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Long-range effects of Na^+ binding in Na,K-ATPase reported by ATP

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Abbreviations: Tris, tris(hydroxymethyl)-aminomethane; K_{Diss}, equilibrium dissociation constant; REDOR, rotational-echo double-resonance; CP-MAS, cross-polarization magic-angle spinning.

ABSTRACT

This paper addresses the question of long-range interactions between the intramembranous cation binding sites and the cytoplasmic nucleotide binding site of the ubiquitous ion-transporting Na,K-ATPase using ^{13}C cross-polarization magic-angle spinning (CP-MAS) solid-state NMR. High affinity ATP binding is induced by the presence of Na^+ as well as of Na-like substances such as $Tris^+$, and these ions are equally efficient promoters of nucleotide binding. CP-MAS analysis of bound ATP with Na,K-ATPase purified from pig kidney membranes reveals subtle differences in the nucleotide interactions within the nucleotide site depending on whether Na^+ or $Tris^+$ are used to induce binding. Differences in chemical shifts for ATP carbon atoms C1′ and C5′ observed in the presence of Na^+ or $Tris^+$ suggest alterations in the residues surrounding the bound nucleotide, hydrogen bonding and/or conformation of the ribose ring. This is taken as evidence for a long-distance communication between the Na^+-filled ion sites in the membrane interior and the nucleotide binding site in the cytoplasmic domain and reflects the first conformational change ultimately leading to phosphorylation of the enzyme. Stopped-flow fluorescence measurements with the nucleotide analog eosin show that the dissociation rate constant for eosin is larger in $Tris^+$ than in Na^+, giving kinetic evidence for the difference in structural effects of Na^+ and $Tris^+$. According to the recent crystal structure of the E_1•AlF_4^-•ADP•3Na^+-form the coupling between the ion binding sites and the nucleotide side is mediated by - among others - the M5-helix.

Na,K-ATPase (EC 3.6.3.9), the first P-type ATPase to be discovered[1], is present in the plasma membrane of animal cells. The enzyme is responsible for transport of 3 Na^+ outward and 2 K^+ inward against their electrochemical gradients at the expense of the energy of 1 ATP-molecule. The mechanism of energy transduction from the hydrolytic site to the transport sites involves transition between two major E_1 and E_2 protein conformations[2,3]. In the E_2-conformation, induced by K^+, the affinity for ATP is low[4,5]. Displacement of K^+ from the transport sites by Na^+ enables ATP binding with high affinity, a signature of the E_1-conformation. Recent crystal structures of Na,K-ATPase in the E_2 forms[6-9] well as E_1 forms[10,11] show that the distance between the ATP binding site in the large cytoplasmic domain and the ion binding sites in the intramembranous domain is about 6 nm. The structural effect of the ion occupying the ion binding site is thus transmitted over a long distance, and this is presumably due to rearrangements related to the very long M5 segment, which connects the ATP binding domain with the ion binding sites in the membrane interior (see Figure 1).

High-affinity nucleotide binding can be induced by several types of cations in addition to Na^+, and these include $Tris^+$, $choline^+$ and $imidazole^+$ (see refs. 4, 5, 12-14). Among these Na^+ is unique, since this ion is transported during ATP hydrolysis. Na^+ can also be occluded within the enzyme by addition of oligomycin[15]. $Tris^+$, $choline^+$ and $imidazole^+$ are not occluded by oligomycin[15] and transport of these ions by Na,K-ATPase has not been reported. The properties of ion binding, occlusion and conformational changes are reviewed in ref. 16. Analysis of the competition between for example $Tris^+$ and K^+ for the induction of high-affinity nucleotide binding suggests that $Tris^+$ does not bind to the transport sites[13]. Since both Na^+ and $Tris^+$ can induce high-affinity binding of nucleotide - but only Na^+ binds to the transport sites - an important question is whether the interactions between the bound

nucleotide and the residues of the nucleotide binding site are different in Na^+ and $Tris^+$. A difference in nucleotide binding mode to Na^+- and $Tris^+$-induced conformations will most probably be due to a structural effect mediated by the Na^+ ion occupying the transport site, which through rearrangements in the protein structure is transmitted to the distant nucleotide site. This rearrangement could be considered the first step mandatory for phosphorylation and provide the structural basis for the strict coupling between ATP hydrolysis and Na^+ transport.

The question of how nucleotide binding differs in the presence of Na^+ or $Tris^+$ remains unanswered. Although recent crystal structures have revealed much about the nucleotide binding domain in the E_1 and E_2 forms, obtaining crystals of the enzyme with ATP *in situ* under non-hydrolysing conditions is a challenge. Solid-state NMR, using cross-polarization magic-angle spinning (CP-MAS), has been shown to be a valuable alternative to X-ray diffraction for elucidating the fine details of nucleotide binding to Na,K-ATPase in the $[Na_3]E_1$ form[17-19]. It is possible to conduct NMR measurements on the non-crystalline, membrane-embedded enzyme with ATP freeze-trapped in the high-affinity nucleotide site.

The molecular conformation of ATP in the binding site, determined by REDOR solid-state NMR measurements of intramolecular carbon-phosphorus distances, is in excellent agreement with the bound ADP conformation that was later determined by X-ray crystallography (Figure 1) and uniquely reveals that the ATP γ phosphate group extends toward the phosphorylation site of aspartate residue 369 (crystal structures with ATP in the binding site are not yet available). Further retrospective analysis of earlier solid-state NMR data also reveals good agreement with the later crystal structure[18,19] : the ^{13}C chemical shifts for uniformly ^{13}C labelled ATP ([U-^{13}C]ATP) are consistent with strong interaction between the adenine ring and the ribose moiety with residues in the binding site in this protein conformation. Marked perturbations in chemical shifts relative to the solution shifts for C2 of

ATP (see Figure 1 for numbering scheme) might reflect changes in the electron density within the adenine ring as a result of π–π or π–cation interactions with amino acid side groups around the binding site[19]. Changes in the shifts for the ribose sites, particularly C4' and C5', are consistent with conformational changes or to partial deformation of the sugar ring as the nucleotide enters the binding site. Moreover, ^{13}C-detected proton spin-diffusion experiments indicated that the adenine ring of ATP is held in close contact with residues in the binding site, whereas the ribose ring occupies a more spacious cavity[18], which again is evident in the crystal structure. Hence solid-state NMR is powerful technique for probing the structure and environment of ATP bound to Na,K-ATPase in atomic-level detail.

Here, we exploit CP-MAS NMR spectroscopy of [U-^{13}C]ATP bound to Na,K-ATPase to seek differences in the bound ATP molecule in the presence of Na^+ or $Tris^+$. Chemical shift differences for the bound nucleotide are consistent with an effect of Na^+ that is different from that of $Tris^+$ on the ATP binding step preceding phosphorylation.We extend the NMR studies of nucleotide binding with analysis of eosin and carboxyeosin binding to the enzyme. These highly fluorescent dyes of the xanthene type have been shown to bind to the nucleotide site of the Na,K-ATPase, with a large enhancement of fluorescence upon binding. Transient kinetic fluorescence experiments are used to monitor rates of dissociation of eosin and carboxyeosin from the binding site. The dissociation rate constant is larger in the presence of $Tris^+$ than in Na^+. This indicates that the structure of the protein surrounding the bound eosin is affected by Na^+ in the transmembrane transport sites, in agreement with the NMR results.

Taken together, these two techniques demonstrate long-range effects of Na^+-binding on the native Na,K-ATPase protein structure, transmitted about 6 nm from the membrane interior to the cytoplasmic domain prior to the phosphorylation and transport events.

