Nucleotide-Binding Kinetics of Na,K-ATPase: Cation Dependence†

Natalya U. Fedosova* and Mikael Esmann

Department of Biophysics, University of Aarhus, Ole Worms Allé 185, DK-8000 Aarhus C, Denmark

Received September 22, 2003; Revised Manuscript Received January 26, 2004

ABSTRACT: Correlation between the Na,K-ATPase affinity to ADP and the cation (its nature and concentration) present in the medium was investigated. In buffer with low ionic strength ($I \approx 1$ mM) high-affinity ADP binding was not observed, while a stepwise increase in the concentrations of added cation (Na^+, $Tris^+$, imidazole$^+$, *N*-methylglucamine$^+$, choline$^+$) induced an increase in the ADP affinity. The effect was fully saturated at 30−50 mM for all of the cations tested. The maximal affinity for ADP was slightly higher in the presence of Na^+, $Tris^+$, or imidazole$^+$ than in the presence of *N*-methylglucamine$^+$ or choline$^+$ (equilibrium dissociation constant K_d 0.2−0.3 vs 0.7 μM). The ADP dissociation rates from its complex with enzyme in the presence of Na^+ or $Tris^+$ were similar, implying identity of the nucleotide-binding enzyme conformations, which therefore are assigned to E_1. The ability to compete with K^+ clearly distinguished Na^+ from other cations, which speaks against the sole involvement of the transport sites in the induction of the ADP-binding E_1 conformation. Since the cations are similar in their mode of induction of the high ADP affinity but they demonstrate a pronounced difference in ability to compete with K^+, their effects cannot be combined within any scheme with only one type of cation-binding sites. We suggest that the high affinity toward nucleotide is induced by cation interactions within the protein or lipid and that these nucleotide-domain-related sites coexist with the transport sites, which bind only Na^+ or K^+.

Na,K-ATPase (EC 3.6.3.9), one of the P-type ATPases, is present in the plasma membrane of animal cells. The enzyme is responsible for transport of $3Na^+$ inward and $2K^+$ outward against their electrochemical gradients at the expense of the energy of 1ATP molecule. The widely accepted hypothesis, describing the mechanism of energy transduction from the hydrolytic site to the transport sites, involves transition between two major protein conformations: E_1[1] and E_2 (*1*, *2*). As an alternative to this mechanical description of ion transport, Scarborough (*3*) has recently suggested a charge transfer model, where electronic rearrangements in the hydrolytic site are transmitted as an electric pulse to the transport sites. Both concepts require an interplay between the hydrolytic (nucleotide-binding) site and the transport sites. The experimental evidence also reveals their close interactions. Thus, Na occupation of the transport sites enables nucleotide binding with high affinity, the protein state being defined as an E_1 conformation. On the contrary, low-affinity nucleotide binding to the E_2 conformation of the enzyme promotes K^+ release from the transport sites. In other words, the presence of either ligand induces significant changes in the protein structure tens of angstroms away (*4*).

An essential property for the enzyme is its selectivity: it is able to distinguish between Na^+ and K^+, despite only a small difference in their ionic radii (0.095 vs 0.133 nm). It was shown (*5*−*7*), however, that other cations (Tris, imidazole, choline, *N*-methylglucamine) have a Na^+-like influence on a number of partial reactions of Na,K-ATPase, including nucleotide binding. The question is if these cations implement their effect through binding to the transport sites.

To elucidate the mechanism of the ion effect, we studied the cation dependence of the enzyme affinity for ADP. We compared the efficiency of different monovalent cations (Na^+, $Tris^+$, imidazole$^+$, *N*-methylglucamine$^+$, choline$^+$) to induce high-affinity nucleotide binding (E_1 conformation) with their ability to compete with K^+. The techniques employed in the present study included both transient and equilibrium measurements of nucleotide binding. The results suggest that the transport sites are not involved in the induction of the enzyme conformation with high affinity to the nucleotide. We refer to these sites, responsible for the induction of the high affinity to the nucleotide, as nucleotide-domain-related cation-binding sites.

MATERIALS AND METHODS

Preparation of Na,K-ATPase. Pig kidney microsomal membranes were treated with SDS and purified by differential centrifugation to a specific activity of 28 μmol (mg of protein)$^{-1}$ min^{-1} at 37 °C (*8*, *9*). The K^+ contamination measured by atomic absorption spectroscopy was less than 3 μmol/μmol of enzyme, and the buffer solutions contained less than 2 μM K^+ (unpublished observations).

Equilibrium-Binding Experiments. Equilibrium binding of ADP was measured in double-labeling filtration experiments essentially as previously described (*10*−*13*). Na,K-ATPase was allowed to equilibrate at 20 °C for 10 min in a buffer

† This work was supported by the Danish Medical Research Council, The Novo Nordisk Foundation, and Aarhus University Science Foundation.

* To whom correspondence should be addressed. Phone: +45 8942 2933. Fax: +45 8612 9599. E-mail: nf@biophys.au.dk.

