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Phosphorylation of Na^+, K^+-ATPase by ATP in the presence of K^+ and dimethylsulfoxide but in the absence of Na^+

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Purified Na^+, K^+-ATPase was phosphorylated by [γ-^{32}P]ATP in a medium containing dimethylsulfoxide and 5 mM Mg^{2+} in the absence of Na^+ and K^+. Addition of K^+ increased the phosphorylation levels from 0.4 nmol phosphoenzyme/mg of protein in the absence of K^+ to 1.0 nmol phosphoenzyme/mg of protein in the presence of 0.5 mM K^+. Higher velocities of enzyme phosphorylation were observed in the presence of 0.5 mM K^+. Increasing K^+ concentrations up to 100 mM lead to a progressive decrease in the phosphoenzyme (EP) levels. Control experiments, that were performed to determine the contribution to EP formation from the P_i inevitably present in the assays, showed that this contribution was of minor importance except at high (20–100 mM) KCl concentrations. The pattern of EP formation and its KCl dependence is thus characteristic for the phosphorylation of the enzyme by ATP. In the absence of Na^+ and with 0.5 mM K^+, optimal levels (1.0 nmol EP/mg of protein) were observed at 20–40% dimethylsulfoxide and pH 6.0 to 7.5. Addition of Na^+ up to 5 mM has no effect on the phosphoenzyme level under these conditions. At 100 mM Na^+ or higher the full capacity of enzyme phosphorylation (2.2 nmol EP/mg of protein) was reached. Phosphoenzyme formed from ATP in the absence of Na^+ is an acylphosphate-type compound as shown by its hydroxylamine sensitivity. The phosphate radioactivity was incorporated into the α-subunit of the Na^+, K^+-ATPase as demonstrated by acid polyacrylamide gel electrophoresis followed by autoradiography.

Introduction

The membrane bound Na^+, K^+-ATPase (the sodium pump) belongs to the category of active ion motive ATPases sometimes called the P-type ATPases [1]. This nomenclature is based on the observation that the hydrolysis of ATP and the coupled cation transport by these ATPases proceeds through the formation of one or more phosphorylated intermediates, EP, where the gamma phosphate residue of ATP has reacted with an aspartic acid residue in the transport system and formed a covalent acylphosphate (see also Nørby [2] for references).

In the reaction schemes for P-ATPases, phosphorylation and dephosphorylation are generally intimately coupled to binding, transport and debinding of the cations [3–6]. Thus formation of EP from ATP is believed to be dependent upon binding and occlusion of Na^+ by Na^+, K^+-ATPase and with binding and occlusion of Ca^{2+} by sarcoplasmic reticulum Ca^{2+}-ATPase.

Recent studies, as well as that reported in the present paper, show, however, that such a coupling is not compulsory. Hara et al. [7] and Polvani and Blostein [8] observed phosphorylation of Na^+, K^+-ATPase (pig kidney and red blood cell enzyme, respectively) from ATP in the absence of Na^+ when pH was 5.0 and 6.2 and they suggest that H^+ can substitute for Na^+ in the reaction. On the other hand, Schuurmans-Stekhoven and co-workers have demonstrated that imidazoleH^+ (but not TrisH^+) and certain other cationic amines can stimulate phosphorylation at neutral pH in the absence of Na^+ [9,10] and they argue that the proton-catalyzed

Abbreviations: Me_2SO, dimethylsulfoxide; PP, pyrophosphate; LDS, lithium dodecylsulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; TNP-ATP, 2′, 3′-*O*-(2,4,6,-trinitrophenyl)-adenosine 5′-triphosphate; *p*-NPP, *p*-nitrophenyl phosphate; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Mes, 4-morpholineethanesulfonic acid; EP, phosphoenzyme.

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phosphorylation may be due to the protonated buffer cations [11].

In the present paper we demonstrate that ATP, acting at an apparently high-affinity site, can phosphorylate Na^+, K^+-ATPase at neutral pH, at low buffer (Tris) concentration, and in the absence of Na^+, provided that the reaction medium contains Mg^{2+} and 20–40% Me_2SO. This process is surprisingly enough stimulated by KCl in concentrations up to 0.5 mM. The observation is in some ways, but not all, analogous to that of Carvalho-Alves and Scofano [12] who demonstrated phosphorylation by ATP of sarcoplasmic reticulum ATPase in the presence of Me_2SO but in the absence of Ca^{2+}.