EXPERIMENTAL PROCEDURES

Preparation of Na,K-ATPase. Pig kidney microsomal membranes were treated with SDS and purified by differential centrifugation to a specific activity of 28 μmoles per mg protein per min at 37 ^{o}C [20, 21]. The K^+-contamination measured by atomic absorption spectroscopy was less than 3 μmoles per 1 μmole enzyme, and the buffer solutions contained less than 2 μM K^+ (unpublished observations). Na,K-ATPase from the salt gland of *Squalus acanthias* was prepared according to the method of Skou and Esmann[22], omitting the saponin treatment.

The Na,K-ATPase was characterized in two types of buffer. For experiments in Na^+, a buffer containing 20 mM NaCl, 10 mM CDTA and 10 mM Tris was adjusted to pH 7.0 with NaOH, giving a final [Na^+] of about 50 mM in 10 mM $Tris^+$, 20 mM Cl^- and 10 mM CDTA. For samples in $Tris^+$ the final composition was 60 mM $Tris^+$, 20 mM Cl^- and 10 mM CDTA (adjusted to pH 7.0 with Tris-base), and this buffer has the same ionic strength as the Na^+-buffer.

Fluorescence Experiments. Equilibrium binding of eosin (0.1 μM) to pig kidney Na,K-ATPase (30 μg/ml) in Na^+ or $Tris^+$ was measured on a SPEX Fluorolog-3 spectrofluorometer at 20 °C, excitation wavelength 530 nm (bandpass 1 nm) and emission wavelength 550 nm (bandpass 10 nm). Addition of 100 μM ADP was used to induce full dissociation of bound eosin. See ref. 14 for further details.

Time-course of eosin (or its analog 6-carboxyeosin) dissociation was monitored on a SX20 stopped-flow spectrofluorometer (Applied Photophysics) using excitation wavelength of 530 nm (bandpass 7 nm) and 550 nm cut-off filter on the emission side. The experiments were performed with both shark and kidney enzymes. Briefly, equilibrium binding of the

fluorescent ligand (0.3 μM) to the enzyme (60 μg/ml) was first reached by the preincubation in Na^+- or $Tris^+$-buffer. Dissociation of eosin or carboxyeosin was induced by mixing equal volumes of an enzyme-containing solution and a corresponding buffer solution containing 200 μM ADP. The average of 5-6 individual traces was used to calculate the dissociation rate constants by regression analysis of a single exponential process.

Equilibrium Binding Experiments. Equilibrium binding of ADP was measured in double-labeling filtration experiments essentially as previously described[12, 23-25]. Na,K-ATPase was allowed to equilibrate at 20 ^oC for 10 min in a Na^+- or $Tris^+$-buffer. The buffer also contained various concentrations of [^{14}C]ADP and [^3H]glucose (both from New England Nuclear) and in some experiments also K^+. One mL of this suspension (usually 0.24 - 0.28 mg protein/mL) was loaded on two stacked Millipore HAWP 0.45 μm filters. Then, without rinsing, filters were separately counted in 4 mL Packard Filtercount scintillation fluid. The amount of nucleotide bound to the protein was calculated by subtracting from the total amount of nucleotide on the (top) filter (bound plus unbound nucleotide) the amount of unbound nucleotide, trapped in the filter together with the wetting fluid; the amount of unbound nucleotide was considered to be proportional to the amount of [^3H]glucose in the same filter. The concentration of free ADP in the suspension was calculated by subtraction of the amount bound to the protein.

Sample preparation for NMR. Na,K-ATPase membrane stock solution (typically 5 mg protein/ml) was diluted 10-fold into a buffer containing - for the Na^+-sample - 22 mM NaCl, 11 mM CDTA and 11 mM Tris, giving a final [Na^+] of about 50 mM in 10 mM $Tris^+$, 20 mM Cl^- and 10 mM CDTA. For the sample in $Tris^+$ the final composition was 60 mM $Tris^+$, 20 mM Cl^-, and 10 mM CDTA. The suspension was centrifuged for 2 h at 20000 rpm at 4 ^oC in a Beckman Ti70 rotor. The pellets were transferred to an Eppendorf vial with about

0.25 ml pellet per vial, and the protein concentration was usually about 50 mg/ml (an enzyme concentration of about 0.13 mM). The reaction with [U-^{13}C]ATP was initiated by addition of a 20-fold concentrated stock solution of Li-salt of the nucleotides. The total concentration of ATP was 0.16 mM, slightly larger than the enzyme concentration. The suspension containing the nucleotide was homogenized in the Eppendorf vial with a Kontes homogenizer, and was kept at about 15 ^{o}C for 10 min before cooling to 0 ^{o}C. The suspension was transferred to a 4 mm NMR rotor and frozen in liquid N_2 [17, 18].

Solid-state NMR experiments. Spectra were obtained on a Bruker Avance 400 spectrometer operating at a field strength of 9.3 T, corresponding to a frequency of 100.13 MHz for ^{13}C. Membrane samples were spun at a MAS rate of 5.1 kHz, and each 1D spectrum was obtained by block-wise averaging of 326,680 transients. Conditions were: a 4.0-μs ^{1}H excitation pulse, two-pulse phase modulated (TPPM) proton decoupling[17] at a field of 85-kHz during signal acquisition and a 1.6-s recycle delay pulse. Cross-polarization was achieved with a 2-ms contact time at a proton field of 65 kHz. Difference spectra showing signals for [U-^{13}C,^{15}N]ATP only were obtained by subtracting in each case a spectrum of kidney membranes from a spectrum of an identical sample containing 0.16 mM [U-^{13}C,^{15}N]ATP. A temperature of -25°C was used throughout. Each spectrum is the result of the accumulation of 100,000-200,000 transients with block averaging, with measurement times of 2-4 days.

Data Analysis was performed using the ORIGIN 6.0 software (Microcal, Amherst, Cal) and KyPlot 2.13 freeware (Koichi Yoshioka, Dept. Biochemistry and Biophysics, Graduate School of Allied Health Sciences, Tokyo, Japan; www.wounded-moon.org/win32/kyplot.html).

RESULTS

Equilibrium ADP Binding. In the presence of 50 mM Na^+ or $Tris^+$ nucleotides such as ADP and ATP bind with high affinity to Na,K-ATPase. Figure 2 shows equilibrium binding curves in the range 0.01 - 10 μM free ADP, and the data are fitted adequately by a single site model with equilibrium dissociation constant K_{Diss} = 0.59 μM in Na^+ and K_{Diss} = 0.81 μM in $Tris^+$. The maximal binding capacity (100% in Figure 2) in Na^+ as well as in $Tris^+$ is about 2.8 nmol/mg protein. ATP binds with a slightly lower K_{Diss} than ADP[4,5].