[1] Abbreviations: E_1, protein conformation of Na,K-ATPase predominant in Na^+-containing media and characterized by high affinity to nucleotides ATP and ADP; E_2, protein conformation of Na,K-ATPase predominant in K^+-containing media; NMG, *N*-methylglucamine; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi035707n CCC: $27.50
Published on Web 03/17/2004

containing 1 mM histidine (adjusted to pH 7.0 with 0.045 mM CDTA) and chloride salts of either Na^+, $Tris^+$, $choline^+$, *N*-methylglucamine$^+$ (NMG^+), or imidazole$^+$ at various concentrations. Approximately 57% of total imidazole is protonated under these conditions. The enzyme was stable in this buffer, also in the absence of added chloride salts, for at least 1 h at 20 °C, the time that is sufficient for the equilibrium-binding experiment. In some experiments KCl was also added to the incubation medium. The buffer also contained various concentrations of [^{14}C]ADP and [^{3}H]-glucose (both from New England Nuclear). One milliliter of this suspension (usually 0.24−0.28 mg of protein/mL) was loaded on two stacked Millipore HAWP 0.45 μm filters. Then, without rinsing, the filters were separately counted in 4 mL of 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 [^{3}H]glucose in the same filter. The concentration of free ADP in the suspension was calculated by subtraction of the amount bound to the protein.

Time-Resolved Measurements of Nucleotide Dissociation. Dissociation rate constants were measured using a rapid filtration system RFS-4 (Bio-logic, Claix, France) as previously described (*10*−*13*). Briefly, 1 mL of Na,K-ATPase, generally at a concentration of 0.1 mg of protein/mL, was loaded onto a single filter, and the filter was manually perfused with the same buffer as described above containing a mixture of [^{14}C]ADP and [^{3}H]glucose to reach equilibrium. Then the adsorbed ATPase was perfused for times ranging between 30 ms and 2 s with the same buffer, containing now 0.02 mM unlabeled ADP. The temperature was 20 °C. Residual nucleotide binding was calculated as described above, but most of the unbound nucleotide together with the [^{3}H]glucose label was actually immediately washed off by the perfusion.

Data analysis was performed using the ORIGIN 6.0 software (Microcal, Amherst, CA) and KyPlot 2.13 freeware (Koichi Yoshioka, Department Biochemistry and Biophysics, Graduate School of Allied Health Sciences, Tokyo, Japan; www.woundedmoon.org/win32/kyplot.html).

RESULTS AND DISCUSSION

Na^+ Requirement for High-Affinity ADP Binding. Binding of ADP (at concentrations up to 20 μM) to Na,K-ATPase is negligible in a medium with very low ionic strength, without added cations (Figure 1). Addition of 5 mM NaCl to the buffer induces a measurable ADP binding. A further increase in the Na^+ concentration causes an increase in the affinity for ADP (compare binding curves at 10 and 35 mM Na^+) until an ionic strength effect on the interaction between the negatively charged ADP and positive charges on the nucleotide-binding site becomes predominant and reverses the increase in affinity. The latter is illustrated by the binding curve at 200 mM Na^+. The data are fitted by single hyperbolic functions; the maximal binding capacity is approximately 2.8 nmol/mg of protein at all Na^+ concentrations tested. At [Na^+] <5 mM ADP-binding curves were difficult to obtain due to the low affinity. In addition, they demonstrated a noticeable deviation from the hyperbolic behavior.

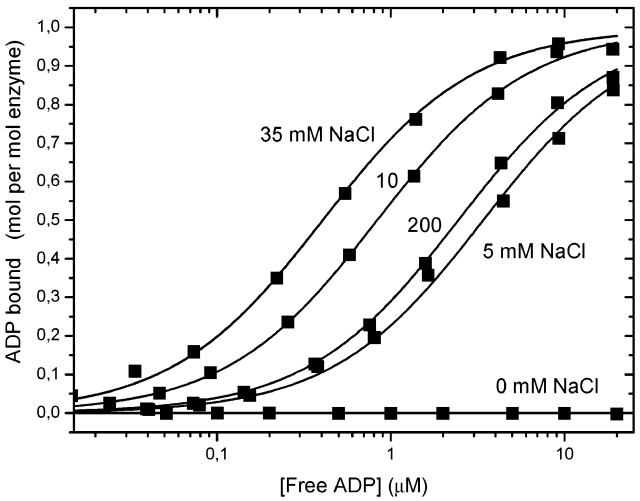

FIGURE 1: Equilibrium binding of [^{14}C]ADP to Na,K-ATPase at different NaCl concentrations.. The amount of [^{14}C]ADP bound is measured with the filtration technique. The full lines represent single hyperbolic functions with a maximal binding of 1 mol/mol of Na,K-ATPase (2.8 nmol/mg of protein).

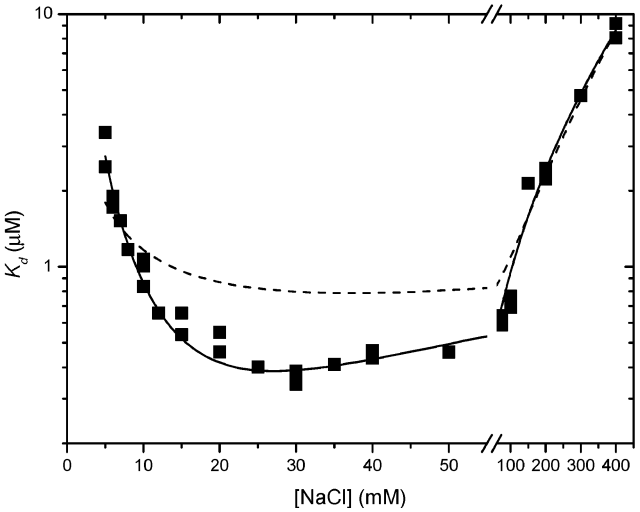

FIGURE 2: Effect of NaCl on the K_d for ADP binding to Na,K-ATPase. Equilibrium dissociation constants determined from experiments such as those shown in Figure 1 are shown on a logarithmic scale as a function of the NaCl concentration. The data are fitted to a model, where compulsory cation binding is described by the Hill equation, with $n = 1$ (dashed line) or $n = 2.3$ (best fit, full line). The other parameters of the best fit are presented in Table 1.

