Nucleotide Binding to Na,K-ATPase: The Role of Electrostatic Interactions†

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ABSTRACT: The contribution of electrostatic forces to the interaction of Na,K-ATPase with adenine nucleotides was investigated by studying the effect of ionic strength on nucleotide binding. At pH 7.0 and 20 °C, there was a qualitative correlation between the equilibrium dissociation constant (K_d) values for ATP, ADP, and MgADP and their total charges. All K_d values increased with increasing ionic strength. According to the Debye−Hückel theory, this suggests that the nucleotide binding site and its ligands have "effective" charges of opposite signs. However, quantitative analysis of the dependence on ionic strength shows that the product of the effective electrostatic charges on the ligand and the binding site is the same for all nucleotides, and is therefore independent of the total charge of the nucleotide. The data suggest that association of nucleotides with Na,K-ATPase is governed by a partial charge rather than the total charge of the nucleotide. This charge, interacting with positive charges on the protein, is probably the one corresponding to the α-phosphate of the nucleotide. Dissociation rate constants measured in complementary transient kinetic experiments were 13 s^{-1} for ATP and 27 s^{-1} for ADP, independent of the ionic strength in the range 0.1−0.5 M. This implies similar association rate constants for the two nucleotides (about 40×10^6 M^{-1} s^{-1} at $I = 0.1$ M). The results suggest that long-range Coulombic forces, affecting association rates, are not the main contributors to the observed differences in affinities, and that local interactions, affecting dissociation rates, may play an even greater role.

Na,K-ATPase (EC 3.6.1.37), present in the plasma membrane of all animal cells, is responsible for the active transport of Na^+ and K^+ at the expense of the energy released from ATP hydrolysis. The enzyme is a P-type ATPase; i.e., ATP hydrolysis proceeds in two steps: first the γ-phosphate from ATP is transferred to the carboxyl group of Asp369; then the acyl−phosphate bond is hydrolyzed. These steps in ATP hydrolysis are thought to be performed by two different conformations of enzyme—E_1[1] and E_2—and the phosphorylation/dephosphorylation steps are coupled to the vectorial transport of Na^+ and K^+ consecutively [for recent reviews, see (*1*)].

This work focuses on the first step in the reaction cycle of Na,K-ATPase: binding of the substrate to the E_1-conformation of the enzyme. When sufficient Na^+ is present to induce the E_1-conformation, Na,K-ATPase exhibits high affinity toward the two adenyl nucleotides ATP and ADP (*2*, *3*). Electrostatic interactions are known to be important contributors to the specificity of enzyme−substrate binding and to stabilization of a charged transition state. In this paper, we therefore address the question to what extent the difference in the affinities of Na,K-ATPase toward ATP and ADP is related to the charges of these ligands.

The techniques employed in the present study include both equilibrium measurements of nucleotide binding and transient kinetic measurements of nucleotide dissociation rates. Changes of ionic strength were used to characterize, in the frame of Debye−Hückel theory (*4*), the electrostatic component of enzyme−substrate interactions. The results of these experiments suggest that the electrostatic interaction between the positively charged binding site of Na,K-ATPase and the nucleotides is imposed by a single negative charge on the nucleotides, presumably located on their α-phosphate, while the specificity of binding, reflected in the K_d values, is determined by short-range interactions.

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 (*5*).

Equilibrium Binding Experiments. Equilibrium binding of nucleotides was measured in double-labeling filtration experiments as previously described for Ca^{2+}-ATPase (*6*−*9*). One milliliter of a suspension of Na,K-ATPase (0.1 mg of protein/mL) in an appropriate buffer was loaded on two stacked Millipore HAWP 0.45 μm filters. The buffer (10 mM histidine at pH 7.0) contained NaCl at various concentrations and either 10 mM CDTA or 10 mM $MgCl_2$; varying NaCl concentrations generated ionic strengths up to 1 M. Radiolabeled nucleotides ([γ-^{32}P]-ATP from Amersham, ^{14}C-

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[1] Abbreviations: E_1, protein conformation of Na,K-ATPase predominant in Na^+-containing media; E_2, protein conformation of Na,K-ATPase predominant in K^+-containing media.