Experimental Procedures

Enzyme preparations. Membrane-bound enzyme was obtained from pig-kidney outer medulla as described by Jørgensen [13] with the modification [14] that includes several washings to minimize contaminating Na^+ and K^+. The contaminating cations were determined by centrifugation of the enzyme stock solution at 100000 $\times g$ for 30 min, and subsequent flame photometry (Eppendorf) on the supernatant. [Na^+] and [K^+] were estimated to be less than 20 μM and 15 μM, respectively. At 37 °C and under standard conditions [15], it had a Na^+, K^+-ATPase activity of 20 U/mg protein, a ouabain binding capacity of 2.3 nmol/mg protein and a maximal phosphorylation level of 2.2 nmol/mg protein. Protein concentration was measured by the method of Lowry et al. [16].

Chemicals. [γ-^{32}P]ATP was prepared according to the method of Glynn and Chapell [17] except that the labelling reaction was stopped by 20 s heating in boiling water and the reaction mixture was diluted six times prior to chromatography on Dowex anion exchange resin. The [γ-^{32}P]ATP obtained was neutralized with Tris and stored at −18 °C. Tris and Mes were purchased from Sigma. Me_2SO (spectroscopic grade) was obtained from Merck.

Determination of phosphointermediates and enzyme activities. For determination of phosphorylation and hydrolytic activity, the enzyme (50–120 μg/ml) was preincubated at 27 °C in an assay medium containing 5 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 40% Me_2SO, and KCl at the concentrations indicated in the figures. Modifications of the assay media are indicated in the figure legends when appropriate. Before starting the reaction, the pH of the medium was adjusted to pH 7.0. Reactions were always initiated by the addition of 0.1 mM [γ-^{32}P]ATP and, unless otherwise specified, stopped after one min. The volume was 0.5 ml.

The phosphorylation reaction was stopped by addition of 3 ml ice-cold solution of 120 mM perchloric acid containing 5 mM P_i and 5 mM PP_i. The resulting mixture was filtered through Millipore filters (0.45 μm pore size) and the protein captured on the filter was washed seven times with 3 ml of stopping solution. Filters were then dried and counted in a scintillation counter. Controls were performed by denaturing the enzyme with perchloric acid before the addition of [γ-^{32}P]ATP to the assay media. The contaminant P_i present in the media was determined in an aliquot by extraction as phosphomolybdate according to De Meis and Carvalho [18].

ATPase activity was stopped by addition of 0.2 ml of 0.4 M perchloric acid to 0.5 ml of reaction medium. [^{32}P]P_i was measured after dilution of the Me_2SO concentration to 5–8% and addition of 0.4 ml of a suspension containing 0.25% (w/v) charcoal activated with 0.1 M HCl [19,20]. This mixture was stirred for 10 s and centrifuged. Aliquots of the supernatant were counted in a liquid scintillation counter. The rate of P_i production was then calculated from the known specific radioactivity of [γ-^{32}P]ATP.

To determine the contribution of EP formation from P_i in the reaction media, phosphorylation by P_i in the presence or absence of ATP was measured in a reaction medium identical to that used for phosphorylation by ATP except that non radioactive ATP (or no ATP) was used. The desired concentration of P_i was obtained by adding $^{32}P_i$ of known specific activity. Radioactivity incorporated into the enzyme was estimated as described for [γ-^{32}P]ATP phosphorylation. [^{32}P] P_i was obtained from the Brazilian Institute of Atomic Energy and was purified according to De Meis and Tume [21].

Hydroxylamine sensitivity. After stopping the phosphorylation reaction as described above with 3 ml of 120 mM perchloric acid containing 5 mM P_i and 5 mM PP_i, the mixture was filtered through Millipore filters, which were washed two times with 3 ml water. The filters were then washed slowly (15 min) with 5 ml of 50 mM acetate buffer (pH 5.5) with or without 0.4 M hydroxylamine. After two additional washings with 3 ml water, filters were dried and counted for ^{32}P in a liquid scintillation counter.

Polyacrylamide gel electrophoresis. The samples (1.25 ml containing 0.2 mg protein/ml) were phosphorylated at 27 °C in the appropriate media. The reaction was stopped with 6 ml of 120 mM perchloric acid containing 5 mM P_i and 5 mM PP_i and the samples centrifuged 5 min at 5000 rpm. The pellets were washed twice with distilled, cold water and the phosphorylated protein was solubilized in 70 μl of a solution (pH 2.4) containing 1% LDS, 4 mM EDTA, 23 mM citric acid, 3.1 mM phosphoric acid, 2.9 mM Tris, 1% mercaptoethanol, 10 μg/ml pyronine Y and 10% glycerol [22]. Aliquots of 10 μl were submitted to electrophoresis in polyacrylamide gels (7.5% w/v) containing lithium dodecylsulfate at acid pH as described by Lichtner and Wolf [22]. The

gels were dried and submitted to autoradiography using XOMAT -X ray film.