The relation between the observed affinity to ADP and the Na^+ concentration is shown in Figure 2. The equilibrium dissociation constant for ADP (K_d) is given on a logarithmic scale as a function of the NaCl concentration. A clear saturation behavior is observed, with the maximal affinity ($K_d \approx 0.3$ μM) at 30−40 mM NaCl. At concentrations above 50 mM the effect of ionic strength becomes predominant, leading to the increasing K_d values (data are shown here for concentrations up to 400 mM NaCl).

Analysis of the data was based on the assumption that only the enzyme form ENa_n is able to bind the nucleotide. We deliberately avoided ascribing the enzyme conformation existing in the buffer with low ionic strength to either E_1 or E_2, since it has characteristic properties of both conforma-

tions. It does not bind ADP with high affinity, but upon addition of Na^+ high affinity for nucleotide is rapidly induced (*14*). The cation binding was approximated by Hill's model (Scheme 1) where several ligand molecules attach simultaneously to the Na,K-ATPase.

Scheme 1

$$E \underset{K_{Na}^n}{\overset{n\mathrm{Na}}{\rightleftharpoons}} E_1{\cdot}\mathrm{Na}_n \underset{K_A}{\overset{\mathrm{ADP}}{\rightleftharpoons}} E_1{\cdot}\mathrm{Na}_n{\cdot}\mathrm{ADP}$$

In our previous study (*13*) the relation between the observed K_d and the ionic strength (I) at saturating Na^+ concentrations ($[Na^+] > 50$ mM) was interpreted in terms of the limited Debye−Hückel model for attraction between the charged ADP molecule (z_{ADP}) and the charge of the binding site (z_E) (*15*). Obviously, the electrostatic screening has its impact in the ADP affinity at all Na^+ concentrations, also below 50 mM.

Therefore, adopting the scheme above and taking into account the ionic strength effect, the constants in the following equation were fitted to the data:

$$K_d = K_A \times 10^{-z_{ADP}z_E I^{1/2}}\left(1 + \frac{K_{Na}^n}{[Na^+]^n}\right) \qquad (1)$$

where K_{Na} is the equilibrium dissociation constant for n ions, $[Na^+]$ is the Na^+ concentration, and K_A is the ADP dissociation constant. The effect of ionic strength in the whole range of ion concentrations is reflected by the term $10^{-z_{ADP}z_E I^{1/2}}$.

The analysis clearly revealed a cooperativity in Na^+ binding. Figure 2 demonstrates that binding of one Na^+ ($n = 1$, dashed line) is not sufficient to describe the observed increase in ADP affinity. The best fit was obtained at $n = 2.3$ (full line), correlating with the number of Na^+ transported per cycle ($n = 3$). The values for the other parameters were $K_A = 0.1$ μM, $K_{Na} = 16$ mM, and $z_{ADP}z_E = -3.1$.

High-Affinity ADP Binding Induced by Other Cations. Tris, imidazole, choline, and *N*-methylglucamine were all found to induce high-affinity ADP binding in a fashion similar to that of Na^+. In all cases the binding process can be described by a single hyperbolic function and has the same maximal binding capacity. Tris behaves in a way quantitatively similar to Na^+, where 20−30 mM cation brings the apparent dissociation constant for ADP down to 0.2−0.3 μM (Figure 3A). Imidazole is as good as Tris in inducing the high-affinity ADP binding. Choline and NMG also increase the ADP affinity in a saturable fashion, but the maximal affinity is lower than that in the presence of Na^+ and Tris (Figure 3B).

Thus, the presence of any of the cations tested increases the Na,K-ATPase affinity to ADP. Their potencies, however, are different, possibly due to the different affinities for the enzyme. It is conceivable that a further decrease in apparent dissociation constant for ADP (potentially possible at higher concentrations of choline or NMG) is masked by the ionic strength effect.

Binding of ADP with high affinity is a characteristic feature of the Na,K-ATPase in the E_1 conformation. The fact that the presence of any of the cations tested significantly increases the affinity for the nucleotide implies that their binding induces formation of the E_1 conformation of the enzyme. This statement raises the following questions: (1) Does the mechanism of induction of high affinity for ADP involve the transport sites? (2) Are the induced conformations the same with respect to the substrate-binding-site structure? Below we will compare effects of Na^+ and other cations with the emphasis on Tris. Our choice of Tris is based on the following considerations: Tris is not transported by the Na,K-ATPase (to the best of our knowledge) but shows the same affinity to the enzyme as Na^+ (see Figure 3A), which specifically binds to the transport site and is transported by the Na,K-ATPase. Also, ADP binding, induced by either Tris or Na^+, is characterized by a similar K_d (Figure 3A).