Addition of K^+ (0.1 mM) to the binding solution has a very marked effect on ADP binding in $Tris^+$, but very little effect on binding in Na^+ (Figure 2, compare filled and open symbols). This has been reported earlier with binding assay conditions similar to the ones used here[23]. The simplest interpretation of the effect of K^+ is outlined in Scheme 1. In the absence of K^+ as well as Na^+ the strong binding of ADP is induced by a sufficiently high ionic strength (here is used 60 mM $Tris^+$), Figure 2. Addition of Na^+ leads to occupation of the intramembranous cation sites (in square brackets), and under these conditions (50 mM Na^+ + 10 mM $Tris^+$) ADP is also bound with high affinity. Addition of K^+ leads to spontaneous occlusion of K^+ in the $(K_2)E_{2,Tris}$ form, which does not bind ADP with high affinity. In the presence of 60 mM $Tris^+$ (and absence of Na^+) a large fraction of the Na,K-ATPase molecules are forced into the $(K_2)E_{2,Tris}$ form by addition of 0.1 mM K^+ (Figure 2), and increasing the K^+ concentration to 50 mM virtually abolishes ADP binding (Figure 2). In contrast to the effect of $Tris^+$, Na^+ is able to compete with K^+ for the ion binding sites[13]. In Scheme 1 this is indicated as an equilibrium between the $(K_2)E_{2,Tris}$-form (with low affinity for ADP) and the $[Na_3]E_{1,Tris}$-form (with high affinity for ADP), and the equilibrium between these depend on

the Na:K ratio. As shown in Figure 2 the effect of 0.1 mM K^+ is negligible in 50 mM Na^+. It should be noted that a binding site for $Tris^+$ has not been kinetically described, and the enzyme form $E_{1,Tris}$ in Scheme 1 is therefore meant to indicate an interaction between $Tris^+$ and the enzyme which could be of purely electrostatic nature.

These binding experiments indicate that the enzyme conformation in the presence of Na^+ is different from that in $Tris^+$, and this is further analyzed by fluorescence and NMR.

Eosin Fluorescence Studies. Eosin and its derivative 6-carboxyeosin bind with high affinity to a site which appears to be the same as the nucleotide binding site of Na,K-ATPase[14, 26]. The bound eosin has a high fluorescence, which is reduced considerably upon ADP-induced displacement of eosin from the site[26]. In Figure 3A the expected decrease in fluorescence occurs upon addition of ADP in Na^+, and a marked decrease in fluorescence is also observed in $Tris^+$ (first and third tracings from left). Figure 3A also shows the effects of K^+ on equilibrium eosin fluorescence in the presence of Na^+ or $Tris^+$. When 0.1 mM K^+ is added in the presence of Na^+ there is no fluorescence change, but following the addition of ADP the effect is similar to that in the absence of K^+ (compare first and second tracings), indicating a lack of effect of this concentration of K^+ on eosin binding in 50 mM Na^+. K^+ at a concentration of 0.1 mM can thus not counteract the Na^+-effect, as also observed with ADP binding (see Fig. 2). In the right hand part of Figure 3A similar experiments in 60 mM $Tris^+$ (and absence of Na^+) are shown. Here the addition of 0.1 mM K^+ leads to a marked decrease in fluorescence, indicating dissociation of a large fraction of bound eosin (fourth tracing from left). Thus, K^+ is a much more potent competitor for $Tris^+$ than for Na^+, and the eosin experiments reveal the same pattern of ion interactions as the ADP-binding experiments (Figure 2).

Transient analysis of the rate of eosin dissociation induced by addition of saturating concentrations of ADP was used to investigate if the binding mode for eosin is different in $Tris^+$ and Na^+. The dissociation rate constant (denoted $k_{off,Na}$ and $k_{off,Tris}$ in Scheme 1, with L symbolising eosin) reflects molecular details of the ligand binding, and differences in dissociation rate constants are better measures of changes in binding mode than differences in K_{Diss}. Figure 3B shows that dissociation of eosin from the pig enzyme is somewhat more rapid in $Tris^+$ than in Na^+, with dissociation rate constants of about 29 ± 1.2 s^{-1} in Na^+ and 34 ± 0.6 s^{-1} in $Tris^+$. We have previously shown that the 6-carboxy derivative of eosin binds in the same manner as eosin, but with a higher affinity[14]. In agreement with this we observe that the dissociation rate constants for 6-carboxyeosin are smaller than for eosin, about 8.7 ± 0.4 s^{-1} in Na^+ and 9.7 ± 0.4 s^{-1} in $Tris^+$, and interestingly with the same difference as for eosin, namely a slower dissociation in Na^+ than in $Tris^+$ (Figure 3B). The transient experiments were also carried out using Na,K-ATPase isolated from shark rectal glands, and with eosin the observed rate constants were about 34 ± 1.0 s^{-1} in Na^+ and 43 ± 0.7 s^{-1} in $Tris^+$. With 6-carboxyeosin we found about 10.0 ± 0.2 s^{-1} in Na^+ and 12.5 ± 0.4 s^{-1} in $Tris^+$. The rate constants for eosin dissociation are always smaller in Na^+ than in $Tris^+$ also with shark enzyme, corroborating the results with the pig kidney enzyme. A statistical analysis of the difference between the dissociation rate constants in Na^+ and $Tris^+$ shows that for shark in eosin p_0=0.00018 (***), and in 6-carboxyeosin p_0=0.00055 (***). For kidney in eosin p_0=0.0033(**), and in 6-carboxyeosin p_0=0.04 (*).

The transient fluorescence experiments with eosin show that the dissociation rate constant for eosin in $Tris^+$ is larger than in Na^+, which means that - in agreement with the NMR results described below - the bound eosin experiences another interaction with the

protein in Na^+ than in $Tris^+$. The larger dissociation rate constant in $Tris^+$ indicates that the eosin binding in $Tris^+$ is not as tight as in Na^+.

NMR analysis of nucleotide binding. ^{13}C CP-MAS NMR spectra of [U-^{13}C]ATP to Na,K-ATPase membranes are shown in Figure 4. Under these conditions the nucleotide site is saturated by ATP and more than 80 % of the total ATP is bound. Hence the ^{13}C resonance lines for ATP represent predominantly the bound nucleotide (approximately 0.13 mM). The two sets of spectra in Figure 4A in Na^+ (upper set) or $Tris^+$ (lower set) show resonances from the added ATP as well as from the endogenous ^{13}C in the membrane proteins, lipids and carbohydrates. The resonances from ATP alone can be observed in subtraction spectra where the signal from the membrane components (absence of ^{13}C-ATP) is subtracted from the spectra with ^{13}C-ATP present. Such subtraction spectra are shown for ATP bound in Na^+ and in $Tris^+$ in Figure 4B.

The measured chemical shifts of the 8 (from 10) observed ^{13}C-atoms are given in Table 1, and the positions are indicated by the dashed lines. Note that the chemical shifts are somewhat different from those reported earlier, which is a consequence of the lower temperature used here (-25°C compared to 4°C of the earlier study[19]). In the present case the lower temperature reduces chemical exchange of ATP in and out of the nucleotide site and the chemical shifts represent bound ATP exclusively. There is a clear difference in the resonance positions for two of the ^{13}C-atoms (C1´and C5´) consistent with structural differences in the interaction between the protein and ATP in the presence of Na^+ and in $Tris^+$. The relatively large chemical shift differences observed for these two sites probably signify differences in the types and strength of the stabilizing interactions (e.g. hydrogen bonding) with binding site residues. Residues L480 and R685 are in close proximity of C5´, and residues R544 and R685 are close to C1´ (residue R685 is behind the ATP molecule in Figure 1B and is thus not

visible in this presentation). One might expect differences in the ribose ring conformation to affect all the chemical shifts of the saccharide carbons, although this cannot be ruled out. By contrast, the adenine chemical shifts vary by less than 0.5 ppm, suggesting that the environment of the aromatic moiety does not change substantially in the presence of $Tris^+$.

DISCUSSION

High affinity nucleotide binding requires a certain ionic strength in the binding buffer. Typically 30 mM of monovalent cations such as Na^+, $Tris^+$, imidazole and choline as chloride salts are sufficient. In Scheme 1 the increased ionic strength in the presence of $Tris^+$ leads to an increase in affinity for ADP (the $E_{1,Tris}$-form) where the enzyme form E present at very low ionic strength does not bind nucleotide with high affinity[13].

