.
16 De Meis, L. and Tume, R. (1977) Biochemistry 16, 4455–4463.
17 De Meis, L. and Carvalho, M.G.C. (1974) Biochemistry 13, 5032–5038.
18 De Moraes, V.L.G. and De Meis, L. (1987) FEBS Lett. 222, 163–166.
19 De Moraes, V.L.G. (1990) Biochim. Biophys. Acta 1026, 135–140.
20 Schwarzenbach, G., Gut, R. and Anderegg, G. (1954) Helv. Chim. Acta 117, 937–957.
21 Fabiato, A. and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505.
22 Glynn, I.M. and Richards, D.E. (1982) J. Physiol. (Lond.) 330, 17–43.
23 Fritzsch, G and Koepsell, H. (1983) J. Theor. Biol. 102, 469–476.
24 Fritzsch, G. (1985) J. Theor. Biol. 117, 397–415.
25 Robinson, J.D. (1974) Biochim. Biophys. Acta 341, 232–247.
26 Klodos, I. and Skou, J.C. (1977) Biochim. Biophys. Acta 481, 667–679.
27 Plesner, I.W. and Plesner, L. (1981) Biochim. Biophys. Acta 648, 231–246.
28 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116.
29 Mårdh, S. and Post, R.L. (1977) J. Biol. Chem. 252, 633–638.
30 Klodos, I. and Nørby, J.G. (1987) Biochim. Biophys. Acta 897, 302–314.
31 Pedemonte, C.H. and Beaugé, L. (1986) Arch. Biochem. Biophys. 244, 596–606.
32 Sachs, J.R. (1988) J. Physiol. (Lond.) 400, 575–591.
33 Robinson, J.D. and Pratap, P.R. (1991) Biochim. Biophys. Acta 1061, 267–278.
34 Nørby, J.G. (1970) Acta Chem. Scand. 24, 3276–3286.
35 Mårdh, S. and Zetterquist, Ö. (1974) Biochim. Biophys. Acta 350, 473–483.

36 Hobbs, A.S., Albers, R.W. and Froehlich, J.P. (1983) J. Biol. Chem. 258, 8163–8168.
37 Kaniike, K., Erdmann, E. and Schoner, W. (1974) Biochim. Biophys. Acta 352, 275–286.
38 Rossi, R.C. and Garrahan, P.J. (1989) Biochim. Biophys. Acta 981, 105–114.
39 Grisham, C.M. (1981) J. Inorg. Biochem. 14, 45–57.
40 Pedemonte, C.H. and Beaugé, L. (1983) Biochim. Biophys. Acta 748, 245–253.
41 Forbush, B., III (1987) J. Biol. Chem. 262, 11104–11115.
42 Sachs, J.R. (1988) J. Physiol. (Lond.) 400, 545–574.
43 Cavarrubias, Y.G.-M., Breitwieser, G.E. and De Weer, P. (1987) J. Gen. Physiol. 90, 14a–15a.
44 Skou, J.C. (1960) Biochim. Biophys. Acta 42, 6–23.
45 Bond, G.H. and Hudgins, P.M. (1979) Biochemistry 18, 325–331.
46 Robinson, J.D. (1974) FEBS Lett. 47, 352–355.
47 Forgac, M.D. (1980) J. Biol. Chem. 255, 1547–1553.
48 Robinson, J.D. (1985) in The Sodium Pump (Glynn, I. and Ellory, C., eds.), pp. 391–398, The Company of Biologists, Ltd., Cambridge, UK.
49 Robinson, J.D. (1989) Biochim. Biophys. Acta 997, 41–48.
50 Tashima, Y., Hasegawa, M., Mizunuma, H. and Sakagishi, Y. (1977) Biochim. Biophys. Acta 482, 1–10.
51 Askari, A. and Huang, W.-H. (1984) J. Biol. Chem. 259, 4169–4176.
52 Cantley, L.C., Cantley, L.G. and Josephson, L. (1978) J. Biol. Chem. 253, 7361–7368.
53 Hegyvary, C. and Jørgensen, P.L. (1981) J. Biol. Chem. 256, 6296–6303.
54 Forbush, B., III (1987) J. Biol. Chem. 262, 11116–11127.
55 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 452–463.
56 The, R. and Hasseibach, W. (1977) Eur. J. Biochem. 74, 611–621.
57 De Meis, L. and Suzano, V.A. (1988) FEBS Lett. 232, 73–77.
58 Post, R.L., Toda, G. and Rogers, F.N. (1975) J. Biol. Chem. 250, 691–701.
59 Rossi, R.C. and Nørby, J.G. (1991) in The Sodium Pump: Recent Developments (Kaplan, J.H. and De Weer, P., eds.), pp.419–422, The Rockefeller University Press, New York.
60 Forbush, B. III (1988) J. Biol. Chem. 263, 7961–7969.
61 Karlish, S.J.D. and Stein, W.D. (1982) J. Physiol. (Lond.) 328, 295–316.
62 Carvalho-Alves, P.C. and Scofano, H.M. (1983) J. Biol. Chem. 258, 3134–3139.