Results

Phosphorylation of Na^+, K^+-ATPase from ATP in the absence of Na^+

The assay solutions used in the experiments to be described contained no more than about 10 μM Na^+ or K^+ as contaminants. These conditions we henceforth denote as 'the absence' of Na^+ or K^+.

First we explored whether Na^+, K^+-ATPase, like Ca^{2+}-ATPase [12,20], could at all be phosphorylated in a medium lacking the usually activating cation (here Na^+) and having a lower water activity because of the presence of up to 40% Me_2SO. Fig. 1 shows that this is indeed the case both at pH 6.0 and pH 7.0. In the preliminary experiments, 0.5 mM KCl was added to inhibit whatever stimulating effect the contaminating Na^+ (see above) may have. The contaminating Na^+ has probably no effect as such (see below) but small concentrations of K^+ were unexpectedly found to stimulate phosphorylation. The experiments to be reported below give a detailed account of the effect of K^+ and will also show that the EP concentrations reached after one minute as in Fig. 1 are probably lower than steady-state levels.

The effect of the K^+ concentration (0–100 mM) on the amount of EP formed by phosphorylating with 0.1 mM [γ-^{32}P]ATP in 40% Me_2SO for one minute is shown in Fig. 2. Concentrations of K^+ below 2–3 mM are 'stimulatory', and the highest values are obtained with 0.2–0.5 mM K^+. Already at this point we want to emphasize that [^{32}P] P_i contaminating the [γ-^{32}P]ATP, or formed during the experiments by hydrolysis of ATP, contributes to EP formation but does not obscure the basic characteristics of the ATP-promoted phosphorylation. Experiments to resolve this problem are reported later.

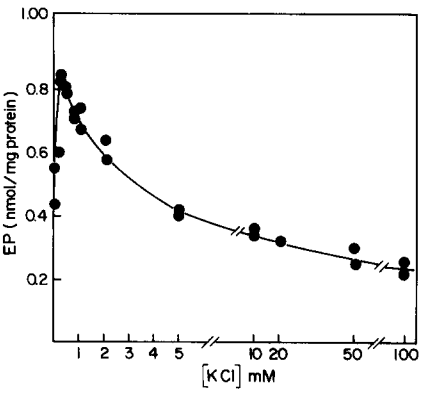

Fig. 2. Effect of K^+ on phosphorylation by ATP. ATPase was phosphorylated for 1 min by 0.1 mM [γ-^{32}P]ATP at 27 °C in the presence of 40% v/v Me_2SO, 5 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$ and increasing concentrations of KCl. No Na^+ was added to the reaction medium.

The time course of the rise in EP is illustrated in Fig. 3. In the reaction medium used here and in the absence of Me_2SO, one must assume that the enzyme is in the E_1-form since these are conditions under which there is a high affinity for ADP, ATP [23] and the ATP 'analog' eosin [24], and a low affinity for P_i [25]. There is no appreciable phosphorylation since the activating Na^+-ion is absent. In 40% Me_2SO the reactivity of the enzyme is changed, and furthermore an effect of 0.5 mM K^+ is observed, increasing the velocity of EP formation. Preliminary data (not shown) reveal that KCl concentrations above 0.5 mM also accelerates the dephosphorylation and that this effect at least is partly responsible for the lower EP level in high KCl.

There is no dramatic effect of pH around the optimum (pH 6.0 to 7.5) but phosphorylation in the presence of 40% Me_2SO and 0.5 mM K^+ is clearly inhibited

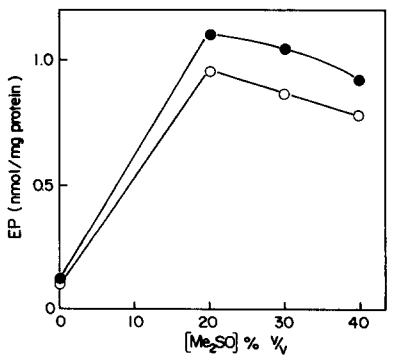

Fig. 1. Effect of Me_2SO on phosphorylation of Na^+, K^+-ATPase by ATP in the absence of Na^+. Purified Na^+, K^+-ATPase was phosphorylated at 27 °C in media containing 0.5 mM KCl, 5 mM $MgCl_2$ and either 5 mM Tris-HCl (pH 7.0) (○) or 5 mM Mes-Tris (pH 6.0) (●) in the presence of different Me_2SO concentrations. The reaction was initiated by addition of 0.1 mM [γ-^{32}P]ATP and stopped after one min. EP was determined according to 'Experimental Procedures'.