Addition of K^+ (or its congeners Rb^+, Cs^+ and Tl^+) leads to formation of the $(K_2)E_2$ form, where the transport sites are occupied with 2 K^+ in the spontaneously formed occluded state, and the nucleotide binding site does not bind ADP with a measurable affinity in the $(K_2)E_2$-form, Figure 2. This effect of K^+ is seen at very low concentrations of K^+ in the presence of for example 60 mM $Tris^+$, Figure 2. $Tris^+$ can thus not compete with K^+ for the transport sites. In terms of Scheme 1, $Tris^+$ is not thought to bind to specific cation sites, but rather - at a concentration of for example 60 mM - facilitate ADP binding by an electrostatic effect on the nucleotide binding domain, giving the $E_{1,Tris}$•ADP form[13].

Filling the transport sites with Na^+ instead of K^+ leads to a rearrangement of the cytoplasmic domain, allowing high affinity nucleotide binding to the $[Na_3]E_{1,Tris}$ form[13]. Occlusion of Na^+ induced by phosphorylation or oligomycin binding[15] is not required for the competition with K^+.

Why does $Tris^+$ probably not bind to the transport sites? The $Tris^+$ ion has an effective ionic radius of about 0.26 nm, whereas that of Na^+ is about 0.1 nm and that of K^+ is about 0.13 nm[27]. $Tris^+$ is thus much larger than the two transported cations and it is inconceivable that the $Tris^+$ can occupy the Na^+-sites I, II and III of the E_1•AlF_4^-•ADP•3Na^+-form[11] or the K^+-sites in the $(K_2)E_2$-form[7] without a major reorganisation of the protein near these sites. The $E_{1,Tris}$-form is thus different from the $[Na_3]E_{1,Tris}$ form as well as the $(K_2)E_{2,Tris}$-form (cf. Scheme 1). We have not performed NMR experiments with other buffer ions such as imidazole or choline, and at present we do not know if the $E_{1,Tris}$-form represents similar enzyme forms induced by the other buffer ions .

The NMR spectrum for ATP bound to the enzyme in $Tris^+$ (Figure 4) represents a non-productive complex that is not capable of phosphorylating Asp-369. The differences in the spectrum seen when $Tris^+$ is substituted with Na^+, reflect changes in the protein structure around the nucleotide site as a result of Na^+ binding to the transport sites I, II and III, i.e. a long range effect of Na^+ before the phosphorylation event. This change in the organisation of the nucleotide binding domain to the $[Na_3]E_{1,Tris}$•ATP form allows subsequent binding of Mg^{2+} and phosphorylation of the enzyme and thus ion transport.

CONCLUSION

The main result is the change in the NMR difference spectrum when $Tris^+$ is substituted with Na^+. We interpret the difference as reflecting how the transport sites are filled. When filled with K^+ there is no ATP binding (the $(K_2)E_2$-form). When filled with Na^+ (the $[Na_3]E_1$-form) we can detect resonances from ^{13}C-atoms of the bound ATP, and the resonance positions are changed when Na^+ is substituted with $Tris^+$. $Tris^+$ is probably not bound to the transport sites

due to the larger volume of $Tris^+$ than Na^+ or K^+. With $Tris^+$ there is high affinity binding of ADP (Figure 2) - but the enzyme is not in a Na-like E_1 form since minute amounts of K^+ can lead to dissociation of the bound ADP. This is seen both with ADP binding and eosin fluorescence. The difference in the organization of the high affinity nucleotide site is thus a result of binding of Na^+ to the transport sites in the membrane interior, about 6 nm away from the nucleotide binding site in the cytoplasmic domain, i.e. a long range effect possibly transmitted by M5 segment (see Figure 1) taking place before phosphorylation of the enzyme.

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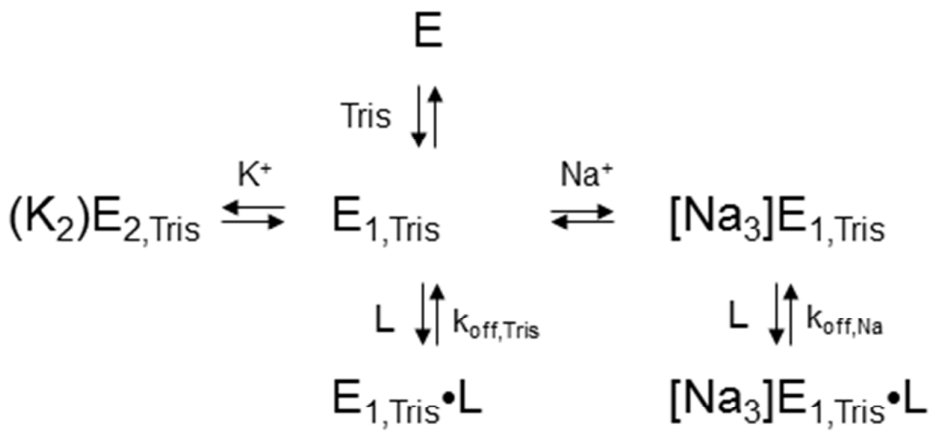
Table 1. Summary of ^{13}C chemical shifts for [U-^{13}C,^{15}N]ATP in a kidney Na,K-ATPase membrane suspension in Na^+ and in $Tris^+$.

Carbon site	Chemical shift (ppm)	
	Na^+	$Tris^+$
C6	154.8	155.0
C2	151.8	152.2
C8	138.5	138.9
C1’	85.7	86.4
C3’	81.5	80.9
C4’	76.8	77.0
C2’	68.8	69.1
C5’	61.7	62.9

Scheme 1. Schematic diagram for interactions of cations and nucleotide binding site ligands L (ADP, ATP or eosin) with Na,K-ATPase. The brackets symbolise the binding site for Na^+ and K^+. See text for more details. All experiments were done in a 10 mM $Tris^+$ buffer which in addition contained 50 mM Na^+ or 50 mM extra $Tris^+$. In some experiments K^+ also was added. The dissociation rate constants for L are $k_{off,Na}$ and $k_{off,Tris}$, respectively. Adapted from ref. 13.



