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IMIDAZOLE CHLORIDE AND TRIS-CHLORIDE SUBSTITUTE FOR SODIUM CHLORIDE IN INDUCING HIGH-AFFINITY Ado*PP*[NH]*P* BINDING TO $(Na^+ + K^+)$-ATPase

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Optimal binding of [2,8-^{3}H]Ado*PP*[NH]*P* to $(Na^+ + K^+)$-ATPase requires 25 mM Na^+ (Cl^-), 50 mM imidazole$^+$ (Cl^-) or 50 mM Tris$^+$ (Cl^-). Chloride is essential as counterion. We conclude that imidazole$^+$ and Tris$^+$ are able to bind to the Na^+ site, and recommend the use of dilute buffers for studying the partial reactions of $(Na^+ + K^+)$-ATPase. In NaCl or the substituting buffers the dissociation constant for the enzyme-Ado*PP*[NH]*P* complex at 0°C and pH 7.25 is 0.4 μM, whereas in millimolar $MgCl_2$ it is about 2 μM. These distinct levels in affinity with $MgCl_2$ as compared to NaCl, together with the $MgCl_2$-dependence of photolabelling of the enzyme with ATP analogues (Rempeters, G. and Schoner, W. (1981) Eur. J. Biochem. 121, 131–137), suggest significant changes within the substrate site of $(Na^+ + K^+)$-ATPase upon binding of $Mg^{2+}(Cl^-)_2$.

Introduction

The formation of a phosphointermediate in $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) catalyzing the active transport of univalent cations through cellular membranes depends on the simultaneous presence of Na^+ and Mg^{2+} [1,2]. For the cation requirement of the preceding ATP binding, however, contradictory results have been reported [3–5]. Nørby and Jensen [3] and Hegyvary and Post [4] report ATP binding to a high-affinity site in plain buffers. Addition of NaCl to the incubation medium was reported to have no significant effect [3] or to stimulate ATP binding slightly [4]. However, Robinson [5] saw with Ado*PP*[NH]*P*, a non-hydrolyzable ATP analogue, that NaCl stimulated its binding significantly. Moreover, 50 μM $MgCl_2$ increased the affinity for Ado*PP*[NH]*P*, although ATP binding is found in the presence of $MgCl_2$ chelators. Millimolar $MgCl_2$ concentrations led to a decrease in the nucleotide affinity [5]. On the other had, we found that millimolar concentrations of $MgCl_2$ enhanced the inactivation of $(Na^+ + K^+)$-ATPase by two different ATP affinity labels [6,7]. Their action was also enhanced by the presence of low concentrations of Na^+.

This confusing situation, that NaCl or $MgCl_2$ may or may not affect ATP binding [3–5], and the effects of these ions on the interaction of the enzyme with protein-reactive ATP analogues [6,7], led us to the suggestion that the experimental conditions used so far may cause those contradictory results. This assumption seems also likely from the finding that Tris buffer or choline induce

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Abbreviations: Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; Hepes; *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid, $pK_a = 7.55$.

the Na^+ form of $(Na^+ + K^+)$-ATPase as detected by trypsinolysis under controlled conditions [8]. Moreover, choline, Mg^{2+} and protonated Tris as well as histidine have been found to show an 'Na^+' effect on the extrinsic fluorescence of eosine maleimide [9]. Consistent with our suggestion, we demonstrate here that the effects of NaCl and $MgCl_2$ on the binding of ATP or Ado*PP*[NH]*P* to $(Na^+ + K^+)$-ATPase are masked or confused by the buffers used previously. We found that the buffer composition and its ionic strength are important in obtaining comparable and reproducible data.

Material and Methods

Chemicals

[2,8-^{3}H]Adenosine 5'-[β,γ-imido]triphosphate ([2,8-^{3}H]Ado*PP*[NH]*P*) was purchased from ICN Chemical and Radioisotope Division (Irvine, CA, USA). Non-radioactive Ado*PP*[NH]*P* was from Boehringer (Mannheim). All other chemicals were obtained from Merck (Darmstadt), Boehringer (Mannheim) or Serva (Heidelberg).

Enzyme and assay

$(Na^+ + K^+)$-ATPase was prepared from pig kidney [10] with a specific activity of 10–14 U/mg. The contamination of the enzyme preparations by K^+ and Na^+ as determined by flame photometry were: 0.43 μmol Na^+/mg protein and 0.03 μmol K^+/mg protein. The enzyme preparation was either allowed to stand at 4°C for 2 weeks or was freed from ATP according to Ref. 11. It was finally adjusted to an activity of 5 U/ml in 1 mM imidazole chloride (pH 7.25)/40 μM EDTA. $(Na^+ + K^+)$-ATPase activity was determined with a coupled optical assay [6]. One unit of $(Na^+ + K^+)$-ATPase is defined as the amount of enzyme hydrolyzing 1 μmol ATP/min at 37°C.