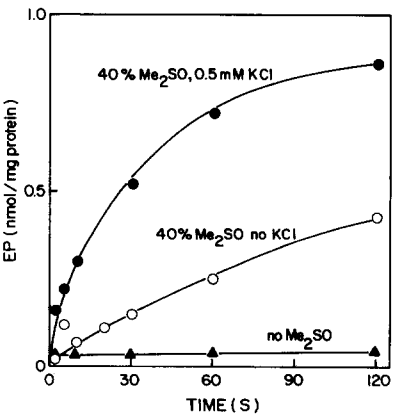

Fig. 3. Time dependence of the phosphorylation reaction. Na^+, K^+-ATPase was phosphorylated at 27 °C by [γ-^{32}P]ATP, in the presence of 5 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.0) in the absence of Me_2SO and with no added monovalent cations (▲) or in the presence of 40% Me_2SO and no KCl (○) or 0.5 mM KCl (●).

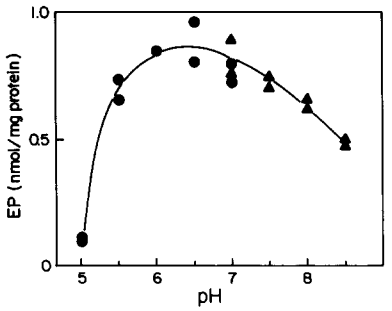

Fig. 4. pH-dependence of phosphoenzyme levels in 40% Me_2SO. Phosphoenzyme levels were measured at 27°C in media containing 40% v/v Me_2SO, 0.5 mM KCl, 5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP, and 5 mM Tris-HCl (▲) or Mes-Tris (●) adjusted to the desired pH.

at pH 5.0 (Fig. 4), where others [7,8,11] find a Na^+-independent phosphorylation in the absence of Me_2SO.

In 40% Me_2SO, low concentrations of Na^+ have no effect on EP-level when there is 0.5 mM K^+ in the medium (Fig. 5). In the absence of K^+ there is a doubling of [EP] when 5 mM NaCl is added, and with a concentration of 100 mM Na^+ or higher, the full phosphorylation capacity is reached both in the presence and absence of K^+. This situation corresponds to 'normal', Na^+-stimulated phosphorylation by ATP in the absence of Me_2SO, and it indicates that also in the presence of Me_2SO the phosphorylation 'rate constant' with Na^+ is much higher than the dephosphorylation 'rate constant'.

The hitherto described experiments were all done with 0.1 mM ATP, and from Fig. 6 it appears that this concentration is much higher than $K_{0.5}$ for ATP both with and without K^+. The experiments in Fig. 6 report the EP concentration after 30 s of phosphorylation and as a first approximation (especially valid for the experiments at zero K^+) the ordinate values may be considered to represent initial rate of EP formation. The

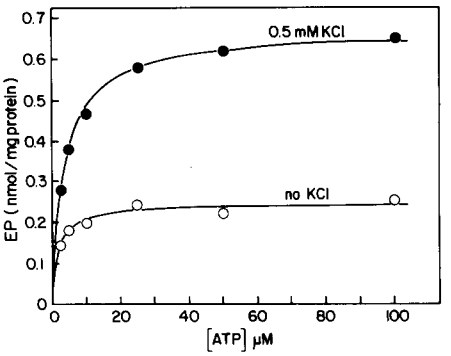

Fig. 6. ATP concentration dependence of the rate of phosphoenzyme formation in 40% Me_2SO. ATPase was phosphorylated in 40% Me_2SO, under the conditions of Fig. 3 at different [γ-^{32}P]ATP concentrations. KCl concentrations were zero (○) and 0.5 mM (●). The phosphorylation time was 30 s.

curves in Fig. 6 are rectangular hyperbolae corresponding to a $K_{0.5}$ for ATP of 3.7 μM (0.5 mM K^+) and 1.8 μM (no K^+), respectively. The apparent affinity for ATP is thus considerably higher than that corresponding to the K_m for ATP hydrolysis under optimal conditions [26], but still lower than the ATP affinity for the high affinity site [26,27].