LEGENDS TO FIGURES

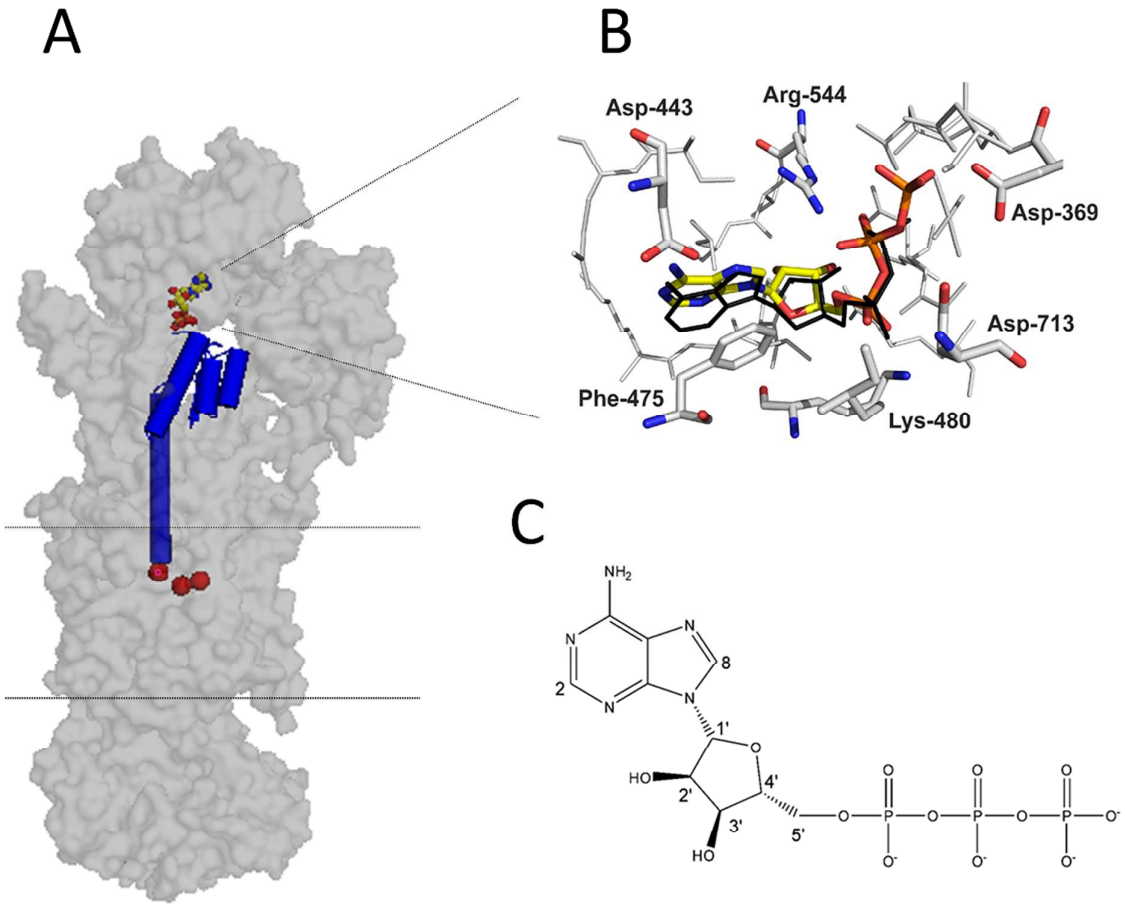
Figure 1. Structural model of ATP bound to pig kidney Na,K-ATPase. **(A)** Outline of the crystal structure of the E_1•AlF_4^-•ADP•3Na^+-form[11] (PDB ID 3WGV) . The three Na^+ sites are marked in red in the transmembrane region and bound ADP is indicated in the cytoplasmic domain. A segment (residues 686-772 of the α-subunit) containing M5 is shown in blue[11]. **(B)** Model of the Na,K-ATPase nucleotide binding site in the $[Na_3]E_1$ form with a representative ssNMR structure of ATP[17] aligned with the bound ADP observed in the crystal structure[11], in colored and black sticks, respectively. Residues possibly interacting with bound nucleotide are indicated. **(C)** The chemical structure of ATP with the usual numbering convention for C-atoms.

Figure 2. Equilibrium binding of [^{14}C]ADP to Na,K-ATPase. The amount of [^{14}C]ADP bound is measured with the filtration technique. Binding was measured in 10 mM CDTA (pH 7.0) and either 60 mM $Tris^+$ (triangles) or 10 mM $Tris^+$ plus 50 mM of Na^+ (squares) in the absence of KCl (filled symbols). In two experiments 0.1 mM KCl was also present (open symbols) in addition to Na^+ or $Tris^+$. In the presence of 50 mM K^+ and 10 mM $Tris^+$ binding is represented by open diamonds. The full lines represent single hyperbolic functions of the type [Bound] = B_{max} • [ADP]/(K_{Diss} + [ADP]) with a maximal binding B_{max} = 2.8 nmol per mg Na,K-ATPase, which is taken as 100%. Equilibrium dissociation constants K_{Diss} (± SD) were 0.59±0.01 μM (Na^+, filled squares) 0.61±0.01μM (Na^+ + K^+, open squares), 0.81±0.02 μM ($Tris^+$, filled triangles) and 3.00±0.13 μM ($Tris^+$ + K^+, open triangles). In the presence of 50 mM K^+ (open diamonds) K_{Diss} is larger than 150 μM. Data points represent the average of 4 individual experiments ± SD.

Figure 3. Eosin fluorescence experiments. **(A)** Equilibrium eosin fluorescence experiments are shown for kidney enzyme in the presence of 50 mM Na^+ (left hand tracings) or 60 mM $Tris^+$ (right hand tracings). ADP leads to a complete reduction in eosin fluorescence in the presence of Na^+, and addition of 0.1 mM K^+ alone has no effect. In 60 mM $Tris^+$ the addition ADP leads to a complete reduction in eosin fluorescence. Addition of 0.1 mM K^+ leads to displacement of a large fraction of eosin, the remaining bound eosin can be displaced by ADP. **(B)** Transient experiments used to determine the rate of dissociation of 6-carboxyeosin (tracings a and b) or eosin (tracings c and d) from the $[Na_3]E_{1,Tris}$•eosin form (tracings a and c) or $E_{1,Tris}$•eosin form (tracings b and d, see Scheme 1 for nomenclature). The curves are averages of 3-6 individual tracings.

Figure 4. Comparison of ^{13}C CP-MAS NMR spectra for [U-^{13}C,^{15}N]ATP in a kidney Na,K-ATPase membrane suspension in Na^+ and in $Tris^+$. **(A)** CP-MAS spectra of Na,K-ATPase membranes before (red lines) and after addition of [U-^{13}C,^{15}N]ATP (black lines) in the presence of 50 mM Na^+ (upper set of spectra) or 60 mM $Tris^+$ (lower set of spectra). The regions of interest are shown on an expanded scale. **(B)** Difference spectra were obtained by subtracting the background spectra of membranes with no added [U-^{13}C,^{15}N]ATP (red lines) from the corresponding spectra with added [U-^{13}C,^{15}N]ATP for Na^+ (top spectrum) and $Tris^+$ (lower spectrum). The peaks were assigned according to ref. 28. Difference spectra were obtained as described in the Methods section. All spectra were collected from hydrated samples at -25°C. Chemical shifts for [U-^{13}C,^{15}N]ATP bound in the presence of Na^+ or $Tris^+$ are summarised in Table 1.

Figure 1


A
B
Asp-443
Arg-544
Asp-369
Asp-713
Phe-475
Lys-480
C
NH2
N
N
8
2
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N
1'
O
HO
2'
4'
3'
5'
HO
O
P
O-

Figure 2.