Binding experiments

[2,8-^{3}H]Ado*PP*[NH]*P* was diluted with non-radioactive Ado*PP*[NH]*P* (imidazole salt) to obtain a specific radioactivity of $7 \cdot 10^3$ Ci/mol and a concentration of $1.4 \cdot 10^{-7}$ Ci/ml. Into centrifugation tubes immersed in ice were pipetted 0.5 ml of this solution, 0.5 ml of buffers, 0.5 ml of enzyme suspension and 0.1–0.5 ml of various concentrations of non-radioactive Ado*PP*[NH]*P* (imidazole salt). The final volume was made up to 2 ml with distilled water. The contents of the tubes were mixed immediately and the enzyme was sedimented at $100\,000 \times g$ and 0°C for 20 min. The supernatant was decanted and the interior of each tube was wiped carefully. The pellet then was solubilized in 0.5 ml 1 M NaOH at 60°C. The solubilized protein was transferred to scintillation vials already containing three drops of concentrated HCl. 8 ml scintillation fluid containing Triton X-100 [3] were added and the radioactivity was meausred in a Tri-Carb® 460 CD (Packard Instruments, Zürich, Switzerland) equipped with the external standard option. The buffers and salts used in this study were contaminated by Na^+ and K^+ as shown in Table I. These impurities were determined by flame photometry.

Calculation of binding capacity and dissociation constant

Each determination of the maximal binding capacity and of the dissociation constant of the enzyme-Ado*PP*[NH]*P* complex is based upon a series of 12 tubes each containing $0.7 \cdot 10^{-7}$ Ci of radioactivity and variable concentrations of total Ado*PP*[NH]*P* (5 nM–10 μM). A concentration of 0.5 mM Ado*PP*[NH]*P* was included as a control; radioactivity bound to the enzyme under this condition was regarded to be non specifically bound and was subtracted from total binding of non-controls. The maximal binding capacity was determined from the ordinate intercept of a Klotz plot. The dissociation constant (K_d) was determined from a Hill Plot.

In cases of very low affinity of the enzyme for Ado*PP*[NH]*P* (dissociation constants above 10–20

TABLE I

CONTAMINATION OF SALT AND BUFFER SOLUTIONS BY Na^+ AND K^+ AS DETERMINED BY FLAME PHOTOMETRY

Solution (0.1 M)	$[Na^+]$ (M)	$[K^+]$ (M)
Imidazole-HCl (pH 7.25)	$5 \cdot 10^{-6}$	$2.5 \cdot 10^{-7}$
Histidine	$< 2 \cdot 10^{-7}$	$< 10^{-6}$
Tris-HCl (pH 7.25)	10^{-5}	$< 10^{-6}$
Choline chloride	$< 2 \cdot 10^{-7}$	$< 10^{-6}$

μM), the maximal binding capacity could not be determined with accuracy. Under these circumstances the dissociation constant was calculated on the basis of the average maximal binding capacity of the enzyme preparation as determined in parallel incubations under suitable conditions.

To judge for variations of Ado*PP*[NH]*P* binding between different enzyme preparations, binding in 100 mM imidazole chloride (pH 7.25) was always measured as a control.

Results

Study on the binding capacity for AdoPP[NH]P under different experimental conditions

The capacity of $(Na^+ + K^+)$-ATPase for binding of Ado*PP*[NH]*P* (pmol Ado*PP*[NH]*P*/U enzyme) varies only slightly for different enzyme preparations, irrespective of the ligands added. Preparations which had been allowed to stand at 4°C for 2 weeks prior to use to degrade ATP had a binding capacity of 105 ± 11 pmol Ado*PP*[NH]*P* /U enzyme ($n = 28$; three different preparations). The washing procedure to remove ATP [11] increased the binding capacity to 128 ± 10 pmol Ado*PP*[NH]*P*/U enzyme ($n = 23$; two different enzyme preparations). This is probably due to the fact that washing decreases the activity of the enzyme, whereas the binding of Ado*PP*[NH]*P* seems to be not equally affected.

The degradation of ATP as judged from competitive binding of [2,8-^{3}H]Ado*PP*[NH]*P* is complete after storage of the enzyme preparations at 4°C for 2 weeks. A contaminating myokinase activity, which is present in trace amounts (Rempeters, G., unpublished data), probably degrades ATP to AMP.

