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Nucleotide Binding to Na,K-ATPase: p*K* Values of the Groups Affecting the High Affinity Site†

Natalya U. Fedosova* and Mikael Esmann

Department of Biophysics, Institute of Physiology and Biophysics, University of Aarhus, Ole Worms Allé 1185, DK-8000 Aarhus C, Denmark

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ABSTRACT: Investigation of the ionic strength effect on the interactions between nucleotides (ATP and ADP) and Na,K-ATPase in a broad pH range was aimed at revealing p*K* values of the charged groups of the interacting species. Ionic strength experiments suggested that an amino acid residue with a p$K > 8.0$ is part of the protein binding site. A combination of equilibrium and transient experiments at various pH values allowed for the characterization of the groups electrostatically involved in either the association process (k_{on}) or the stability of the preformed complexes (k_{off}). Two groups ($pK_1 = 6.7$ and $pK_2 = 8.4$) appear to be important for the proper organization of the binding site and, therefore, the association reaction. Moreover, deprotonation of the basic group completely precludes association. pH dependencies of the dissociation rate constants for ATP and ADP are very different. An increase in pH from 5 to 9.5 induces a 9-fold *increase* in k_{off} for ATP, whereas k_{off} for ADP *decreases* 4-fold between pH 5 and 8, and decreases further in the alkaline region. A comparison of the pH dependencies for k_{off} for ATP and ADP suggests two effects: (1) at acidic pH, the value of the total negative charge of the nucleotide determines the tightness of binding; and (2) short-range interactions involving the terminal phosphate group are important for nucleotide dissociation from the site. The difference in the pH dependencies of k_{off} for the nucleotides suggests the existence of positive charges in close proximity to Asp369, relieving the repulsion between the γ-phosphate of ATP and Asp369.

Na,K-ATPase (Na-pump) is an integral protein of the plasma membrane that maintains the electrochemical gradients of Na^+ and K^+ at the expense of energy released by ATP hydrolysis. The minimal structural unit required for the pumping activity is the $\alpha\beta$-dimer. As every studied P-type ATPase does, the enzyme adopts two major conformations, E_1[1] and E_2, with very different functional characteristics and structural arrangements. Their interconversions underlie ion transport. The existing extensive body of literature explicitly describes functional properties of the pump, but the lack of high-resolution structural data hinders a detailed understanding of its transport mechanism. The observed effects are often interpreted in the frame of Ca-ATPase structure, as justified by the 30% amino acid sequence identity between the catalytic subunits of these proteins and pervasive similarities in the functional cycles.

Kinetics of the nucleotide binding reaction and the structural organization of the intracellular loop containing both nucleotide binding and phosphorylation domains have been subjects of intense investigations (*1*−*9*). Unfortunately, when isolated, the intracellular loop binds nucleotides with low affinity (*1*, *2*), the N-domain alone has even lower affinity (*3*), and the obtained high-resolution structures of the nucleotide binding domain either do not include bound nucleotide (*4*) or are related to a conformational state with low affinity to the substrate (*3*). Therefore, the mechanism of high affinity substrate binding remains unknown. Results of chemical modification studies presented a list of amino acid residues comprising the ATP-binding pocket, among them Lys480, Lys487, Lys501, Lys589, Lys605, Lys618, Lys622, Lys719 (see ref *5* for a review), together with Cys577 (*6*) and Gly502 (*7*). An analysis of the functional consequences of mutations within the putative nucleotide binding site suggested several amino acid residues to be in close contact with bound nucleotides, some of them exhibiting substrate-specific effects. Thus, the $Asp^{369}Ala$ mutation tremendously strengthens ATP affinity but has almost no effect on ADP binding (*8*). The results suggest electrostatic repulsion between Asp369 and the γ-phosphate of ATP. The positive charge of another amino acid residue, Arg544, appears to have an even more localized effect: its mutation to Gln decreases affinities to both ADP and ATP, whereas an $Arg^{544}Lys$ mutation decreased ADP affinity by a factor of 15, without any change in ATP affinity (*9*). The authors interpreted the observed effect in terms of orientation and distance of the positive charge on the amino acid residue relative to the negatively charged β- and γ-phosphate groups of the nucleotides.

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

[1] Abbreviations: E_1, the protein conformation of Na,K-ATPase predominant in Na^+-containing media; E_2, the protein conformation of Na,K-ATPase predominant in K^+-containing media; k_{on}, bimolecular binding rate constant; k_{off}, dissociation rate constant; K_d, equilibrium dissociation constant; z_E, z_A, charges of the protein binding site and ligand, respectively; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid.