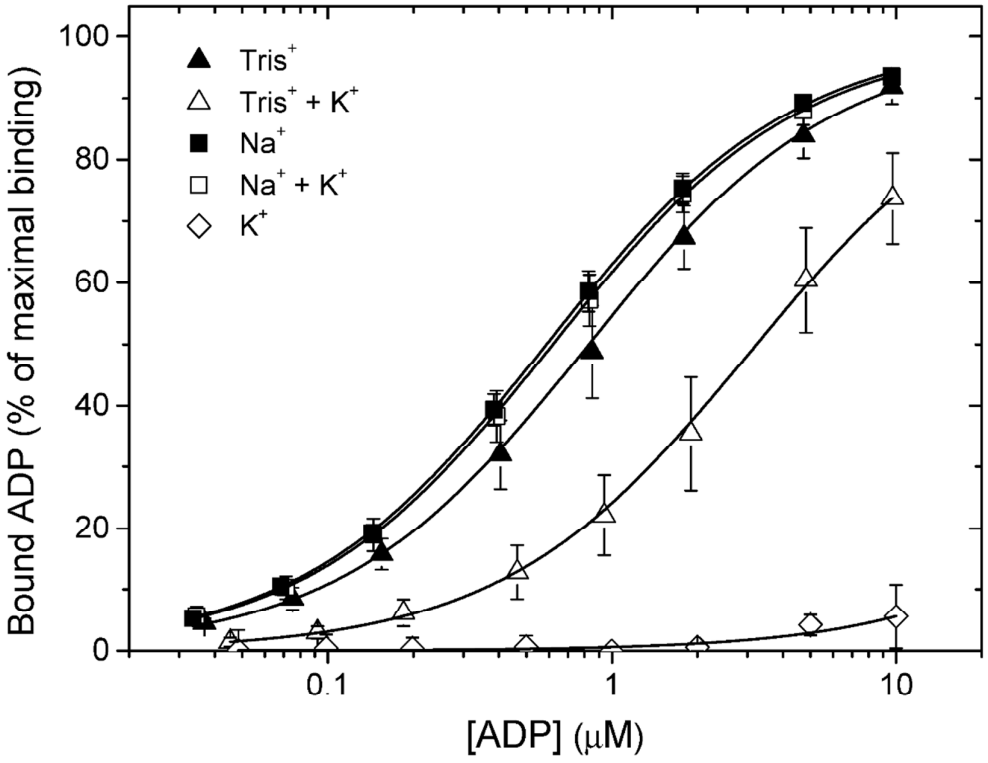

Bound ADP (% of maximal binding)
100
80
60
40
20
0
0.1
1
10
[ADP] (μM)
Tris+
Tris+ + K+
Na+
Na+ + K+
K+

Figure 3.

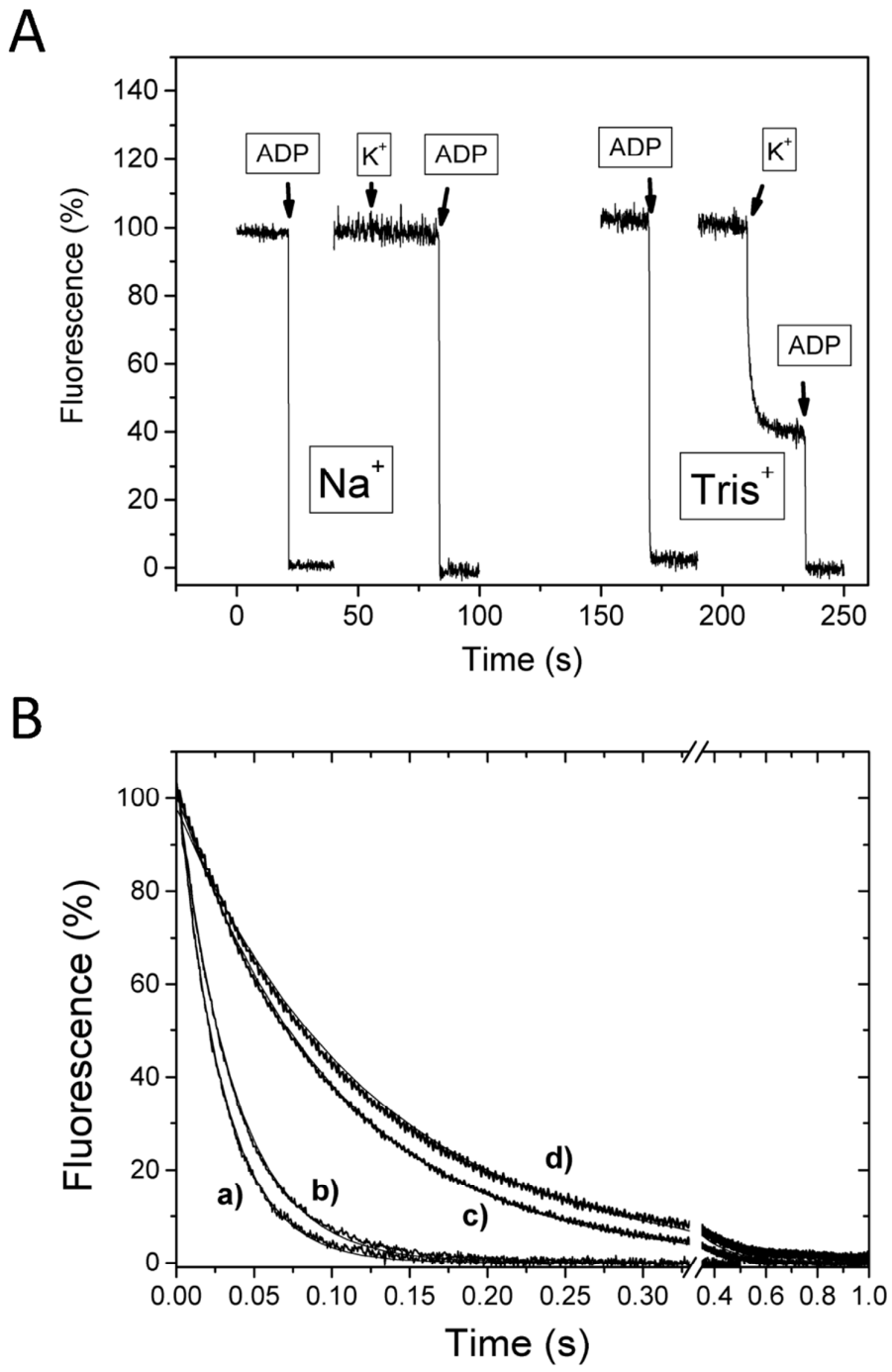
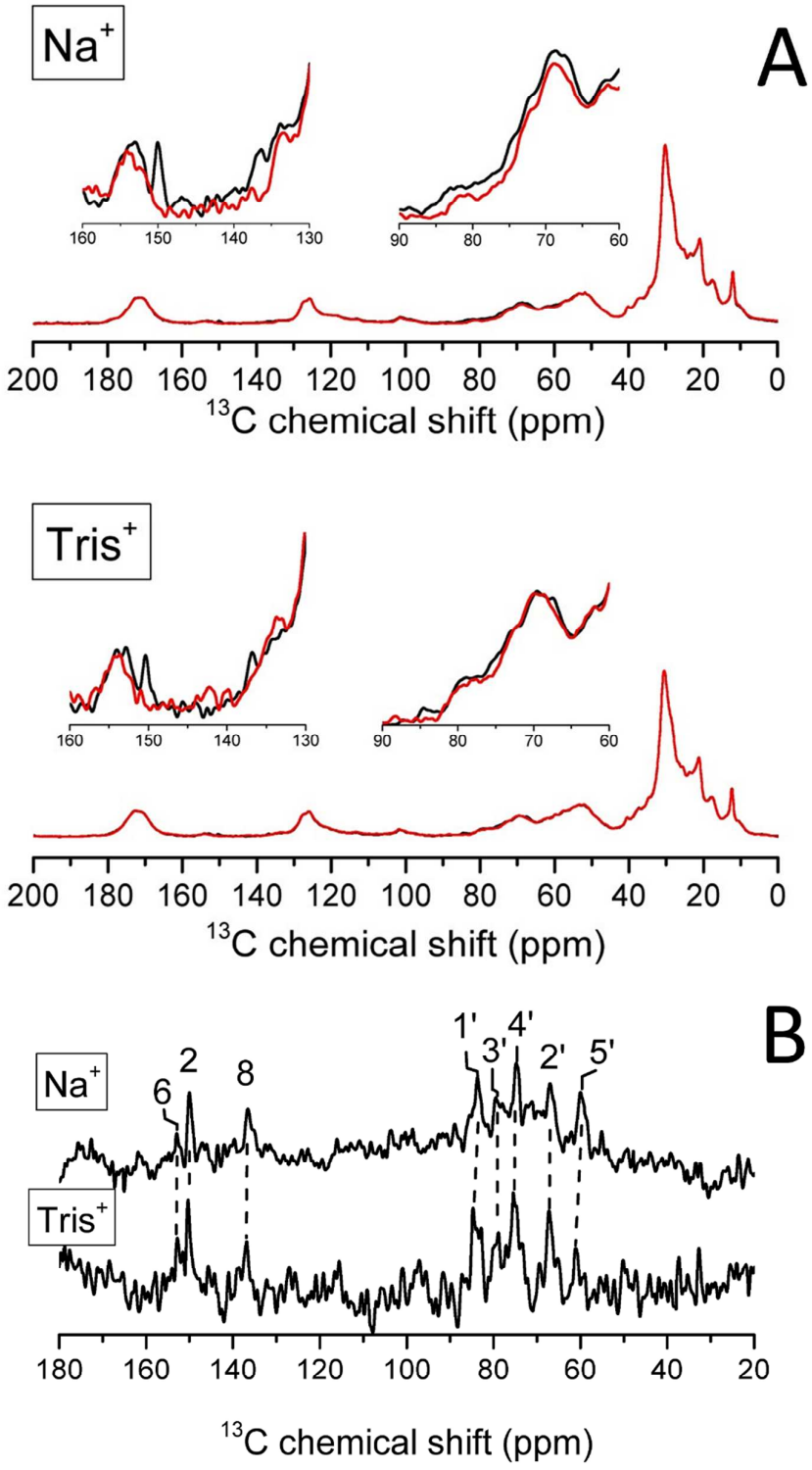