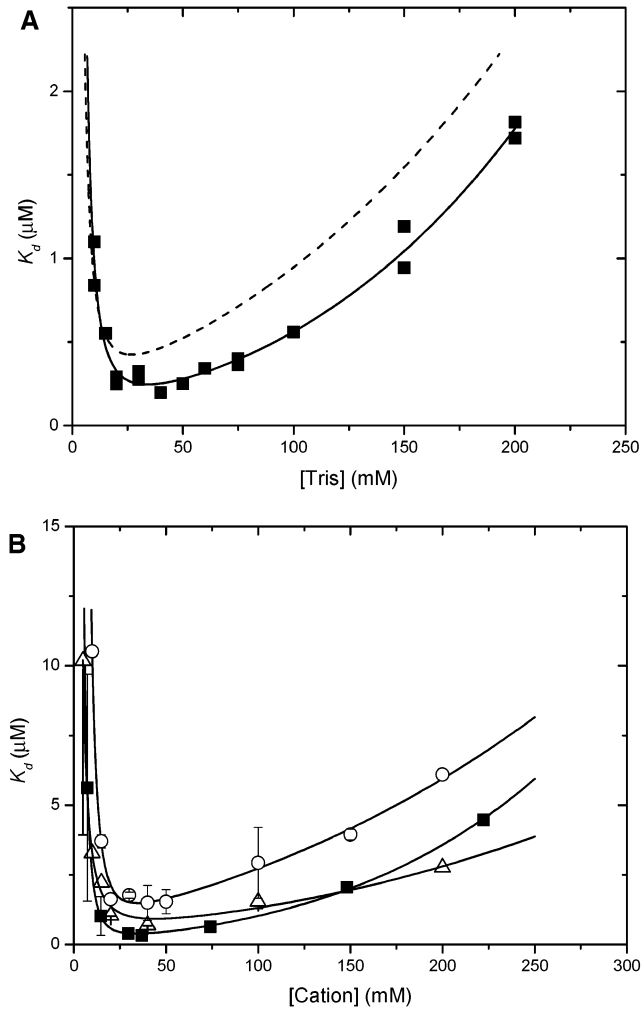

FIGURE 3: Effect of different cations on the K_d for ADP binding to Na,K-ATPase. (A) K_d for ADP as a function of the Tris concentration. The data are fitted to the Hill equation. The equilibrium dissociation constant for ADP as a function of NaCl concentration, derived from Figure 2, is included as a dashed line for comparison. (B) K_d for ADP as a function of cation concentrations: imidazole (squares), choline (triangles), and *N*-methylglucamine (circles). The parameters for the best fit of each data set are presented in Table 1.

A fit of Hill's model to the Tris data produced the following parameters: $z_{ADP}z_E = -3.9$, $K_{Tris} = 25$ mM, $K_A = 0.03$ μM, and $n = 2.6$ (Figure 3A, Table 1). Thus, the set of parameters describing Tris interaction with the enzyme is similar to that for Na^+. Binding of either cation occurs with similar affinities and induces comparable ADP affinities. The charge products are also reasonably close.

Table 1: Kinetic Parameters (±SE) Obtained by Fitting the Experimental Results to the Hill Equation (Figures 2 and 3)

parameter	NaCl	Tris	imidazole	choline	NMG
K_{cation}	16 ± 2	25 ± 5	25 ± 23	31 ± 14	21 ± 2
K_A	0.10 ± 0.02	0.03 ± 0.01	0.05 ± 0.09	0.16 ± 0.15	0.40 ± 0.11
n	2.3 ± 0.2	2.6 ± 0.3	3.1 ± 1.6	2.0 ± 0.1	3.4 ± 0.2
$z_{ADP}{\cdot}z_E$	−3.1 ± 0.1	−3.9 ± 0.3	−4.2 ± 2.0	−2.7 ± 0.9	−2.6 ± 0.3

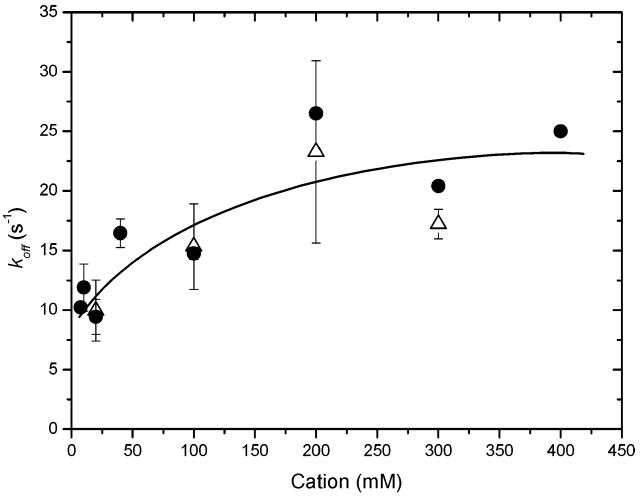

FIGURE 4: Effect of Na^+ (circles) and $Tris^+$ (triangles) concentrations on ADP dissociation from the nucleotide-binding site of Na,K-ATPase. The experimental details are described in Materials and Methods. The data points represent the average of at least three experiments ± SE. The line is drawn to guide the eye.

Imidazole, NMG, and choline data were also fitted by eq 1 (Table 1). Taken together, the results speak for the conclusion that all cations tested behave in the same way, i.e., bind to the same sites on Na,K-ATPase, and their binding has analogous effects on the ADP-binding site. Thus, either all ions can bind to the transport sites or these binding sites are not the transport sites.

The similarity of the equilibrium dissociation constants for ADP in the presence of Tris or Na^+ suggests that the structure of the nucleotide-binding site, induced by the cations, is the same. The K_d value, however, is not the best criterion for comparison of the induced sites. Since it is a function of binding and dissociation rate constants, changes in both constants may have a compensating effect. The dissociation rate constant is a much better parameter, since it depends only on the properties of the complex between the ligand and the binding site. Below, it was used to compare the complexes formed in the presence of Na^+ or Tris.

Figure 4 shows dissociation rate constants for ADP (k_{off}) as a function of either Na^+ or Tris. It is clear that ADP dissociates from the complex with Na,K-ATPase with the same rate constants, independently of the cation present (at a given cation concentration). The fact, that k_{off} shows saturation behavior (i.e., first increases with the ionic strength but levels off at a value of about 27 s^{-1}) has been described previously (*13*). The observed k_{off} independence of ionic strength (at I >100 mM) is in line with Debye−Hückel theory.