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We have previously characterized the electrostatic component of the nucleotide−Na,K-ATPase interactions by studying the effect of ionic strength on the binding process. It revealed the importance of electrostatic forces for the interaction process and suggested (according to the Debye−Hückel theory) that the nucleotide binding site and its ligand have effective charges of opposite signs. We have also shown that the short-range interactions make a major contribution to the specificity of protein−ligand interaction, and the difference in affinities toward the ligands at a given ionic strength is mainly determined by the dissociation rate constant (*10*). The purpose of the present investigation is to reveal the p*K* values of the charged groups involved in the binding of the substrate and the groups involved in its retention in the binding site. The results characterize electrostatic interactions within the high affinity nucleotide binding site of Na,K-ATPase.

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 μmol per mg protein per min at 37 °C (see refs *11* and *12* for experimental details).

Nucleotide Binding Experiments. The techniques employed in the present study include both equilibrium measurements of radiolabeled nucleotide binding and transient kinetic measurements of the nucleotide dissociation rate (*10*). The experiments were all performed at 20 °C in 10 mM CDTA, 10 mM histidine (in the pH range 5.0−7.5), or 10 mM Tris (pH range 8.0−9.5). A wide range of ionic strengths was created by the addition of NaCl. The lowest concentration of Na^+ used was 50 mM, which together with contributions from the buffer components resulted in ionic strengths of about 0.08 M. (This value varies slightly between the Tris and histidine buffers, and the actual ionic strength was calculated from the composition of each binding medium.) We used [γ-^{32}P]ATP, [^{14}C]ATP, and [^{14}C]ADP with specific radioactivities of about $1.5 \cdot 10^{10}$ Bq/mmol for the [^{32}P]- and $2 \cdot 10^9$ Bq/mmol for the [^{14}C]-containing substrates.

Equilibrium Binding Experiments. Equilibrium binding of nucleotides was measured in double-labeling filtration experiments essentially as previously described (*10*). Na,K-ATPase (0.1 mg) in the above buffer was loaded on two stacked Millipore HAWP 0.45 μm filters; the enzyme remained adsorbed on the top filter, whereas the bottom one served as a control. Then the filters were manually perfused with a solution containing radiolabeled nucleotides, together with [^{3}H]glucose in the same buffer as that used for the enzyme, so that complete enzyme equilibration with nucleotide was achieved at all nucleotide concentrations. Then, without rinsing, filters were counted separately 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 also proportional to the amount of [^{3}H]glucose in the same filter.

The data were fitted to a hyperbolic function, and the resulting K_d values were subjected to further analysis.

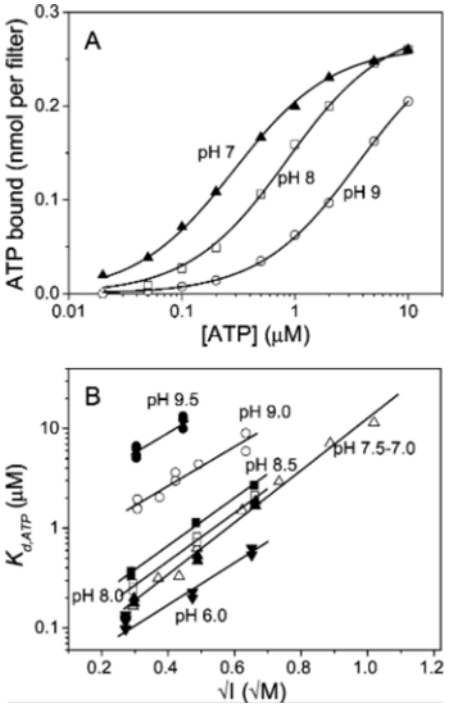

FIGURE 1: Ionic strength dependencies of the K_d value for ATP binding in the pH interval 6.0−9.5. (A) Equilibrium binding of ATP to Na,K-ATPase at pH 7, 8, and 9 in the presence of 202 mM Na^+. The full lines represent single hyperbolic functions with a maximal binding capacity of about 2.8 nmol/mg protein. (B) Obtained K_d values plotted as a function of ionic strength and fitted by eq 1. (●) pH 9.5; (○) pH 9.0; (■) pH 8.5; (□) pH 8.0; (▲) pH 7.5; (△) pH 7.0; and (▼) pH 6.0.