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ATP and ^{14}C-ADP from NEN) were mixed with ^{3}H-glucose (NEN) in the same buffer as that used for loading the enzyme onto the filter. The filter was then manually perfused with two to six 0.5 mL aliquots of the radioactive nucleotide solution, at a flow rate of about 1 mL/s, to ensure complete equilibration with nucleotide at all nucleotide concentrations. The temperature was 20 °C. Then, without rinsing, filters were counted in 4 mL of Packard Filtercount scintillation fluid. The amount of nucleotide bound to the protein was calculated by subtracting the amount of unbound nucleotide, trapped in the filter together with the wetting fluid, from the total amount of nucleotide on the filter (bound plus unbound nucleotide); the amount of unbound nucleotide was considered to be proportional to the amount of ^{3}H-glucose in the same filter. The fact that the protein remained fully adsorbed on the first filter was established both by counting the second filter and by checking for the absence of protein in the filtrate.

Time-Resolved Measurements of Nucleotide Dissociation. To determine nucleotide dissociation rates, a rapid filtration system was used (RFS-4, Bio-logic, France) (*7*, *10*, *11*). First, Na,K-ATPase was loaded onto a single filter, and the filter was manually perfused with a buffer containing radiolabeled nucleotide, ^{3}H-glucose, and varying concentrations of NaCl as described above, to reach equilibrium. Second, the adsorbed ATPase was perfused for times ranging between 30 ms and 2 s with the same buffer, containing now 0.02−2 mM unlabeled nucleotide. 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. In control experiments in which unlabeled nucleotide was not included in the final perfusion solution, a large fraction of the labeled nucleotide apparently dissociated very slowly (i.e., on the second time scale), but this was presumably artifactual and due to rebinding of labeled nucleotide to Na,K-ATPase during the perfusion period; such rebinding was prevented by our diluting out the nucleotide specific radioactivity with unlabeled nucleotide. A similar observation had previously been made when dissociation of $^{45}Ca^{2+}$ from sarcoplasmic reticulum Ca^{2+}-ATPase had been measured in the absence of calcium chelator under neutral conditions, where the ATPase affinity for Ca^{2+} is also submicromolar (Champeil and Henao, unpublished results). The possible existence of a nucleotide effect other than the dilution, e.g., additional binding to Na,K-ATPase at a putative second site, was ruled out by complementary experiments, as follows: equilibrium binding experiments showed that there was no additional binding up to about 0.07 mM nucleotide (the highest tested), although 0.02 mM unlabeled nucleotide added to the dissociation medium already had a pronounced effect on the apparent dissociation kinetics.

THEORY: DEBYE−HÜCKEL APPROACH

The interaction between two molecules, A (adenine nucleotide) and E (enzyme), described by the classical scheme

$$E + A \leftrightarrow EA \tag{1}$$

can be characterized by the equilibrium constant, K_d:

$$K_d = \frac{[E]\cdot[A]}{[EA]} \tag{2}$$

If species A, E, and EA carry net charges under the conditions of the experiment, the interaction between E and A has an electrostatic component, and K_d is therefore affected by the ionic strength of the reaction medium. Characterization of this electrostatic component in terms of the Debye−Hückel theory (*4*) leads to

$$\log(K_d/K_{d,0}) = -z_E\cdot z_A\cdot\sqrt{I} \tag{3}$$

where $K_{d,0}$ is the thermodynamic dissociation constant for the EA complex at $I = 0$ and z_E and z_A are the charges of E and A, respectively. The equation implies that a plot of log(K_d) vs $\sqrt{I}$ is a straight line, the slope of which allows empirical evaluation of the charge product ($z_E\cdot z_A$), and—if the charge z_A is known—of the corresponding value of z_E. This equation was initially derived to characterize the effect of ionic strength on the interaction between two point charges, but it can also be used for the description of ligand−protein interactions: in this case, since one of the interacting species is a large protein molecule, the calculations assume that certain charges in the reaction site are more important than distant charged groups on the protein. Thus, the effective point charge at the binding site of the protein (z_E) represents a sum of the protein charges that are steering ligand attraction. The applicability of the Debye−Hückel law for ligand binding to a protein molecule has been discussed previously (*12*).

The calculations of the total charges of the ligands were based on their corresponding p*K* values (*13*). Note that the ionic strength effect on the protonation stability constants was neglected, because the charge of the ligand increases by maximally 10% under the conditions of experiments (pH 7.0) when the ionic strength changes from 0.1 to 1.0 M. The charge of the protein binding site was also assumed constant at all ionic strengths, because the nucleotide affinity of Na,K-ATPase is rather constant at pH 5.8−7.6 (*2*), implying that the p*K* values of the essential groups involved in binding are very different from 7.0, the pH value for the present experiments. An ionic strength-induced shift in p*K*'s would have practically no effect on the protonation state of these groups and, therefore, no effect on the charge of the binding site. In addition, a small increase in the negative charge of the ligand at high ionic strengths and a small decrease in the positive charge of the protein binding site would cancel each other, at least partially, in the charge product ($z_E\cdot z_A$).