Influence of different incubation conditions on the dissociation constant of the enzyme complex with AdoPP[NH]P

In contrast to the binding capacity, the dissociation constant of the enzyme-Ado*PP*[NH]*P* complex is severely affected by alterations of the ionic strength of the incubation medium, the nature of the buffer and the presence of variable ligands (Fig. 1, Table II). The K_d varies only slightly, however, with different enzyme preparations.

Effect of the buffer. The dissociation constant of the enzyme-Ado*PP*[NH]*P* complex depends on the kind of buffer as well as upon its concentration. In 10 mM imidazole chloride (pH 7.25), the dissociation constant is about 20 μM and decreases to 0.4 μM in 100 mM imidazole chloride. Upon a further rise in the imidazole chloride concentration the dissociation constant again increases (data not

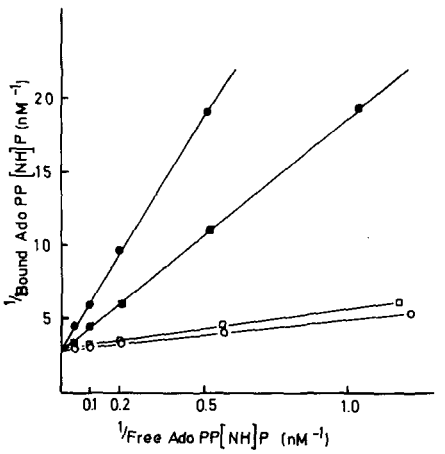

Fig. 1. Klotz-plot of Ado*PP*[NH]*P* binding to $(Na^+ + K^+)$-ATPase. Binding was measured in 100 mM imidazole chloride (pH 7.25) (□), 100 mM imidazole chloride (pH 7.25)+5 mM $MgCl_2$ (■), 100 mM Tris-HCl (pH 8.25) (○) and 100 mM Tris-HCl (pH 8.25)+5 mM $MgCl_2$ (●).

TABLE II

Ado*PP*[NH]*P* BINDING TO $(Na^+ + K^+)$-ATPase IN VARIOUS BUFFERS (pH 7.25) OF ABOUT 50 mM IONIC STRENGTH

The dissociation constants (K_d) were determined as described under Material and Methods. Binding in 100 mM imidazole chloride (pH 7.25) was run as a control with each enzyme preparation. The number of determinations (n) and the standard deviation (S.D.) of the K_d are as indicated.

Buffer	Concentration (mM)	Ionic strength (mM)	$K_d \pm$S.D. (μM)
Imidazole chloride	100	47	0.40 ± 0.08 ($n = 5$)
Tris-HCl	50	46	0.37 ± 0.05 ($n = 3$)
Histidine/Tris-HCl [a]	50	50	1.10
Triethylamine-CO_2	50	50	0.87
Hepes-triethylamine	50	50	4.65

[a] 50 mM histidine/Tris-HCl contains 50 mM histidine and 50 mM Tris.

shown). In Tris-HCl (pH 7.25), optimal binding is achieved with a buffer concentration of 50 mM. The corresponding dissociation constant is 0.34 μM. In histidine/Tris-HCl (pH 7.25), binding is weaker. The optimal dissociation constant of 1.1 μM is observed at a concentration of 50 mM (not shown).

Taking into account the different degree of ionization at pH 7.25 of imidazole, Tris and histidine with pK_a values of 7.2, 8.3 and 6.2, respectively, it becomes evident that in all these buffers optimal affinity is reached at an ionic strength of 50 mM (Fig. 2). Table II lists the affinity of the enzyme for Ado*PP*[NH]*P* at an ionic strength of 50 mM in these and other buffers. It is obvious that the highest affinity is reached in imidazole chloride and Tris-HCl. The minimal ionic strength of imidazole chloride and Tris-HCl required for a

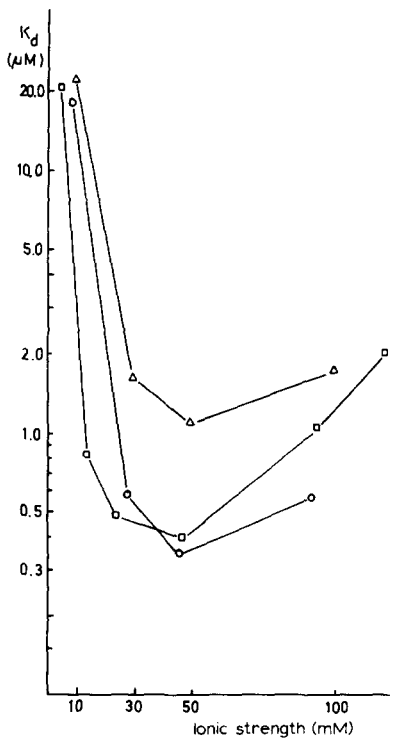

Fig. 2. Effect of the ionic strength of different buffers on the dissociation constant (K_d) of the enzyme-Ado*PP*[NH]*P* complex. Binding was measured in imidazole chloride (pH 7.25) (□), Tris-HCl (pH 7.25) (○) and histidine-Tris-HCl (pH 7.25) (△). Histidine/Tris-HCl buffer contains equimolar concentrations of histidine and Tris, i.e., 10 mM histidine/Tris-HCl contained 10 mM histidine and 10 mM Tris.