ATP is hydrolyzed slowly during the course of the experiments (in Me_2SO, without Na^+). Although the rate of hydrolysis is small (see below) it is clear from Fig. 7 that 0.5 mM K^+ exerts a stimulating effect on hydrolysis. With 0.5 mM K^+ the K_m for ATP (assuming simple kinetics) is around 4.6 μM and V_{max} is 2.4 nmol/mg protein per min which is more than three orders of magnitude lower than V_{max} under optimal conditions for this enzyme preparation (Experimental Procedures) and about two orders of magnitude lower than the Na^+-ATPase activity. The ATPase activity under the circumstances described is inhibited by 2 μM

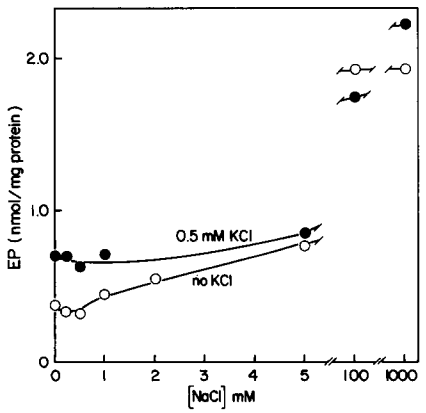

Fig. 5. Effect of Na^+ on the phosphorylation by ATP in the presence of 40% Me_2SO. Na^+, K^+-ATPase was phosphorylated by 0.1 mM [γ-^{32}P]ATP, in the presence of 40% Me_2SO, 5 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.0), NaCl at the indicated concentrations, and zero (○) and 0.5 mM (●) KCl.

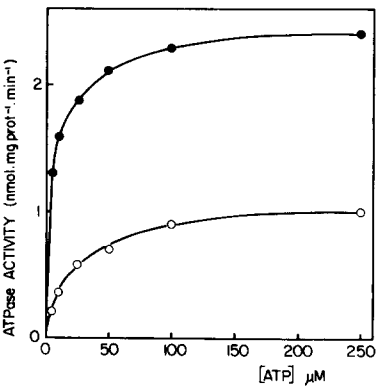

Fig. 7. ATP concentration dependence of the initial rate of ATP hydrolysis in 40% Me_2SO and in the absence of sodium. Hydrolysis of [γ-^{32}P]ATP was measured under the same conditions as in Fig. 6. The P_i liberation was linear at least up to 60 min of reaction. The KCl concentrations were zero (○) and 0.5 mM (●). The reaction time was varied between 10 and 60 min to attain 5 to 10% of hydrolysis.

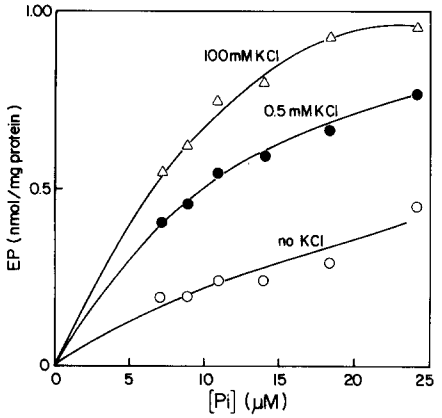

Fig. 8. Phosphorylation by [^{32}P]P_i in the presence of 0.1 mM ATP. Na^+, K^+-ATPase was phosphorylated during 60 s by different concentrations of [^{32}P]P_i added to the media which contained 40% Me_2SO, 5 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.0), 0.1 mM non-radioactive ATP, and zero (○), 0.5 (●) or 100 mM (△) KCl. The P_i concentration was the sum of contaminant P_i in ATP (0.3 to 1% of total ATP, i.e., 0.3–1 μM), P_i from ATP hydrolyzed in 60 s by the ATPase (e.g. 0.5 μM at 100 mM KCl), P_i contained in the $^{32}P_i$ used as a tracer (approx. 2 μM), and non-radioactive P_i (added up to the desired concentration).

ouabain down to 0.6 nmol/mg protein per min (at ATP > 100 μM).

To characterize specifically the phosphorylation from [γ-^{32}P]ATP it was necessary to determine the contribution to EP formation from the small amounts of [^{32}P] P_i inevitably present in the reaction mixtures. In the presence of ATP there is an antagonism between ATP and P_i in EP formation (data not shown) and, as shown in Fig. 8, where all experiments have 0.1 mM ATP, this antagonism is dependent upon the K^+ concentration (at least when there is 40% Me_2SO in the medium), i.e. the higher the K^+ concentration the higher the EP formation from P_i. Knowing the concentration and specific activity of [^{32}P] P_i in the reaction media, it is possible to correct the EP-levels for contribution by P_i and thus estimate specifically how much EP that was formed by phosphorylation with ATP. This is done as follows: The total amount of [^{32}P] P_i in the media is the sum of [^{32}P] P_i from the stock-solution of labelled ATP (0.3 to 1% of ATP determined as described in Experimental Procedures) and [^{32}P] P_i from enzymatic hydrolysis during the experiment from Fig. 7 and similar data (not shown) at the various KCl concentrations. From this sum, the amount of phosphorylation by P_i in the presence of 0.1 mM ATP is taken from Fig. 8 and similar experiments (data not shown) with the appropriate assay conditions. Fig. 9A shows the total phosphorylation (data from the 1 min experiments in Fig. 2), the calculated phosphorylation from P_i and the difference between these, which is the specific phosphorylation from ATP. The results show that the pattern of phosphorylation and the ef-