The Debye−Hückel theory of electrostatic interactions can also be used to describe the rate of binding in such an elementary reaction (eq 1). The association rate constant, k_{on}, has the same exponential dependence on $\sqrt{I}$ as K_d (*14*):

$$\log(k_{on}/k_{on,0}) = z_E\cdot z_A\cdot\sqrt{I} \tag{4}$$

Note that the slope is $+z_E\cdot z_A$ for k_{on} (eq 4) but $-z_E\cdot z_A$ for K_d (eq 3). Since

$$K_d = k_{off}/k_{on} \tag{5}$$

it follows that $k_{off} = k_{on}\cdot K_d = k_{on,0}\cdot K_{d,0}$ is independent of ionic strength.

These effects of ionic strength on a diffusion-controlled reaction can be rationalized by considering that the rate of association is affected by long-range electrostatic forces, while the rate of dissociation is only dependent on short-range interactions, including hydrogen bonds, salt bridges, hydrophobic interactions, van der Waals forces, etc.

RESULTS

Measurements of nucleotide binding to Na,K-ATPase at equilibrium were performed by a filtration method at 20 °C (*6, 7, 15*). Compared with dialysis or centrifugation techniques, this technique minimizes ATP hydrolysis artifacts, and the presence of a second filter may serve as an internal control for the completeness of protein adsorption on the first filter. In addition, filtration techniques make it possible to perform both equilibrium and transient kinetic experiments under identical experimental conditions.

Equilibrium Binding of Nucleotides. To measure equilibrium binding of ATP to Na,K-ATPase, it was necessary to fully prevent phosphorylation of Na,K-ATPase from ATP, and therefore to chelate contaminating Mg^{2+} and other divalent cations. 10 mM CDTA in the buffer was appropriate for this purpose, as confirmed by the direct measurement of virtually zero levels of phosphorylation and ATPase hydrolytic activity (not shown). Under such conditions, the apparent dissociation constants for ATP obtained in experiments performed with [γ-^{32}P]-ATP and ^{14}C-ATP were identical (not shown), confirming that phosphorylation did not occur during the time of the equilibrium measurement. Binding measurements performed at various concentrations of Na^+ revealed that the K_d for this binding was minimal at 60 mM Na^+ (not shown). The effect of the high concentrations of Na^+ was attributed to the effect of high ionic strength, while the effect of the low concentrations of Na^+ was interpreted as implying that in the presence of 10 mM CDTA, concentrations of Na^+ lower than 60 mM were not sufficient for full saturation of the Na,K-ATPase transport sites and induction of the ATPase E_1-conformation. Thus, the lower limit for the ionic strength in our experiments was about 80 mM (from salts and buffer constituents). From a methodological point of view, it is worth pointing out that the equilibrium dissociation constants determined here at 20 °C were found in the same submicromolar range as those obtained earlier by dialysis (*2, 3*), centrifugation (*12*), or filtration methods (*16*) at lower temperatures.

Figure 1 shows the influence of ionic strength on equilibrium nucleotide binding to Na,K-ATPase. The binding curves were fitted by a single hyperbolic function:

$$[EA] = \frac{[EA]_{max} \cdot [A]}{K_d + [A]} \quad (6)$$

The maximal binding $[EA]_{max}$ (approximately 2.8 nmol/mg of protein) was independent of the ionic strength and the type of nucleotide. Comparison of the ATP binding curves at $I = 87$ mM and $I = 388$ mM shows that K_d significantly increases with ionic strength. The effect of ionic strength on ATP affinity is, therefore, similar to that described previously for ADP affinity (*12*). It is also clear from the data in Figure 1 that the affinity for ATP is approximately 3-fold higher than that for ADP both at $I = 87$ mM and at $I = 388$ mM.