reliable measurement of Ado*PP*[NH]*P* binding is 5–10 mM. Under this conditions the dissociation constant is about 20 μM. The dissociation constant can be expected to increase further beyond this value upon decreasing the concentration of buffer, and Ado*PP*[NH]*P* binding would approach zero. To ensure a sufficient buffering capacity and to have a reliable starting point for variation of other ligands a miminal buffer concentration of 10 mM was therefore maintained in all binding experiments.

Effects of choline chloride, NaCl and $MgCl_2$. In 10 mM imidazole chloride, increasing concentrations of NaCl decrease the dissociation constant of the enzyme Ado*PP*[NH]*P* complex from about 20 μM without NaCl to 0.4 μM in the presence of 25 mM NaCl (Fig. 3). The half-maximal effect was observed with 0.6 mM NaCl. There appears to be a second Na^+-binding site, as is evident from the reciprocal plot (Fig. 3, inset) and the upwards bending of the K_d values above 30 mM ionic strength.

Above 25 mM NaCl the dissociation constant for Ado*PP*[NH]*P* again increases. The optimal ionic strength under these conditions thus is about 30 mM. This value agrees with that found in the presence of choline chloride. This substance shows a high $K_{0.5}$ value (Fig. 3).

The effect of 50 mM NaCl in the presence of variable concentrations of Tris-HCl (pH 7.25) is demonstrated in Fig. 4. The nearly perfect coincidence of the curves 'dissociation constant versus ionic strength of the incubation medium' for Tris buffer and Tris buffer + 50 mM NaCl (Fig. 4) indicates that the action upon the enzyme in inducing Ado*PP*[NH]*P* binding might be the same for Tris-HCl and NaCl. Similar effects of imidazole chloride and NaCl have been found (not shown). The optimal dissociation constants induced by NaCl, imidazole chloride and Tris-HCl all amount to $K_d \approx 0.4$ μM.

The apparent affinity of the enzyme for Na^+ is higher than for imidazole$^+$ and Tris$^+$ (cf. Fig. 2 to Fig. 3), which indicates that Na^+ is the 'natural' ligand.

Effect of the counterion for Na^+. The effect of inducing high-affinity Ado*PP*[NH]*P* binding by NaCl may be attributable to Na^+ as well as to Cl^-. The effect of Na^+ is evident from experi-

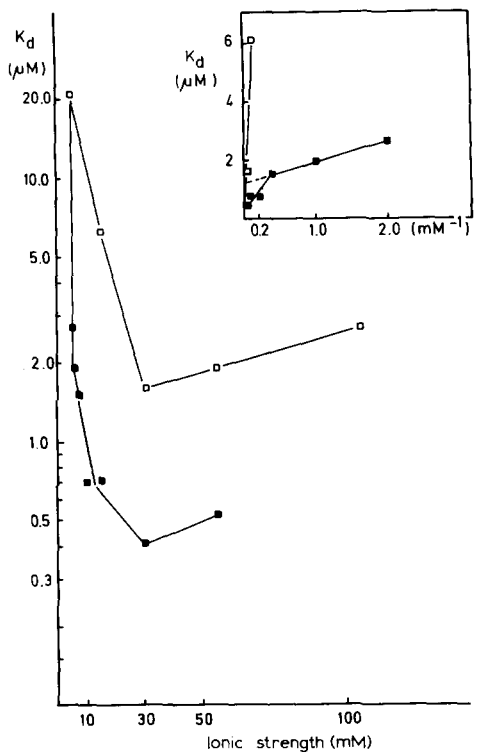

Fig. 3. Dependence on NaCl or choline chloride of the dissociation constant (K_d) of the enzyme-Ado*PP*[NH]*P* complex. Binding was measured in 10 mM imidazole chloride (pH 7.25) with variable concentrations of NaCl (■) or choline chloride (□). The inset shows the dependence of K_d on the reciprocal concentration of NaCl or choline chloride: $K_{0.5}$ for NaCl 0.6 mM.