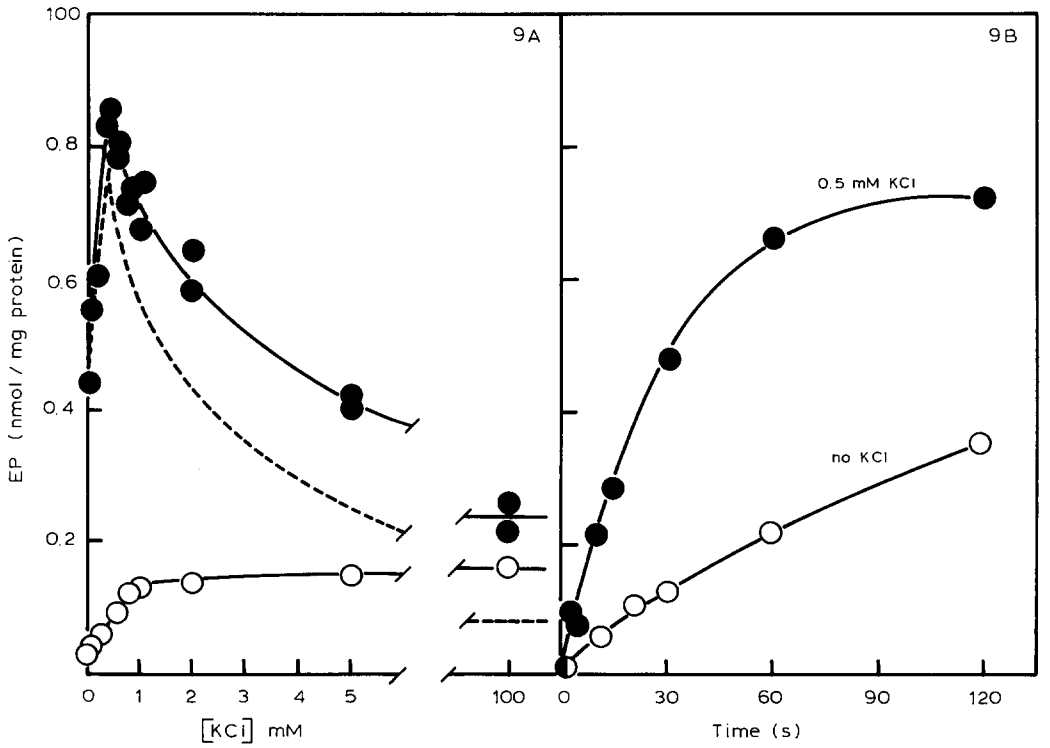

Fig. 9. Phosphorylation of Na^+, K^+-ATPase by [γ-^{32}P]ATP in 40% Me_2SO corrected for the contribution by contaminating [^{32}P]P_i. (A) Phosphorylation as a function of the KCl concentration. The values for total phosphorylation (●) are taken from Fig. 2 and those for phosphorylation by P_i (○) are calculated using the data in Fig. 8 as described in the text. The difference, indicating the specific phosphorylation by ATP, is shown as the dotted line. (B) Time course of specific phosphorylation by ATP in the presence of 0 mM KCl (○) or 0.5 mM KCl (●). The values shown are the data of Fig. 3 corrected for the phosphorylation by P_i, see Figs. 8 and 9A.

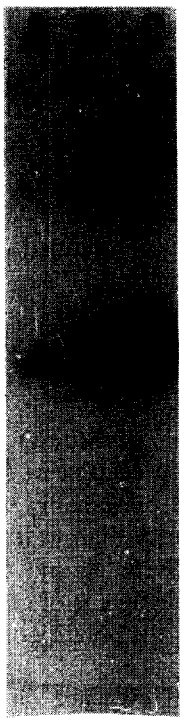

Fig. 10. Dodecylsulfate-polyacrylamide gel electrophoresis of phosphorylated Na^+, K^+-ATPase. Na^+, K^+-ATPase was phosphorylated in 40% Me_2SO for 1 min at 27°C as in Fig. 3, in the presence of zero (slot A) or 0.5 mM KCl (slot B). As a reference enzyme phosphorylated in the absence of Me_2SO and KCl but in the presence of 100 mM NaCl (5 s at room temperature) was run in slot C. Electrophoresis in the presence of LDS and autoradiography were performed according to Experimental Procedures.