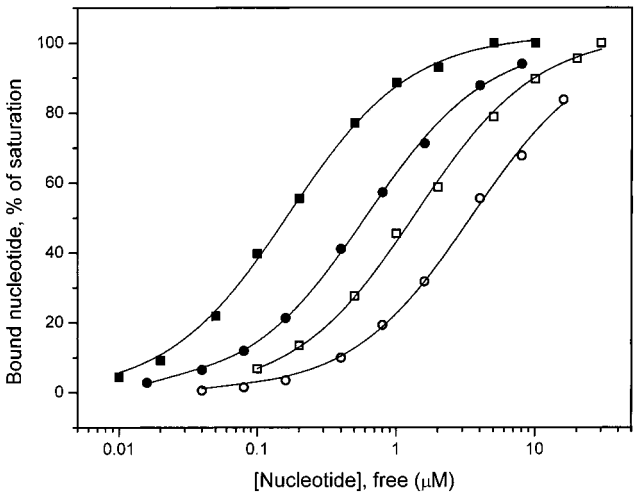

FIGURE 1: Equilibrium binding of ^{14}C-ATP (squares) and ^{14}C-ADP (circles) to Na,K-ATPase at different ionic strengths: filled symbols, 87 mM ([Na^+] = 57 mM); open symbols, 388 mM ([Na^+] = 357 mM). The full lines represent single hyperbolic functions with a maximal binding of 100% equal to about 2.8 nmol/mg of protein.

The large effect of ionic strength on the nucleotide binding affinity is in line with the idea that electrostatic interactions play a significant role, and that the charges on the ligand and enzyme are of opposite sign. However, more detailed quantitative characterization of the electrostatic component of the interaction relies on the dependence of $\log(K_d)$ on $\sqrt{I}$ (cf. eq 3). Linearity of such a plot proves the applicability of the Debye−Hückel theory and allows estimation of the charge product for the interacting species ($z_E \cdot z_A$, see Theory section). Dissociation constants for ATP and ADP, obtained at different ionic strengths as shown in Figure 1, are plotted as $\log(K_d)$ vs $\sqrt{I}$ in Figure 2. For ATP (Figure 2, panel A), the slope of the fitted line was 2.61 ± 0.08. Since the total charges of ATP and ADP are different ($z_{ATP} = -3.8$ and $z_{ADP} = -2.8$ at pH 7.0), whereas the charge of the binding site (z_E) should in principle not depend on the ligand, the slope of the fitted line, equal to the product ($-z_E \cdot z_A$), was expected to be different for ATP and ADP. However, for ADP (Figure 2, panel B), the slope of the fitted line was 2.54 ± 0.06; i.e., it was practically identical to that for ATP, although a difference of 25−30% would be expected from the difference in the total charges of these nucleotides. Assuming that the total charges of the ligands must indeed be taken into account, values of $z_E = +0.91$ and $z_E = +0.69$ would be derived from the ADP data and the ATP data, respectively.

To further investigate this unexpected finding, we also studied binding of MgADP to Na,K-ATPase. Complexation of ADP with a divalent cation changes the total charge of this ligand from −2.8 for ADP to −0.8 for the MgADP complex, i.e., by a factor of 3.5. Assuming that the charge of the nucleotide is the only parameter affected, this was expected both to reduce the affinity for the ligand (i.e., increase K_d) and to cause a significant decrease in the charge product, and therefore minimize the dependence on ionic strength. K_d for MgADP was indeed higher than that for free ADP over the whole Na^+ concentration range (Figure 2, panel B), although only moderately. But the slope of log-