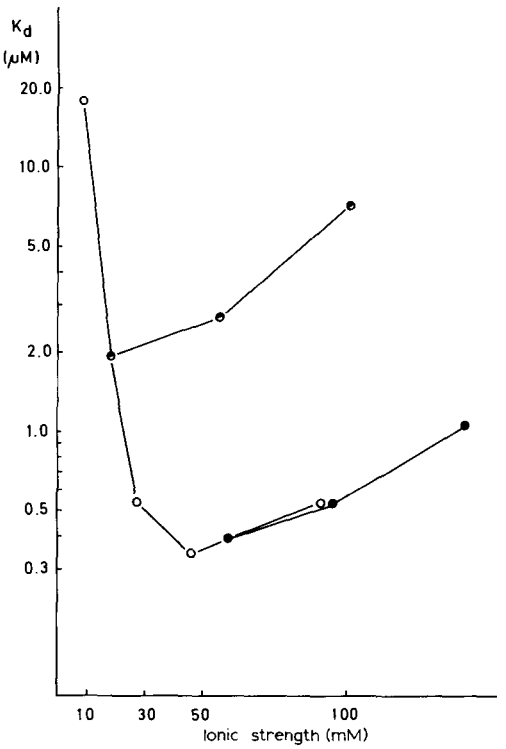

Fig. 4. Dependence of the dissociation constant (K_d) of the enzyme-Ado*PP*[NH]*P* complex on the ionic strength of the incubation medium containing variable concentrations of Tris-HCl (pH 7.25) buffer or additionally NaCl or $MgCl_2$. Binding was measured in variable concentrations of Tris-HCl (pH 7.25) (○), Tris-HCl (pH 7.25) plus 50 mM NaCl (●) or Tris-HCl (pH 7.25) plus 5 mM $MgCl_2$ (◐).

ments with choline chloride, showing a specific Na^+ effect (Fig. 3). An effect of the nature of the counterion on the binding of Ado*PP*[NH]*P* is evident from experiments with Na_2SO_4, $NaNO_3$ and NaSCN (Table III). With none of the respective anions did the affinity of the enzyme for Ado*PP*[NH]*P* reach the same level as with NaCl. With NaSCN the affinity was extremely low.

Effect of $MgCl_2$. Increasing the concentation of $MgCl_2$ up to 5 mM leads to a dissociation constant of about 2 μM. This final value is reached in 10 mM Tris-HCl (pH 7.25) as well as in 50 mM imidazole chloride (pH 7.25). The $K_{0.5}$ for $MgCl_2$ in 10 mM Tris-HCl is 0.14 mM (Fig. 5, inset A). In 50 mM imidazole chloride no exact $K_{0.5}$ could be determined, since the corresponding plot is non-linear (Fig. 5, inset B). The concentration of $MgCl_2$ producing the half-maximal effect can be estimated to be about 0.7 mM.

In 10 mM Tris-HCl, $MgCl_2$ decreases the dissociation constant, whereas in 50 mM imidazole

TABLE III

THE EFFECT OF THE COUNTERION FOR Na^+ ON BINDING OF Ado*PP*[NH]*P* TO ($Na^+ + K^+$)-ATPase

The binding was measured with two different enzyme preparations. Ligand concentrations were 50 mM except for Na_2SO_4 (16.6 mM); ionic strength, 50 mM in all cases.

Ligand	K_d (μM)	
	mean	range
NaCl	0.39	0.36– 0.43
Na_2SO_4	0.80	0.60– 1.01
$NaNO_3$	1.77	1.62– 1.93
NaSCN	22.20	16.10–28.20

chloride, $MgCl_2$ increases the dissociation constant due to the different starting points (Fig. 5).

The incubations in 5 mM $MgCl_2$ and variable concentrations of imidazole chloride and Tris-HCl show that the dissociation constant is 2–3 μM as far as the ionic strength of the incubation medium does not exceed a value of 50 mM. At an ionic strength above 50 mM the dissociation constant again increases (Fig. 4).

Effect of pH. The effect of pH was tested in Tris-HCl buffer of 50 mM ionic strength. The concentration of Tris-HCl was 50 mM at pH 7.25, 100 mM at pH 8.25 and 500 mM at pH 9.25. Fig. 6 shows that the affinity of the enzyme for Ado*PP*[NH]*P* is reduced above pH 8.25. This constrasts to the finding of Hegyvary and Post [4], who found with buffers of varying ionic strength a sharp rise of the K_d value above pH 7.8.