K^+ Effect on Cation-Induced High-Affinity ADP Binding. The structure of the nucleotide-binding site in the presence of any of these cations seems to be the same and corresponds to what is generally described as the E_1 conformation. But how does the presence of a cation induce the E_1 conformation and what is the mechanism of transformation of the low-affinity ADP-binding site in the absence of cations into a high-affinity site in their presence? Scheme 1 gives an adequate description of the data (Figures 2 and 3), and the parameters obtained suggest analogous behavior for all cations tested. But is the effect accomplished through binding of the cations to the transport sites of Na,K-ATPase? That conclusion seems reasonable, since the number of bound cations, suggested by the fitting procedure, is around 3. To find an experimental test for the assumption, we compared the ability of the cations under investigation to compete with K^+, which, like Na^+, is transported by Na,K-ATPase. Namely, we studied the effect of K^+ at different concentrations on cation-induced ADP binding (*16*, *17*). The conditions for ADP binding in the absence of K^+ were optimal for each cation; i.e., ADP binding was induced by the presence of 30 mM cation in 1 mM histidine and 0.045 mM CDTA, pH 7.0.

In the presence of 30 mM NaCl addition of 1 mM KCl leads to a 3-fold decrease in ADP affinity (Figure 5A). An increase in K^+ concentration brings about further decrease in ADP affinity, and at 7 mM K^+ it decreases 100-fold (up to 30 μM). This is considered a limit for the binding measurements performed by our filtration method. [Note that a deviation of the single hyperbolic fit of the experimental data becomes observable in the presence of K^+, as earlier described by Jensen et al. (*18*). Since the detailed description of the ADP-binding process in the presence of K^+ and either cation is beyond the scope of our paper, we characterized nucleotide affinity by a half-saturation value, $K_{0.5}$, obtained from the single hyperbolic fits shown.]

The inhibiting effect of K^+ on ADP binding induced by the other cations at 30 mM (Tris, imidazole, NMG, choline) was much more pronounced. Figure 5B illustrates the change in the nucleotide-binding properties, caused by the presence of 50 μM K^+. In this case, the ADP affinity decreases drastically (10−30-fold). Figure 6 explores the difference between the cations and compares the effect of different K^+ concentrations on the $K_{0.5}$ for ADP in the presence of 30 mM Tris or 30 mM NaCl. Again, it is clear that Na^+ is 30-fold more efficient in K^+ replacement than Tris.

Cation Interactions with Na,K-ATPase. It has been established for years that a high concentration of a protonated buffer favors the E_1 conformation of the Na,K-ATPase. Thus, choline, protonated Tris, and histidine were shown to have a Na-like effect on eosin fluorescence; choline and protonated Tris also affected the intrinsic fluorescence of the enzyme (*5*). However, only Na^+ became occluded in the presence of oligomycin (*19*), clearly manifesting the high cation selectivity of the pump. In the following years extensive work was done to characterize and to compare Na^+ with buffer cations and various amines in terms of their effect on phosphorylation and dephosphorylation of Na,K-ATPase (*6*,

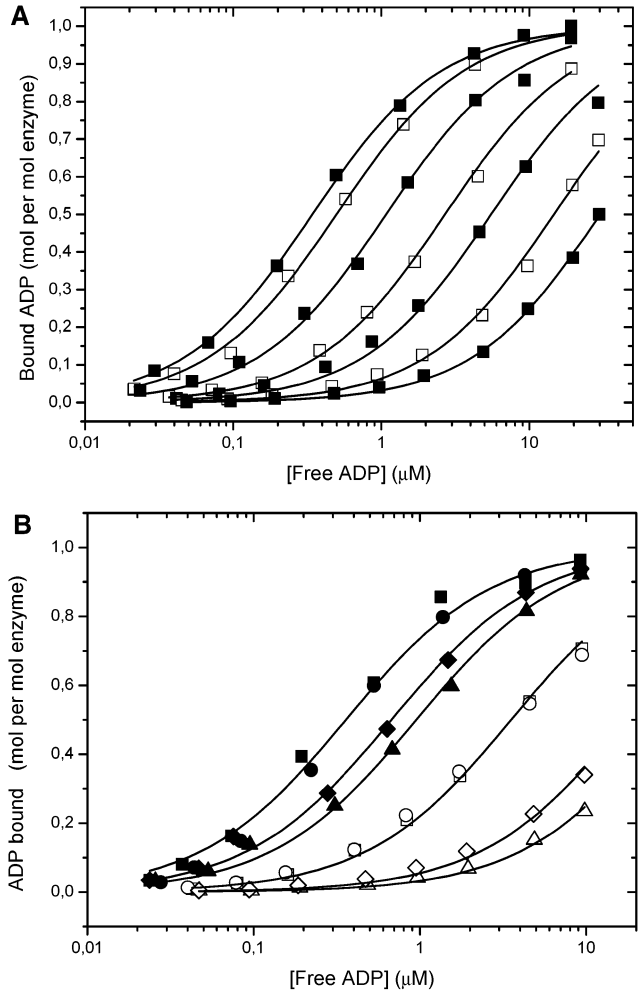

FIGURE 5: Effect of different KCl concentrations on ADP binding to Na,K-ATPase in the presence of different cations. (A) The ADP binding was measured in 1 mM histidine, 0.045 mM CDTA (pH 7.0), 30 mM NaCl, and increasing concentrations of KCl (from left to right): 0, 0.5, 1, 2, 3, 5, and 7 mM. (B) The ADP binding was measured in 1 mM histidine, 0.045 mM CDTA (pH 7.0), and 30 mM cations in the absence (filled symbols) or presence of 50 μM KCl (open symbols): Tris (squares), imidazole (circles), choline (diamonds), and NMG (triangles).