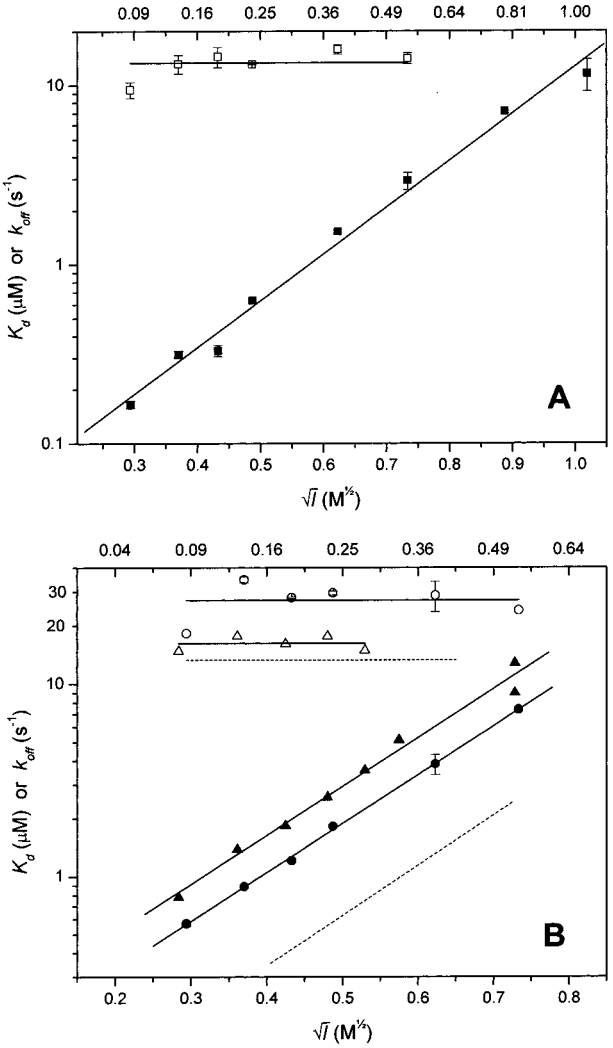

FIGURE 2: Effect of ionic strength on the equilibrium dissociation constant (filled symbols) and the dissociation rate constants (open symbols, see individual examples in Figure 3 below) for ATP (panel A) and ADP (panel B) bound to Na,K-ATPase. The abcissa (lower scale) is the square root of ionic strength; the data are plotted with a logarithmic scale on the ordinate. For convenience, the upper scale on the abscissa indicates the corresponding values of the ionic strength. Each point is the average of two to four experiments, and the error bars give the SEM. Panel A: The data on equilibrium dissociation constants for ATP are fitted to the Debye−Hückel model with a slope of 2.61 ± 0.08. The ordinate intercept corresponds to $K_{d,0} = 0.031$ μM. The rate constants for dissociation of ATP are also shown, with an average of 13.3 s^{-1}. Panel B: The data on equilibrium dissociation constants for ADP (circles) and MgADP (triangles) are fitted to the Debye−Hückel model with slopes of 2.54 ± 0.06 and 2.54 ± 0.12, respectively. The ordinate intercept corresponds to $K_{d,0} = 0.101$ μM for ADP and $K_{d,0} = 0.158$ μM for MgADP. The rate constants for dissociation of ADP (circles) and MgADP (triangles) are also shown, with averages of 27.2 s^{-1} for ADP and 16.2 s^{-1} for MgADP. The equilibrium dissociation constants for ATP as function of ionic strength, as well as the corresponding dissociation rate constants, all derived from panel A, are included as dotted lines for comparison.

(K_d) vs $\sqrt{I}$ obtained for the MgADP complex was about the same as for ADP, with a charge product for the MgADP−enzyme complex of 2.54 ± 0.12. Assuming again that the total charge of the MgADP ligand ($z_{MgADP} = -0.8$) must be taken into account, a value of $z_E = +3.21$ would be derived (instead of $z_E = +0.91$ for ADP binding). This will be discussed below.

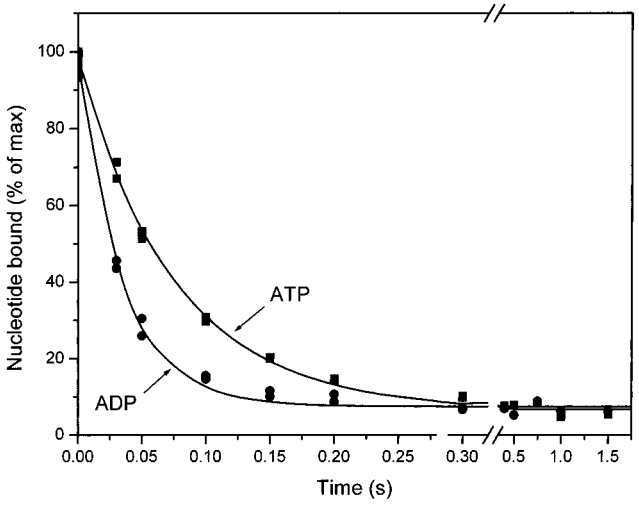

FIGURE 3: Kinetic measurement of the rates of dissociation of the enzyme−ATP (squares) and enzyme−ADP (circles) complexes at $I = 235$ mM. The curves are the single-exponential fits of the experimental data with $k_{off} = 13.2$ s^{-1} (ATP) or 28.4 s^{-1} (ADP).