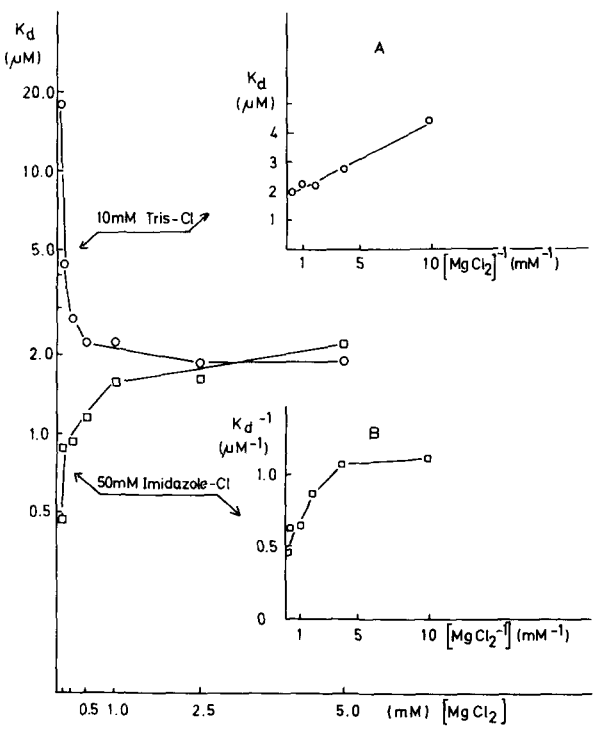

Fig. 5. Dependence on $MgCl_2$ of the dissociation constant (K_d) of the enzyme-Ado*PP*[NH]*P* complex. Binding was measured in 50 mM imidazole chloride (pH 7.25) (□) or 10 mM Tris-HCl (pH 7.25) (○). The insets show the plot of K_d in 10 mM Tris-HCl (pH 7.25) ($K_{0.5} = 0.14$ mM) or K_d^{-1} in 50 mM imidazole chloride (pH 7.25) (B) versus the reciprocal concentration of $MgCl_2$.

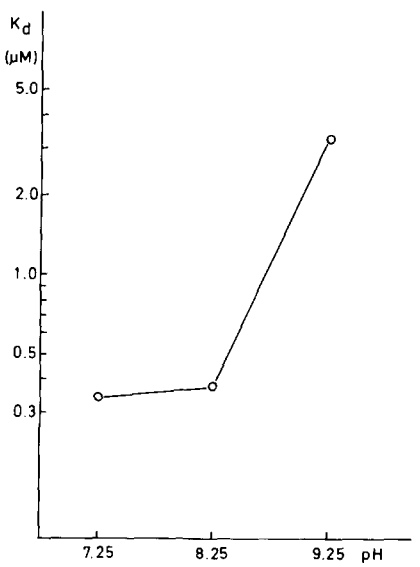

Fig. 6. Dependence on pH of the dissociation constant (K_d) of the enzyme-Ado*PP*[NH]*P* complex. Binding was measured in Tris-HCl buffer of 50 mM ionic strength. The concentration of Tris-HCl was 50 mM at pH 7.25, 100 mM at pH 8.25 and 500 mM at pH 9.25.

Discussion

As demonstrated in this work optimal binding of Ado*PP*[NH]*P* to ($Na^+ + K^+$)-ATPase requires an ionic strength of the incubation medium of about 50 mM. The cations should be Na^+, imidazole$^+$ or Tris$^+$. The apparent affinities of imidazole$^+$ and Tris$^+$ are lower than of Na^+ (compare Fig. 2. with Fig. 3), which reflects that Na^+ is the 'natural' cation. The anion should be Cl^- (Table III). With NaCl, imidazole chloride or Tris-HCl at pH 7.25 an optimal dissociation constant of the enzyme-Ado*PP*[NH]*P* complex of about 0.4 μM is reached.

Since imidazole chloride and Tris-HCl show Na^+-like effects by inducing high-affinity binding of Ado*PP*[NH]*P* and probably also of ATP to ($Na^+ + K^+$)-ATPase (Figs. 2,3), an effect of NaCl on the affinity for nucleoside triphosphates has probably been overlooked in previous studies [3,4]: Nørby and Jensen [3] used a high ionic strength of 73 mM and saw no effect of NaCl on ATP binding (Table III). Hegyvary and Post [4] using buffers of lower ionic strength (Table IV) saw a slight increase in ATP binding; but Robinson [5] observed

a significant effect of NaCl. Although the ionic strength of the buffer and its exact composition under his experimental conditions are unknown, they were certainly suboptimal (Table IV). This is also indicated by our own results with histidine/Tris-HCl buffer (Fig. 2) and the published K_d for Ado*PP*[NH]*P* of 4.2 μM [5].