fects of K^+ shown in the previous figures are characteristic of the ATP-promoted phosphorylation. The phosphorylation by P_i will become more expressed at longer assay times since P_i formed by ATP hydrolysis will accumulate. In Fig. 9B the data from Fig. 3 are corrected for phosphorylation by P_i and comparison of the two figures reveal that this contribution from P_i has little effect on the results. (To correct the data of Fig. 3, phosphorylation by P_i was determined in the presence of 0.1 mM ATP – as described above, e.g. Fig. 8 – at various concentrations of P_i and KCl and at various times of phosphorylation, data not shown)

Finally, the experiment in Fig. 10 reveals that the phosphorylation from [γ-^{32}P]ATP is in the alpha-peptide, i.e. at the same position on the gel as phosphorylation with Na^+ in the absence of Me_2SO. It is also important (Table I) that the EP formed is almost 100% sensitive to hydroxylamine, indicating that the enzyme-phosphoryl compound is an acid anhydride, and not, for example, an ester [28,29].

TABLE I

The effect of hydroxylamine on phosphoenzyme stability

Na^+, K^+-ATPase was phosphorylated for 1 min at 27°C by 0.1 mM [γ-^{32}P]ATP in 40% Me_2SO, 5 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.0), and with 0 mM KCl, 0.5 mM KCl or 0 mM KCl+100 mM NaCl. Phosphorylated enzyme was treated with hydroxylamine or with acetate buffer pH 5.5 as described in Experimental Procedures.

	EP remaining (nmol/mg protein)		
	no KCl	0.5 mM KCl	100 mM NaCl
Control	0.33	0.90	2.08
+sodium acetate buffer (pH 5.5)	0.30	0.91	
+0.4 M Hydroxylamine (pH 5.5)	0.03	0.10	0.21

Discussion

In the present communication we have shown that Na^+, K^+-ATPase can be phosphorylated by ATP in a low ionic strength Tris-buffer, at neutral pH, with 5 mM $MgCl_2$ present but in the absence of Na^+, provided that the medium contains 20–40% (v/v) Me_2SO (Fig. 1). This is an expansion of the previous conditions for phosphorylation: the presence of Na^+ (for a review, see Ref. 30) or amine buffer cations (at pH 5–6) that have been shown to catalyze phosphorylation in the absence of Na^+ [9,10]. The phosphorylation site is on the alpha-peptide (Fig. 10). Furthermore the EP formed is acid stable but highly sensitive to hydroxylamine (Table I), there is competition between ATP and P_i in EP formation, and the phosphorylation by ATP is completely inhibited by ouabain (not shown). There is thus good reason to believe that the phosphorylation site is at the aspartyl residue normally involved in formation of the phosphointermediates of Na^+, K^+-ATPase [2].

It is generally considered that the catalytic subunit of Na^+, K^+-ATPase can adopt to one of two major classes of protein conformations depending upon its liganded state. These are denoted by subscripts 1 and 2, and the dephosphoenzymes E_1 and E_2 as well as the phosphointermediates E_1P and E_2P are essential components in the reaction scheme (for definitions and references see Refs. 5 and 31). Also Ca^{2+}-ATPase goes through two major conformational changes during the catalytic cycle [6].

Normally, the enzyme has to be in the E_1-form to be phosphorylated by ATP. With the reaction medium used in the present study in the absence of Me_2SO (and K^+) one would assume that the enzyme was in the E_1-form (Results). Addition of Me_2SO to the medium changes the reactivity of the enzyme. Is has previously been shown that Me_2SO inhibits the ATPase activity [32–35]. The ATPase activity in the present study (no Na^+, $\pm K^+$) is likewise very low (Fig. 7). The enzyme reacts with ATP to form acid stable, phosphorylated intermediates (Fig. 1), but the build-up of the EP-pool

(Fig. 3) is also extremely slow compared to what is seen under optimal conditions [36,37]. The apparent affinity for ATP is high and of the size of that for Na^+ catalyzed phosphorylation [37] i.e. 10^6 M^{-1} to 10^5 M^{-1}. This is slightly lower than the affinity in equilibrium binding experiments with the E_1 form of the enzyme [27,38] and than $1/K_m$ for Na^+-ATPase activity (39) but much higher than the binding affinity in the presence of K^+ (E_2-form) and in the Na^+, K^+-ATPase reaction [39].

One of the problems one encounters when trying to assign the properties studied to a particular conformation of the catalytic peptide is the fact that both for the dephospho- and for the phosphoenzymes there are multiple (sub) conformations [40,41]. From the observed ATP affinity in phosphorylation and hydrolysis just discussed, one might be inclined to think that it is an E_1-like conformation that is phosphorylated.