Transient Kinetic Measurements of Nucleotide Dissociation. Evaluation of the dissociation rate constant, k_{off}, in transient dissociation experiments performed under the same conditions as the K_d measurements provides additional information about the mechanism of binding and the origin of specificity in protein−nucleotide interactions (see Theory section). Therefore, the dissociation rate constants for nucleotides were also determined over a large range of ionic strengths. Examples of such dissociation experiments at $I =$ 235 mM are shown in Figure 3 for ATP and ADP. Under these conditions, the dissociation of the nucleotide was described by single-exponential functions with rate constants k_{off} of 13.2 s^{-1} for ATP and 28.4 s^{-1} for ADP.

The measured dissociation rate constants for ATP, ADP, and MgADP were independent of ionic strength, as expected from the Debye−Hückel theory (cf. Theory section; see open symbols in Figure 2, panel A for ATP and panel B for ADP and MgADP). In the absence of magnesium, the dissociation rate for ADP is 2−3-fold faster compared to that for ATP (Figure 3). The correlation between dissociation rate constants and equilibrium dissociation constants observed for ATP and ADP implies that the bimolecular rate constant for association (k_{on}) is about the same for the two nucleotides (cf. eq 5). The value of this association rate constant, which depends on ionic strength, can be calculated to be about 40 × 10^6 M^{-1} s^{-1} for both nucleotides at $I = 127$ mM, and decreases to about 4 × 10^6 M^{-1} s^{-1} at $I = 500$ mM.

Note, however, that the rate of dissociation of MgADP was slower than that of free ADP (Figure 2, panel B), whereas the K_d for MgADP was somewhat larger than that of ADP. Consequently, k_{on} is about 2-fold lower for MgADP than for the nonliganded nucleotide, and decreases from about 20 × 10^6 M^{-1} s^{-1} at $I = 127$ mM to about 4 × 10^6 M^{-1} s^{-1} at $I = 340$ mM (the highest ionic strength tested).

DISCUSSION

Quantification of the ionic strength dependence of the K_d for binding of different nucleotides to Na,K-ATPase led to the surprising observation that the slope of the straight line describing this dependence was similar for ATP, ADP, and

MgADP. In terms of the Debye−Hückel approach, this implies that the charge product for the nucleotide−enzyme interaction is the same in all three cases. Since the total charge of the nucleotide varies between −0.8 for MgADP and −3.8 for ATP, it is clear that with an essentially constant slope ($-z_E \cdot z_A$) of about +2.6 either z_E must vary widely or z_A is not equal to the total charge of the ligand. Note that both z_E and z_A, the charges determining the ionic strength sensitivity of both the equilibrium constant K_d (eq 3) and the association rate constant k_{on} (eq 4), reflect the charges "steering" the nucleotide into place at the binding site.

We shall first address the hypothesis that z_A is equal to the total charge of the ligand. The charge in the binding site, z_E, is then +3.2 in the case of MgADP, very different from $z_E = +0.91$ in the case of ADP (cf. Figure 2, panel B) or $z_E = +0.69$ in the case of ATP (cf. Figure 2, panel A). Since the difference between z_E's obtained with ADP and ATP is relatively small, we restrict the discussion to the results concerning ADP binding in the absence or presence of Mg^{2+} ($z_E = +0.91$ vs +3.2). An obvious explanation would be that a Mg^{2+}, in addition to the one complexed with ADP, binds to the nucleotide binding site of the enzyme. Such binding would increase z_E from about +1 to about +3, compensating for the decrease in the total charge of the nucleotide ($z_{ADP} = -2.8$, $z_{MgADP} = -0.8$), and therefore would lead to a charge product similar to the one observed for ADP binding.

However, evidence from the literature seems to contradict the possibility that such a Mg^{2+} binding site contributes to the effective charge on the enzyme steering the ATPase−nucleotide association.

First, the Mg^{2+} binding site seems to be localized in close proximity to the phosphorylation site, Asp369 in the case of Na,K-ATPase, rather than in the nucleotide binding region. This conclusion is based on the functional role of the divalent cation: the positive charge of Mg^{2+} is thought to diminish the repulsion between the negative charge of the ATP γ-phosphate and the carboxyl group of Asp369 at the transphosphorylation step (*17*). Structural evidence arising from data on the metal-catalyzed oxidative cleavage of Na,K-ATPase (*18*) also suggest that Mg^{2+} is bound close to Asp369 and involved in coordination with Asp710 and Asp714. This is also in line with results of a comparative analysis of the three-dimensional structures of the proteins ascribed to the HAD (haloacid dehalogenase) superfamily, where a conserved pair of aspartate residues was assigned as a Mg^{2+} binding site in P-type ATPases (*19*).