NaCl induces binding of Ado*PP*[NH]*P* with a half-maximal effect at 0.6 mM, but binding of NaCl to lower affinity sites also seems to be involved (Fig. 3, inset). This value compares well with data for ^{22}NaCl binding at 0°C in centrifugation experiments of $K_d = 0.23$ mM [12] and $K_d = 0.26$ mM [13] and with data where the K_d value of 0.6 mM for Na^+ has been calculated from competition with K^+ binding [14]. In the paper of Kaniike et al. [12], NaCl binding to lower affinity sites with a K_d of approx. 18 mM was also apparent. In these experiments, low ATP (under 1 mM) did not affect binding, whereas 50 mM choline chloride interfered with binding at the low-affinity sites exclusively. Kaniike et al. [12] regarded binding to these sites to be nonspecific. Induction of suboptimal Ado*PP*[NH]*P* binding and decrease of the affinity for Ado*PP*[NH]*P* above 50 mM ionic strength, as caused by choline chloride (Fig. 3), then must be due to binding outside the Na^+ site. Induction of optimal binding by NaCl or imidazole chloride or Tris-HCl, however, might be due to binding to the Na^+ site.

The Na^+-like effects of imidazole$^+$, Tris$^+$, histidine$^+$ and choline$^+$ could be due:
(a) to contaminations of the buffers and solutions used by Na^+; This explanation can be excluded (Table I);
(b) to a substituting effect of these substances for Na^+; or
(c) to a displacement of K^+ from the enzyme as a contaminant of the enzyme preparation by any of the above salts. It is well established that ATP binds with high affinity to the E_1 form of the α-subunit of $(Na^+ + K^+)$-ATPase, while binding to the E_2 form is weak [15–17]. Since K^+ and ATP as well as Na^+ stabilize the alternative protein conformations E_2 and E_1 [8], binding of K^+ and of ATP (or Na^+) are antagonistic at non-saturating concentrations. Displacement of K^+ from the E_2 form by any cation showing itself no K^+ effect on the E_1-E_2 conformational states might therefore mimic Na^+ in its effect.

To consider the two latter possibilities (b) and (c) one may evaluate the concentrations of K^+ and Na^+ as contaminants of the enzyme protein in the Ado*PP*[NH]*P*-binding assay. These concentrations are 80–100 μM Na^+ and 5–7 μM K^+. Considering the dissociation constants of the enzyme-sodium complex of 0.23–0.26 mM [12,13] and of the enzyme-potassium complex of 11 μM [14] one can calculate that a fraction of 0.24–0.30 of the Na^+ sites and a fraction of 0.31–0.39 of the K^+ sites of $(Na^+ + K^+)$-ATPase are occupied. It therefore appears that almost identical amounts of the enzyme are in the Na^+-(E_1) form and in the K^+-(E_2) form. Since the transition between these two forms is possible without the addition of ATP, as has been shown by trypsinolysis under controlled conditions [8] and by fluorescence studies [9], the additional presence of Tris$^+$, imidazole$^+$, histidine$^+$ and choline$^+$ can induce the Na^+ form. This also has been proven by trypsinolysis under

TABLE IV

RELATION BETWEEN THE BUFFER USED AND THE APPARENT EFFECT OF NaCl ON THE AFFINITY OF $(Na^+ + K^+)$-ATPase for ATP or Ado*PP*[NH]*P*

Ref.	Buffer	pH	Incubation medium Ionic strength (mM)	Nucleotide	Effect of NaCl on K_d
3	88 mM imidazole chloride 12 mM Tris-HCl	7.4	73	ATP	no effect
4	30 mM Tris-CDTA	7.6	25	ATP	slight increase
5	30 mM histidine/HCl-Tris	7.8	?	Ado*PP*[NH]*P*	significant increase

controlled conditions [8] as well as by fluorescence studies [9]. Although choline$^+$ and Tris$^+$ displace K^+ from its site (like Na^+) [14], this latter observation might also be due to an alteration of the E_2-E_1 equilibrium by binding of choline$^+$ and Tris$^+$ to the Na^+ site. Obviously, discrimination between the both above-mentioned possibilities (b) and (c) is not possible so far. It needs more information on the effects of imidazole$^+$, Tris$^+$, histidine$^+$ and choline$^+$ on the Na^+ binding to $(Na^+ + K^+)$-ATPase.

If imidazole$^+$ (Cl^-) or Tris$^+$ (Cl^-) are indeed able to interact at the Na^+ site, consequences not only for nucleotide bindng but also for the succeeding partial reactions of $(Na^+ + K^+)$-ATPase, involving Na^+-binding at sites with $K_d < 1$ mM Na^+ [16,17], must be expected. Therefore, any re-evaluation of Na^+ effects on partial reactions of $(Na^+ + K^+)$-ATPase should take into account the nature and the ionic strength of the buffer and should preferably be done in dilute buffers.