On the other hand, the observation that the K^+-pNPPase reaction of Na^+, K^+-ATPase is stimulated by Me_2SO has led to the proposal that Me_2SO shifts the equilibrium between E_1 and E_2 and E_1P and E_2P towards the E_2 conformations [32–35]. It is indeed quite likely that the principal conformation of the enzyme when it catalyzes *p*-NPP hydrolysis is E_2 [42–44]. Also the greatly increased affinity for P_i in Me_2SO-solutions [25] points towards an E_2 conformation although there might be other explanations, see below.

An interesting part of the present study in connection with the discussion concerning conformations are the unexpected results on the effect of K^+ on the EP level when the enzyme is phosphorylated from ATP (Figs. 2, 3, 6 and 9), and the modest stimulation by small concentrations of Na^+ (Fig. 5). K^+ has a dual effect on the ATP-produced EP level: EP is doubled when K^+ is increased from 0.015 mM (contaminating) to about 0.5 mM, but further addition (100 mM is the largest concentration assayed) decreases EP down to less than 5% (Fig. 2 and 9). The latter 'inhibition' is half-way completed at 2–3 mM K^+, and this range of $K_{0.5}$ is equal to that obtained in K^+-titrations of E_1-eosin fluorescence [24] and of fluorescein labeled Na^+, K^+-ATPase [41] in the presence of 5 mM $MgCl_2$ (but in the absence of Me_2SO). These titrations convert the E_1-form to the E_2-form. A $K_{0.5}$ of 2–3 mM is also found for K^+-activation of the K^+-pNPPase activity of Na^+, K^+-ATPase both in the absence and the presence of 10% Me_2SO [34]. We have yet to explore in detail the effect of KCl on the rate of dephosphorylation, but preliminary data (not shown) suggest that an increased rate at high KCl could be in part responsible for the lower EP levels.

The activating effect of K^+ (Figs. 2, 3, 6, and 9) which has a $K_{0.5}$ of 0.1 mM or less, must be a consequence of K^+-binding to a site different from the inhibitory site(s) discussed above. If we assume that with K^+-concentrations higher than 0.5 mM, the enzyme approaches the E_2 conformation also in the presence of 40% Me_2SO, then we might hypothesize that the stimulating effect of K^+ takes place on an E_1-like enzyme form. Another interesting possibility is, that Me_2SO changes the microenvironment of the ATP site in such a way that it corresponds neither to the E_1 nor to the E_2 state, as these states are defined in water. In this form the site might recognize ATP as well as P_i.

On the basis of the present experiments it is difficult to draw conclusions regarding the molecular role of Me_2SO in the (K^+-stimulated) formation of EP from ATP in the absence of Na^+. It is generally acknowledged that addition of Me_2SO to the medium will increase the partition coefficient for water soluble substances like P_i (and presumably also ATP) between a phase of lower polarity than water - and water [45], for references see also Refs. 46 and 47. Whether this phenomenon plays a role here is of course dependent upon, among other things, the polarity of the ATP-site relative to that of water.

The experimental evidence on the polarity (hydrophobicity) of the ATP-binding site on Na^+, K^+-ATPase is sparse. Skou and Esmann [48] concludes from eosin-binding experiments, that the ATP site on E_1 has a 'low polarity' comparable to that of ethyl alcohol. Binding experiments with TNP-ATP show "..a relative polar environment similar to that of methanol and glycerol.." [49], and suggest that there is little difference between the Na^+-form (E_1) and the K^+-form (E_2) in the polarity of the site. The conclusion must be, that addition of Me_2SO to the system might well increase the partition of ATP into the site, but whether or not this plays a role here is uncertain (see also above concerning the different affinities for ATP).

In Me_2SO, the binding of ATP is followed by P-transfer. This is not the case in the absence of Me_2SO and Na^+. It might be that Me_2SO, which almost certainly can affect the tertiary state of proteins [50], positions the gamma phosphate of the bound ATP in a favorable state relative to the accepting Asp-COOH group. How (or whether) this is enhanced by K^+ (see above) and how it can be accomplished in water (no Me_2SO) with certain cationic buffers, is impossible to say at the moment.

Additionally one might also hypotesize that Me_2SO in the solution in the absence of Na^+ renders the environment of the catalytic site more 'gas phase like'. In water, the ΔG-values for hydrolysis of -COOP and ATP are practically the same [47], i.e. the equilibrium constant between EATP and E-P(ADP) is practically one [51]. If bound ATP and bound-COOP are analogous to PP and acetylphosphate [47] then in a 'gas phase like' environment there would probably be an increased tendency to formation of EP (i.e., -COOP) from ATP.

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