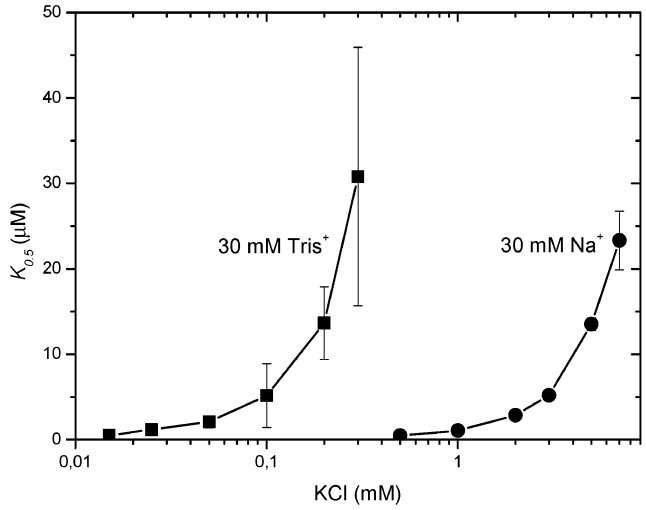

FIGURE 6: Effect of different KCl concentrations on the $K_{0.5}$ for ADP binding to Na,K-ATPase. The ADP binding was measured in 1 mM histidine, 0.045 mM CDTA (pH 7.0), 30 mM NaCl (circles) or 30 mM Tris (squares), and increasing concentrations of KCl.

7, *20*, *21*). The results were ascribed to the effects on the E_1-E_2 equilibrium and, at least partly, to diverse E_1 conformations stabilized by different cations. Experiments with trypsin clearly showed that inactivation of Na,K-ATPase was biphasic when partial digestion was performed in the presence of Na^+, Tris, or choline (*22*), confirming the existence of the cation-induced E_1 conformation. The rate of chymotryptic inactivation reveals, however, a structural difference between the Na- and Tris-bound enzyme (*23*).

Another group of cations interacting with Na,K-ATPase was designed with the purpose of labeling and mapping of cation-binding sites. Two types of organic compounds were synthesized: aryl bis(guanidinium) derivatives and tris-(isothiouronium) derivatives (*24*, *25*). Kinetic analysis showed that these divalent and trivalent cations competitively inhibit Na^+ or Rb^+ occlusion, stabilize the E_1 conformation of the enzyme, and block Na,K-ATPase activity. However, they are not occluded by the enzyme. These findings led to a concept of an entry port of the (cytoplasmic) transport site and a two-step binding process for Na^+ (*26*). The relatively large synthetic compounds are thought to execute their effects through binding to the entry port.

The effects of the protonated buffers, described earlier, may have the same explanation. Due to the low specificity they might be able to bind to the entry port and induce an E_1 conformation of the enzyme. Thus, the identity of the dissociation rate constants for ADP from its complex with enzyme (Figure 4) in the presence of Tris or Na^+ implies that binding of either cation induced an analogous protein conformation at the nucleotide site. Competition with K^+ for the transport sites clearly differentiates Na^+ from the rest of the cations, which could be bound to the entry port. This fact fits the idea that Na^+ binding to the entry port is followed by another step, occlusion. Existence of the second step in the Na^+-binding process improves the affinity for Na^+ compared to the affinities for the cations bound only to the entry port and makes Na^+ a more powerful competitor to K^+.

However, a serious inconsistency appears when we compare experiments performed in the absence of K^+ and in its presence. The similarity of the cations in the mode of induction of high ADP affinity and, at the same time, their pronounced difference in the ability to compete with K^+ cannot be combined within any kinetic scheme with only one type of cation-binding sites. The ability of Na^+ to be occluded by the enzyme can explain its potency in K^+ competition, but it would also cause an increase in the apparent affinity for Na^+ and distinguish Na^+ from other cations in the absence of K^+. However, binding of Na^+ or other cations to the enzyme, estimated from the induction of high-affinity nucleotide binding, occurs with similar affinities. Table 1 presents only marginal differences in K_{cation}. The various cations increase the rate of deocclusion of K^+, but high concentrations (>0.1 M) are necessary to observe the effect (*27*). The described 2-fold variations in the rates of deocclusion (*27*) are too small to account for the 30-fold difference between Na^+ and the other cations for K^+ replacement (Figures 5 and 6).

Since none of the models with only one type of binding sites can fit both sets of the data, we conclude that the cation-

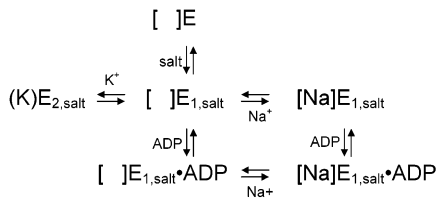

FIGURE 7: Schematic diagram for interactions of cations with Na,K-ATPase. See text for details.

binding sites involved in formation of the protein conformation with high ADP affinity are *not* the transport sites. We term these sites nucleotide-domain-related sites. Their number could also be higher than that stated in Table 1, since fitting to the Hill equation gives the minimal number of cations bound.