$MgCl_2$ leads in the case of imidazole chloride (pH 7.25) and Tris-HCl (pH 7.25) to a dissociation constant of the enzyme Ado*PP*[NH]*P* complex of 2–3 μM if the ionic strength of the incubation medium does not exceed 50 mM (Fig. 4). Half-maximal effects of $MgCl_2$ were found at 0.14 mM in 10 mM Tris-HCl (pH 7.25) and 0.7 mM in 50 mM imidazole chloride (pH 7.25) (Fig. 5). Thus it appears that higher concentrations of imidazole chloride also affect binding of Mg^{2+} (Cl_2^-). This may be an analogy to the reported antagonism between binding of Na^+ and Mg^{2+} [12,18,19].

$MgCl_2$ continuously decreases or increases the K_d value for the enzyme-Ado*PP*[NH]*P* complex, depending on the starting conditions (Fig. 5). Robinson, however, saw in his experiments a jump in the K_d value for Ado*PP*[NH]*P* from 4.2 μM down to 2.2 μM by the addition of 50 μM $MgCl_2$ (Ref. 5, compare his Fig. 2) which was followed by a gradual rise in the K_d value with increasing $MgCl_2$ concentrations. He concluded from that jump that the ternary complex of enzyme-Ado*PP*[NH]*P*-Mg has a tighter binding than the binary complex of enzyme-Ado*PP*[NH]*P*. Drawing this conclusion, Robinson did not consider that 0.1 mM EDTA was included in the control value but was left out in the values for systems containing $MgCl_2$. Since Robinson's finding contradicted our results (Fig. 5), we looked to see whether EDTA might produce such jump effects. Indeed, omission of 0.1 mM EDTA from 30 mM histidine/Tris-HCl (pH 7.25) decreased the K_d for Ado*PP*[NH]*P* from 3.4 μM to 1.6 μM. This finding not only sheds some doubt on Robinson's interpretation, but also confirms the finding of Klodos and Skou [20] that EDTA as such affects $(Na^+ + K^+)$-ATPase.

Binding of Ado*PP*[NH]*P* to $(Na^+ + K^+)$-ATPase in the presence of $MgCl_2$ is significantly different from binding in the presence of NaCl and its substitutes. The K_d for Ado*PP*[NH]*P* drops maximally to 2 μM with increasing $MgCl_2$ concentration, but to 0.4 μM with NaCl and its substitutes. These different maximal levels in affinity are rather stable up to 50 mM ionic strength. $MgCl_2$ effectively counteracts the effects of imidazole chloride, Tris-HCl (Fig. 4) and also of NaCl (Table V) and adjusts the K_d for Ado*PP*[NH]*P* to about 2 μM, when the ionic strength is below 50 mM. The fact that $MgCl_2$ is the most potent effector under consideration is reflected by the low concentration of 0.14 mM $MgCl_2$ required for the half-maximal effect as compared to that of 0.6 mM for NaCl.

Further evidence of significant changes in the interaction between the enzyme and the nucleotide upon binding of $MgCl_2$ to the enzyme we have gained from studies with protein reactive ATP analogues [7] or photoreactive ATP analogues. 3′-*O*-[3-(2-Nitro-4-azidophenyl)propionyl]-ATP effectively photoinactivates $(Na^+ + K^+)$-ATPase only in the presence of millimolar $MgCl_2$ [6]. The

TABLE V

DISSOCIATION CONSTANTS FOR BINDING OF Ado*PP*[NH]*P* TO $(Na^+ + K^+)$-ATPase IN 10 mM IMIDAZOLE CHLORIDE (pH 7.25) AND IN THE PRESENCE OF THE LIGANDS INDICATED

The ionic strength was kept constant at 50 mM with choline chloride.

Final concentrations of ligands (mM)	K_d (μM)
5 $MgCl_2$	1.64
25 NaCl	0.48
5 $MgCl_2$ + 25 NaCl	2.24

same was observed with 8-azido-ATP (Rempeters, G., unpublished data). By contrast, the affinity of the enzyme for both ATP analogues is much higher in NaCl or the substituting buffers; just as with Ado*PP*[NH]*P* (Rempeters, G., unpublished data). Since these photoaffinity labels carry their photoreactive azido groups at different parts of the ATP molecule, namely at the ribose and purine moiety, respectively, major changes within the ATP binding site upon binding of Mg^{2+} $(Cl^-)_2$ have to be assumed; e.g. exclusion of water from the substrate cavity or exposition of cysteine residues.

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