Second, mutagenesis studies, involving substitution of the negatively charged Asp369 with the neutral Ala (*20*), similarly suggest that the charges situated in the area around the phosphorylation site do not contribute to z_E. A change in z_E, i.e., of those charges that are involved in the long-range electrostatic interactions with the nucleotides, should affect the association of all charged nucleotides with the binding site, and therefore should change the K_d values for both ATP and ADP. However, substitution of Asp369 with Ala increases the affinity for ATP 30-fold, but does not affect the ADP affinity (*20*). Thus, the removal of the negative charge of Asp369 does not seem to alter the steering charge z_E, and consequently Asp369 is not part of the steering charge (although it definitely contributes to the local environment, changing specific short-range interactions and the dissociation rate constant k_{off} for ATP). Following this line of reasoning, introduction with Mg^{2+} of additional positive charges close to Asp369 probably does not contribute to z_E either, since this area does not participate in the steering of the nucleotide ligands. Finally, for Ca-ATPase, the three-dimensional structure implies that the nucleotide binding domain and phosphorylation site (together with the putative Mg binding site) are spatially well-separated in the E_1-conformation of the ATPase (*21*). Thus, the similar values for the charge products obtained with ADP and MgADP are probably not explained by the presence of Mg^{2+} in the binding site.

An alternative hypothesis is that the steering charge of the protein binding site z_E is the same for all ligands, and that the effective charge of the ligand z_A must be identical for all nucleotides tested, independent of their total charge. Thus, the association of these ligands would be favored by local charges on both the ligand and protein binding sites, instead of total charges. For the nucleotides, this effective charge z_A is presumably equal to −0.8, since this is the minimal negative charge of the ligands used (i.e., that of MgADP at pH 7.0). The effective charge of the binding site, calculated from the slope of the K_d vs $\sqrt{I}$ curves, must be close to +3.

The suggestion that the effective charge of the ligand responsible for the electrostatic attraction is different from its total charge is supported by our previous experiments with eosin and its analogues. Thus, binding of eosin was shown to be sensitive to the ionic strength (*12*), but the values for the equilibrium dissociation constants (K_d), obtained for the eosin derivatives under identical conditions, showed no correlation with their total charge (*22*). In fact, 5-carboxy- and 6-carboxy-eosins, characterized by the same total charge (−3), exhibited 3-fold difference in affinities. Therefore, the effective negative charge steering the interaction was again not the same as the total charge. For nucleotides, the most probable candidate for the charged group common to all nucleotide ligands and responsible for the observed dependence on ionic strength is the α-phosphate group of the nucleotide, since this group is not involved in coordination with Mg^{2+} in the MgADP complex (*23, 24*).

The fact that only one of the charges on ADP or ATP participates in electrostatic steering contains information about the regions interacting with Na,K-ATPase, as only charges in close proximity to those regions will influence the binding process. Detailed interpretation of our results in terms of orientation of the ligand during its approach toward the binding site (the reaction governed by k_{on}) is not possible at the moment, since the high-resolution structural information on Na,K-ATPase is not yet available. Nevertheless, the ATP binding site of Na,K-ATPase is probably very similar to the ATP binding site of the homologous Ca-ATPase, the crystal structure of which has been determined recently (*21*). The ATP binding site of Ca-ATPase is situated in a cleft, where electrostatic guidance for the diffusion and orientation of a charged substrate is expected to be very important. The binding pocket is positively charged and includes, among other residues, Lys515 and Lys492, that have been already identified as critical for binding of the negatively charged ATP (see ref *21* for discussion). Independently, previous structural characterization of the nucleoside 5′-di- and 5′-triphosphates by rotating-frame nuclear Overhauser effect

Table 1: Equilibrium Dissociation Constants and Dissociation Rate Constants for Some of the Ligands Interacting with the Nucleotide Binding Site of Na,K-ATPase

ligand	K_d, μM	k_{off}, s^{-1}
formycin triphosphate[a]	1	33
formycin diphosphate[a]	3.5	112
eosin[b]	0.25	21
5-carboxyeosin[b]	0.29	30
6-carboxyeosin[b]	0.11	7
ATP[c]	0.3	13
ADP[c]	0.9	27