Our understanding of the interactions between the enzyme, cations, and nucleotide is envisaged in Figure 7. In the absence of added cations and at very low buffer concentration the Na,K-ATPase exists in a form, (E_0), with low affinity to ADP (parentheses indicate empty transport sites). Addition of cation (as its chloride salt) induces a rapid transition to a conformation with high affinity toward nucleotide, and this conformation is therefore assigned to E_1. Occupation of the transport sites with Na^+ (if the salt contained Na^+ as cation) has no additional effect on ADP affinity. However, if the added salt also contained K^+, it binds to the transport sites and causes transition to (K)E_2. Only Na^+ is able to compete with K^+ for those sites, shifting equilibrium toward the [Na]-E_1 conformation.

Considering the nature of the cation interactions with Na,K-ATPase in the nucleotide-domain-related sites, we should keep in mind their relatively high affinity for the enzyme: the effect (induction of high-affinity ADP binding) is fully manifested at 30−50 mM salt. That does not fit to the generally accepted definition of nonspecific interactions, leading to Hofmeister effects (*28*−*30*), charge screening (*13*, *31*), and phase transitions in the lipid bilayer (*32*). It was recently reported, however, that moderate concentrations of NaCl (below 100 mM) have pronounced effects on both structural and dynamic properties of a lipid bilayer (*33*). According to this study, interaction of Na^+ (and, possibly, other cations) with the carbonyl oxygens of the lipids leads to a decrease in lateral diffusion coefficients for lipids and thickens the bilayer by approximately 2 Å. [Note that tris-(isothiouronium) derivatives, considered to be Na^+ antagonists, also alter fluidity of the membrane and have a local effect on its hydrophobic thickness (*34*, *35*).] It is conceivable that these changes in lipid environment stabilize the enzyme conformation with high affinity to nucleotide, i.e., that enzyme in the presence of nontransported cations rests primarily in the E_1 conformation. Binding of K^+ to the transport sites, followed by its occlusion, shifts the equilibrium toward E_2. Since Na^+ is the only cation tested which binds to the transport sites and chases K^+ out, it shows more potency in K^+ competition experiments.

In conclusion, it seems that induction of a protein conformation with a high-affinity ADP-binding site requires binding of cations to a set of sites which are different from the transport sites. These nucleotide-domain-related sites are not selective toward any of the tested cations (although their apparent affinities vary). Occupation of the transport sites by Na^+, which occurs concomitantly with binding to the nucleotide-domain-related sites, does not affect the nucleotide affinity, while occupation of the transport sites with K^+ leads to a conformational change to (K)E_2 with a very low affinity toward nucleotide.

ACKNOWLEDGMENT

The excellent technical assistance of Ms. Angielina Damgaard and Ms. Birthe Bjerring Jensen is acknowledged. We thank Philippe Champeil for helpful discussions and one of the reviewers for numerous insightful suggestions for improvement.

REFERENCES

1. Albers, R. W. (1967) Biochemical aspects of active transport, *Annu. Rev. Biochem. 36*, 727−756.
2. Post, R. L., Kume, S., Tobin, T., Orcutt, B., and Sen, A. K. (1969) Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase, *J. Gen. Physiol. 54*, 306s−326s.
3. Scarborough, G. (2002) Molecular Mechanism of the P-Type ATPases, *J. Bioenerg. Biomembr. 34*, 235−250.
4. Toyoshima, C., Nakasako, M., Nomura, H., and Ogava, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution, *Nature 405*, 647−655.
5. Skou, J. C., and Esmann, M. (1980) Effects of ATP and protons on the Na: K selectivity of the (Na^+ + K^+)-ATPase studied by ligand effects on intrinsic and extrinsic fluorescence, *Biochim. Biophys. Acta 601*, 386−402.
6. Schuurmans Stekhoven, F. M. A. H., Swarts, H. G. P., de Pont, J. J. H. H. M., and Bonting, S. L. (1985) Na^+-like effect of imidazole on the phosphorylation of (Na^++K^+)-ATPase, *Biochim. Biophys. Acta 815*, 16−24.
7. Schuurmans Stekhoven, F. M. A. H., Swarts, H. G. P., de Pont, J. J. H. H. M., and Bonting, S. L. (1986) Sodium and buffer cations inhibit dephosphorylation of (Na^++K^+)-ATPase, *Biochim. Biophys. Acta 855*, 375−382.
8. Jørgensen, P. L. (1974) Purification and characterization of (Na^+ plus K^+)-ATPase. 3. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate, *Biochim. Biophys. Acta 356*, 36−52.
9. Klodos, I., Esmann, M., and Post, R. L. (2002) Large-scale preparation of sodium−potassium ATPase from kidney outer medulla, *Kidney Int. 62*, 2097−2100.
10. Champeil, P., and Guillain, F. (1986) Rapid filtration study of the phosphorylation-dependent dissociation of calcium from transport sites of purified sarcoplasmic reticulum ATPase and ATP modulation of the catalytic cycle, *Biochemistry 25*, 7623−7633.
11. Dupont, Y. (1984) A rapid-filtration technique for membrane fragments or immobilized enzymes: measurements of substrate binding or ion fluxes with a few-millisecond time resolution, *Anal. Biochem. 142*, 504−510.
12. Lacapère, J. J., and Guillain, F. (1993) The reaction mechanism of Ca^{2+}-ATPase of sarcoplasmic reticulum. Direct measurement of the Mg.ATP dissociation constant gives similar values in the presence or absence of calcium, *Eur. J. Biochem. 211*, 117−126.
13. Fedosova, N. U., Champeil, P., and Esmann, M. (2002) Nucleotide binding to Na, K.-ATPase: the role of electrostatic interactions, *Biochemistry 41*, 1267−1273.
14. Esmann, M., and Fedosova, N. U. (1997) Eosin as a probe for conformational transitions and nucleotide binding in Na,K-ATPase, *Ann. N.Y. Acad. Sci. 834*, 310−321.
15. Debye, P., and Hückel, E. (1923) Zur Theorie der Elektrolyte. I. Gefrierpunktsniedrigung und verwandte Erscheinungen, *Phys. Z. 24*, 185−206.
16. Hegyvary, C., and Post, R. L. (1971) Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase, *J. Biol. Chem. 246*, 5234−5240.
17. Nørby, J. G., and Jensen, J. (1971) Binding of ATP to brain microsomal ATPase. Determination of the ATP-binding capacity and the dissociation constant of the enzyme-ATP complex as a