A
Fluorescence (%)
Time (s)
ADP
K+
ADP
ADP
K+
ADP
Na+
Tris+
B
Fluorescence (%)
Time (s)
a)
b)
c)
d)

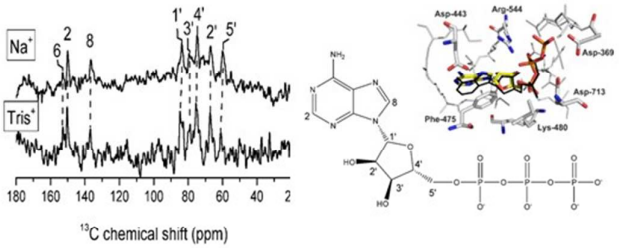
Figure 4.

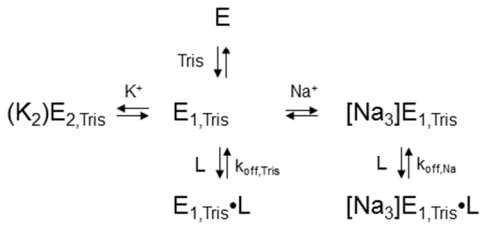

Na+
A
160 150 140 130
90 80 70 60
200 180 160 140 120 100 80 60 40 20 0
13C chemical shift (ppm)
Tris+
160 150 140 130
90 80 70 60
200 180 160 140 120 100 80 60 40 20 0
13C chemical shift (ppm)
B
Na+
Tris+
6
2
8
1'
3'
4'
2'
5'
180 160 140 120 100 80 60 40 20
13C chemical shift (ppm)

For Table of Contents use only

Title : Long-range effects of Na^+ binding in Na,K-ATPase reported by ATP

Authors : David A. Middleton, Natalya U. Fedosova, and Mikael Esmann





Scheme 1. Schematic diagram for interactions of cations and nucleotide binding site ligands L (ADP, ATP or eosin) with Na,K-ATPase. The brackets symbolise the binding site for Na^+ and K^+ . See text for more details. All experiments were done in a 10 mM $Tris^+$ buffer which in addition contained 50 mM Na^+ or 50 mM extra $Tris^+$. In some experiments K^+ also was added. The dissociation rate constants for L are $k_{off,Na}$ and $k_{off,Tris}$, respectively. Adapted from ref. 13.
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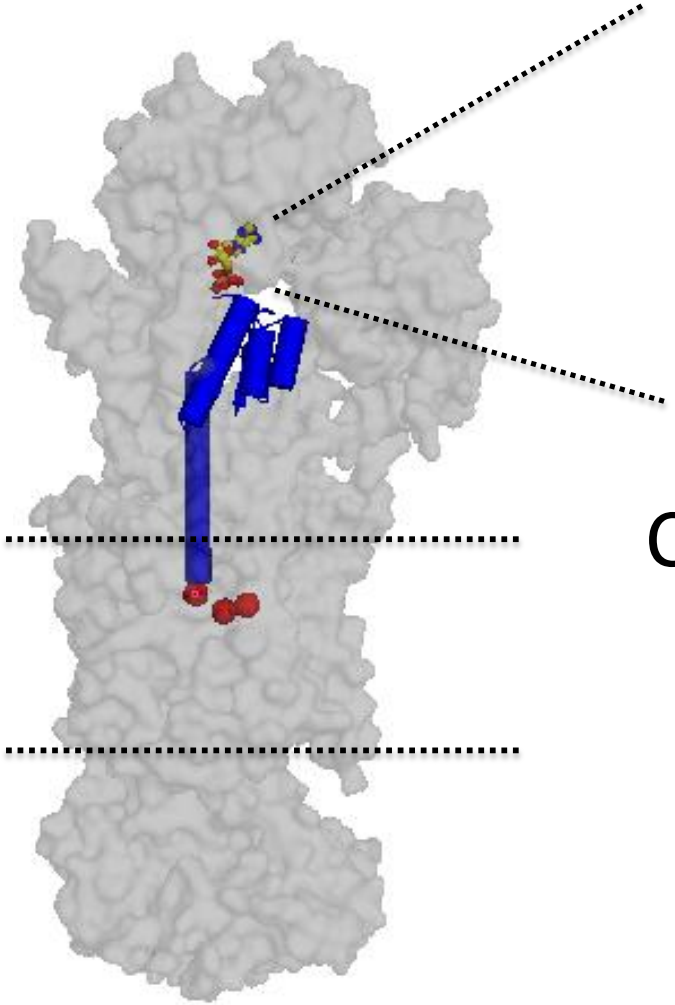
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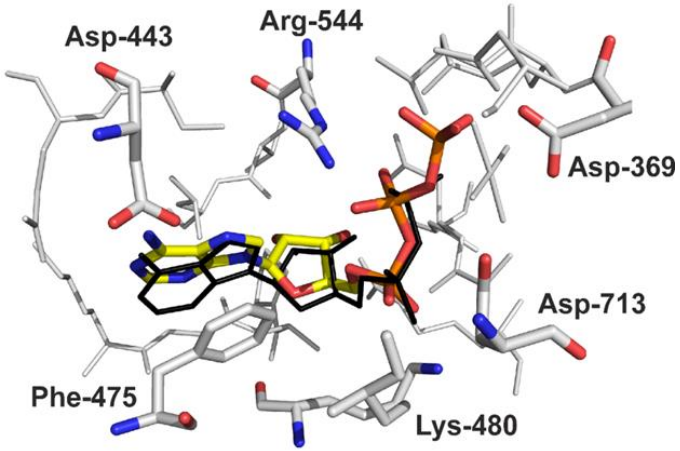
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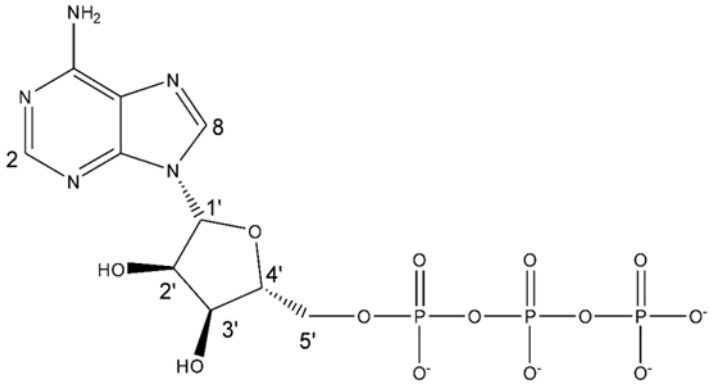
Asp-369
Asp-713
Phe-475
Lys-480