[a] $I \sim 150$ mM; data from Table 4 (*28*). [b] $I \sim 40$ mM; data from (*22*). [c] $I \sim 150$ mM; data from Figure 2, this paper.

spectroscopy (ROESY) NMR techniques showed that whereas ADP exhibits significant conformational flexibility, AMPPCP and presumably ATP in solution predominantly adopt an *anti*-glucoside/adenine conformation with the phosphates lying parallel to the adenine ring (*25*); NMR experiments suggested a similar conformation for the nucleotide bound to Na,K-ATPase (*26*). The overall U-shape of ATP during its approach toward its binding site implies that the charge of the α-phosphate would be located close to the kink region, which might be important for the structural complementarity of the ligand molecule and the binding site of the enzyme.

Our direct measurements of the kinetic rate constants for dissociation of ATP and ADP from Na,K-ATPase are the first ones performed with this enzyme using a rapid filtration technique. The dissociation rates we measure with Na,K-ATPase fall in the same range as those previously obtained with Ca^{2+}-ATPase [k_{off} values of 9−25 s^{-1} at pH 7 and 5 °C, depending on the presence or absence of Ca^{2+} (*11*), or 20−25 s^{-1} at pH 6 and 20 °C, in the absence of Ca^{2+} and potassium (Orlowski and Champeil, unpublished results)]. When combined with the values of the corresponding equilibrium constants, the k_{off} values determined for Na,K-ATPase also allow estimation (eq 5) of the bimolecular rates of ATP binding (k_{on}): about 40×10^6 M^{-1} s^{-1} at $I = 127$ mM and about 4×10^6 M^{-1} s^{-1} at $I = 500$ mM. Note, that these calculated values for ATP association with Na,K-ATPase again have the same order of magnitude as the values previously determined for Ca^{2+}-ATPase at low ionic strength: $(3-7.5) \times 10^6$ M^{-1} s^{-1} at pH 7 and 5 °C, depending on the presence or absence of Ca^{2+} (*11*), and $(1.3-1.7) \times 10^6$ M^{-1} s^{-1} at pH 6 and 20 °C in the absence of Ca^{2+} and potassium (Orlowski and Champeil, unpublished results). The rate constants calculated for ATP binding to Na,K-ATPase are also consistent with those directly measured for the fluorescent ATP-analogue eosin [e.g., 31×10^6 M^{-1} s^{-1} at 6 °C and $I = 0.125$ M (*27*)]. All these association rate constants fall well below the values expected for diffusion-controlled reactions, which is consistent with the idea of ATP binding into a cleft for both enzymes.

Comparison of the ATP and ADP affinities for Na,K-ATPase and their dissociation rate constants suggests that the higher affinity of ATP is mainly due to a slower dissociation rate: for both ADP and ATP, dissociation rate constants are independent of the ionic strength in the range 100−600 mM, and the dissociation rate constant for ADP is about 2-fold larger than for ATP (Figure 2) while the affinities for ADP are on average 3.1-fold lower than for ATP. It can therefore be concluded (eq 5) that over the range of ionic strengths tested, the association rate constant for ATP is quite similar to that for ADP. The idea that the dissociation rate constant is a major factor determining affinity is also consistent with the results of experiments with formycin nucleotides (*28*) and eosin derivatives (*22*); see Table 1. Since the presence of γ-phosphate in the nucleotide molecule has a stabilizing effect on the enzyme−nucleotide complex, as reflected by the dissociation rate, it appears that specific interactions between the γ-phosphate of the nucleotide and the protein contribute significantly to the binding energy.

Conclusion. The presented observations lead to the following conclusions: in the case of Na,K-ATPase, Coulombic forces are important for the binding of nucleotides in the substrate binding site, which is positively charged. Nevertheless, the product of effective charges for protein−ligand interaction does not depend on the total charge of the ligand, and, therefore, the effective charge of the nucleotide is probably different from its total charge. In addition, short-range interactions make a major contribution to the specificity of protein−ligand interactions, and the affinity to the ligand at a given ionic strength is mainly determined by the dissociation rate constant (see Table 1). The conclusion of the present work—that the effective charge of the ligand, responsible for the electrostatic component in the binding reaction, is not necessarily equal to its total charge—is probably of general applicability in the description of the role of electrostatics in many cases of protein−ligand interactions.

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