function of K^+ concentration, *Biochim. Biophys. Acta 233*, 104−116.
18. Jensen, J., Nørby, J. G., and Ottolenghi, P. (1984) Binding of sodium and potassium to the sodium pump of pig kidney evaluated from nucleotide-binding behaviour, *J. Physiol. 346*, 219−241.
19. Esmann, M., and Skou, J. C. (1985) Occlusion of Na+ by the Na,K-ATPase in the presence of oligomycin, *Biochem. Biophys. Res. Commun. 127*, 857−863.
20. Schuurmans Stekhoven, F. M. A. H., Swarts, H. G. P., 't Lam, G. K., Zou, Y. S., and de Pont, J. J. H. H. M. (1988) Phosphorylation of (Na^++K^+)-ATPase; stimulation and inhibition by substituted and unsubstituted amines, *Biochim. Biophys. Acta 937*, 161−176.
21. Forbush, B., III (1988) The interaction of amines with the occluded state of the Na, K-pump, *J. Biol. Chem. 263*, 7979−7988.
22. Jørgensen, P. L. (1975) Purification and characterization of (Na^+,K^+)-ATPase. V. Conformational changes in the enzyme. Transitions between the Na-form and the K-form studied with tryptic digestion as a tool, *Biochim. Biophys. Acta 401*, 399−415.
23. Jørgensen, P. L., and Petersen, J. (1985) Chymotryptic cleavage of alpha-subunit in E1-forms of renal (Na+ + K+)-ATPase: effects on enzymatic properties, ligand binding and cation exchange, *Biochim. Biophys. Acta 821*, 319−333.
24. David, P., Mayan, H., Cohen, H., Tal, D. M., and Karlish, S. J. D. (1992) Structure−activity relations of amiloride derivatives, acting as antagonists of cation binding on Na^+/K^+-ATPase, *J. Biol. Chem. 267*, 1141−1149.
25. Hoving, S., Bar-Shimon, M., Tijmes, J. J., Goldshleger, R., Tal, D. M., and Karlish, S. J. D. (1995) Novel aromatic isothiouronium derivatives which act as high affinity antagonists of alkali metal cations on Na/K-ATPase, *J. Biol. Chem. 270*, 29788−29793.
26. Or, E., David, P., Shainskaya, A., Tal, D. M., and Karlish, S. J. D. (1993) Effects of competitive sodium-like antagonists on Na, K.-ATPase suggest that cation occlusion from the cytoplasmic surface occurs in two steps, *J. Biol. Chem. 268*, 16929−16937.
27. Forbush, B., III (1987) Rapid release of ^{42}K and ^{86}Rb from an occluded state of the Na,K-pump in the presence of ATP or ADP, *J. Biol. Chem. 262*, 11104−11115.
28. Post, R. L., and Suzuki, K. (1991) A Hofmeister effect on the phosphoenzyme of Na, K.-ATPase, *in: The Sodium Pump: Structure, Mechanism and Regulation* (Kaplan, J. H., and De Weer, P., Eds.) pp 201−209, Rockefeller University Press, New York.
29. Suzuki, K., and Post, R. L. (1997) Equilibrium of phosphointermediates of sodium and potassium transport adenosine triphosphatase: action of sodium ion and Hofmeister effect, *J. Gen. Physiol. 109*, 525−526.
30. Ganea, C., Babes, A., Lüpfert, C., Grell, E., Fendler, K., and Clarke, R. (1999) Hofmeister effects of anions on the kinetics of partial reactions of the Na^+,K^+-ATPase, *Biophys. J. 77*, 267−281.
31. Nørby, J. G., and Esmann, M. (1997) The effect of ionic strength and specific anions on substrate binding and hydrolytic activities of Na,K-ATPase, *J. Gen. Physiol. 109*, 555−570.
32. Klodos, I., Post, R. L., and Forbush, B., III (1994) Kinetic heterogeneity of phosphoenzyme of Na,K-ATPase modeled by unmixed lipid phases. Competence of the phosphointermediate, *J. Biol. Chem. 269*, 1734−1743.
33. Bockmann, R., Hac, A., Heimburg, T., and Grubmuller, H. (2003) Effect of sodium chloride on a lipid bilayer, *Biophys. J. 85*, 1647−1655.
34. Shinitzky, M. (1984) Membrane fluidity and cellular functions, in *Physiology of membrane fluidity* (Shinitzky, M., Ed.) CRC Press, Boca Raton, FL.
35. Johannsson, A., Keightley, C. A., Smith, G. A., Richards, C. D., Hesketh, T. R., and Metcalfe, J. C. (1981) The effect of bilayer thickness and *n*-alkanes on the activity of the (Ca^{2+} + Mg^{2+})-dependent ATPase of sarcoplasmic reticulum, *J. Biol. Chem. 256*, 1643−1650.

BI035707N