C
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2
N
N
1'
O
HO
2'
4'
3'
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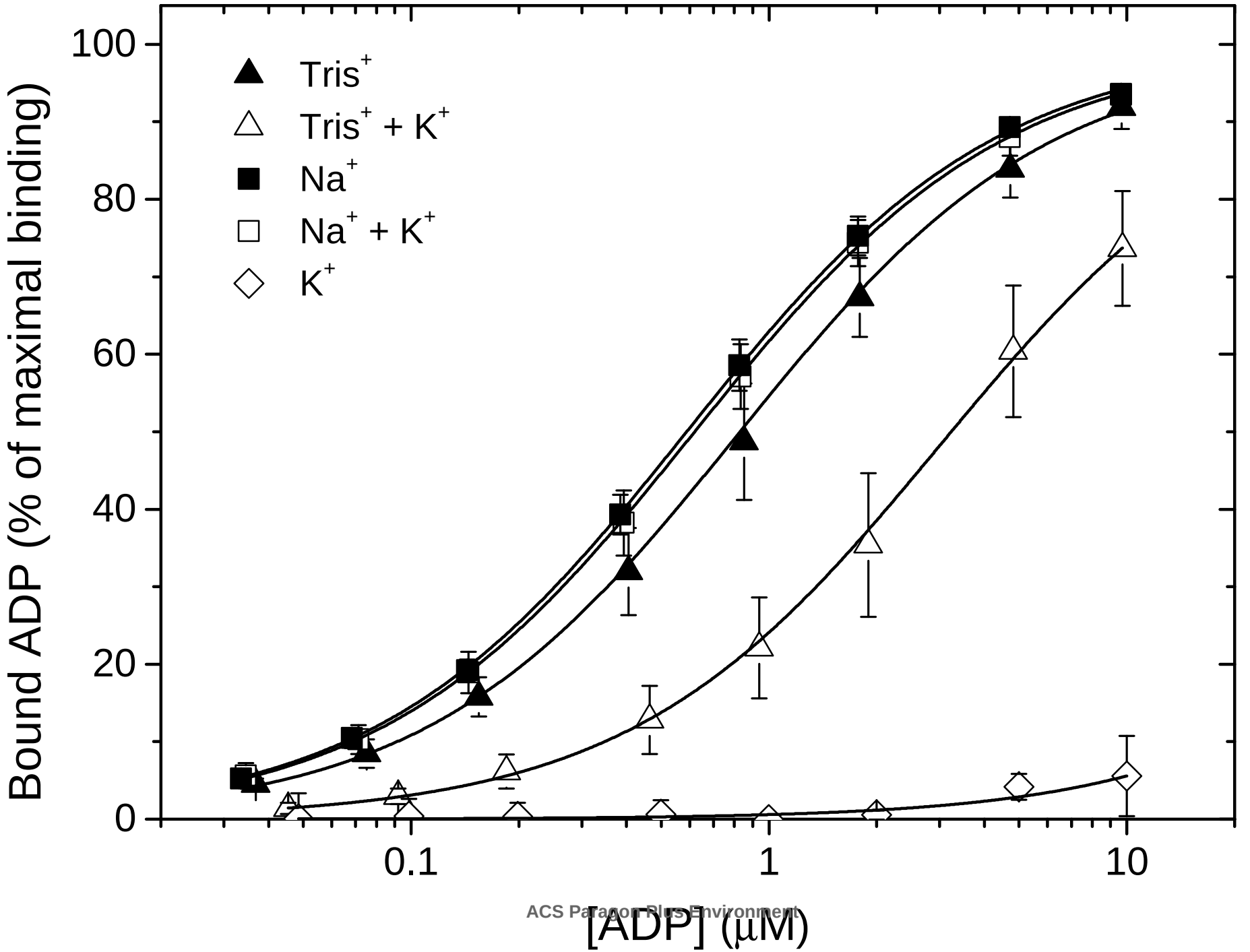
HO
O
P
O-



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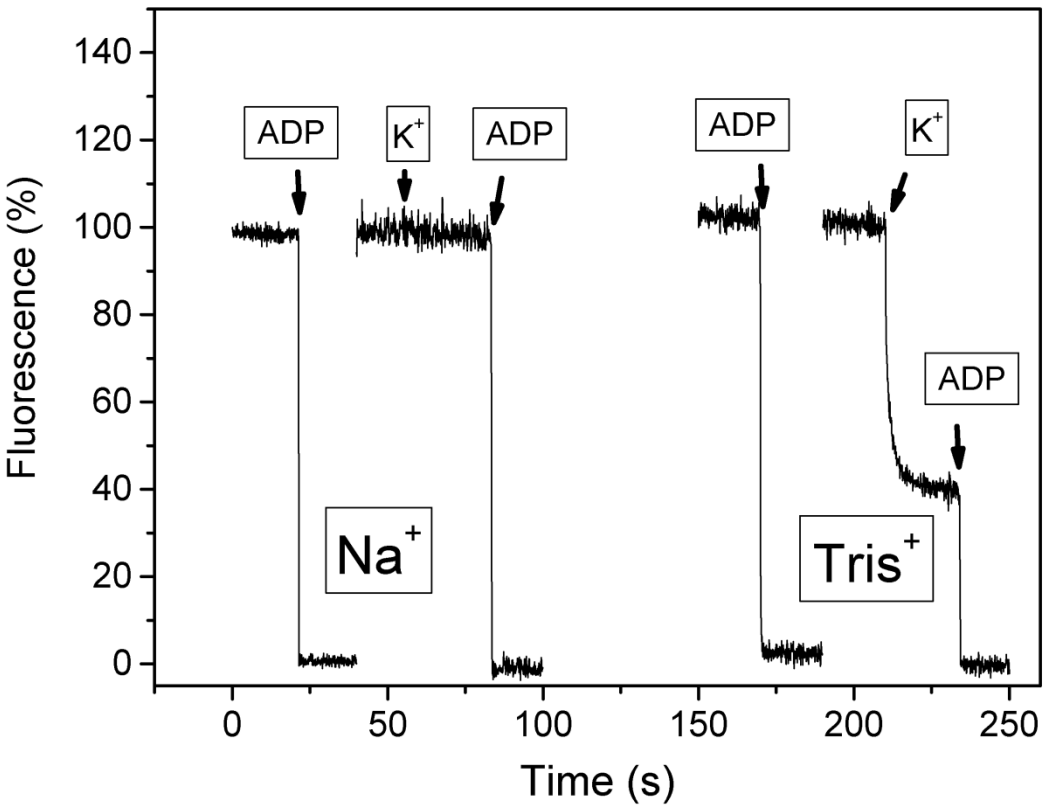

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