Time-Resolved Measurement of Nucleotide Dissociation Rates. To determine dissociation rates of the nucleotides, a rapid filtration system was used (RFS-4, Bio-logic, France) (*10*, *13*, *14*). In all cases, 0.1 mg of Na,K-ATPase was loaded onto a single filter. The filter with the adsorbed ATPase was perfused with buffer containing [^{3}H]glucose and radiolabeled nucleotide (at a concentration of 1 μM) as described above to reach equilibrium. Then the buffer was changed to a radioactivity-free buffer, supplemented with 0−0.05 mM non-radioactive nucleotide, and perfusion was continued for various periods (ranging between 30 ms and 2 s). The data were fitted to a single-exponential function, giving the value for the dissociation rate constant.

Curve fitting for nucleotide binding and dissociation was performed using Origin 6 software (Microcal, Amherst, CA).

RESULTS

Effect of pH on Equilibrium Binding of ATP and ADP. Debye−Hückel Analysis of Nucleotide−Enzyme Interactions. Equilibrium binding of nucleotides to Na,K-ATPase was studied at pH values from 6.0 to 9.5 over a large range of ionic strengths, created by various concentrations of NaCl (Figures 1and 2). Because Na^+ is necessary to induce high affinity nucleotide binding, its concentration must be sufficient for enzyme saturation at all pH values. We have previously shown that 25 mM NaCl gives the highest affinity at pH 7.0 (*15*). The lowest Na^+ concentration used in the present experiments was 50 mM, which together with the buffer components gives ionic strength values in the range of 0.07−0.08 M. In the analysis of the data, we assume that

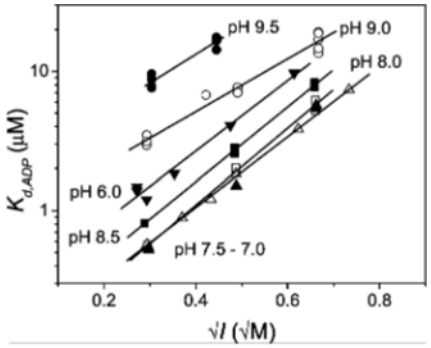

FIGURE 2: Ionic strength dependencies of the K_d for ADP binding in the pH interval 6.0−9.5. (●) pH 9.5; (○) pH 9.0; (■) pH 8.5; (□) pH 8.0; (▲) pH 7.5; (△) pH 7.0; and (▼) pH 6.0. The data were fitted by eq 1.

the Na^+ concentration is saturating over the whole range of pH and ionic strength because we observe increases in K_d with increases in ionic strength at all pH values. The K_d values presented do not exceed 15 μM at any pH, which is considered a limit for reliable determination by the method used.

The linear relationship between log K_d and $\times \bar{I}$ observed at all pH values (Figures 1B and 2) suggests an application of the limiting Debye−Hückel model (*16*), where

$$\log K_d = \log K_{d,0} - z_E z_A \cdot \times \bar{I} \qquad (1)$$

Visual analysis of the data leads to the following conclusions: (a) three ATP-binding experiments performed at 202 mM Na^+ (Figure 1A) show a clear tendency, K_d values increase upon alkalinization of the medium; (b) this holds for all ionic strengths (Figure 1B); (c) equivalent experiments with ADP as a binding ligand yield similar results (Figure 2), except for the fact that K_d at a given ionic strength does not increase monotonically with pH as in the case of ATP (compare Figures 1B and 2). The highest ADP affinities, that is, the lowest K_d values, are observed at pH 7−7.5, while binding becomes weaker at the extremes of the range, that is, pH 6 and pH > 8. Note, that under the same pH and ionic strength conditions the affinity of Na,K-ATPase for ADP is always lower than that for ATP.

Because the parameters in the Debye−Hückel equation (the charge product, $z_A z_E$, and the equilibrium dissociation constant at zero ionic strength, $K_{d,0}$) reflect different aspects of charge involvement in ligand binding, the pH dependencies of both parameters obtained upon data fitting are presented (Figure 3).

Variations in $K_{d,0}$ for ATP are small up to pH 7.5, but the values increase steeply as the medium becomes alkaline (Figure 3A). In contrast, $K_{d,0}$ for ADP decreases, reaching its minimum at pH 7.5, and then increases again, in parallel with that for ATP.

The charge product $z_A z_E$, that is, the slope of the line describing the relationship between logK_d and $\times \bar{I}$, varies between −2 and −2.8 for both nucleotides (Figure 3B). There appears to be little change in the charge product between pH 7.0 and □8.0, whereas a small decrease of its numerical value is observed at the alkaline part of the titration curve, correlating with the drastic increase in $K_{d,0}$ (Figure 3A).

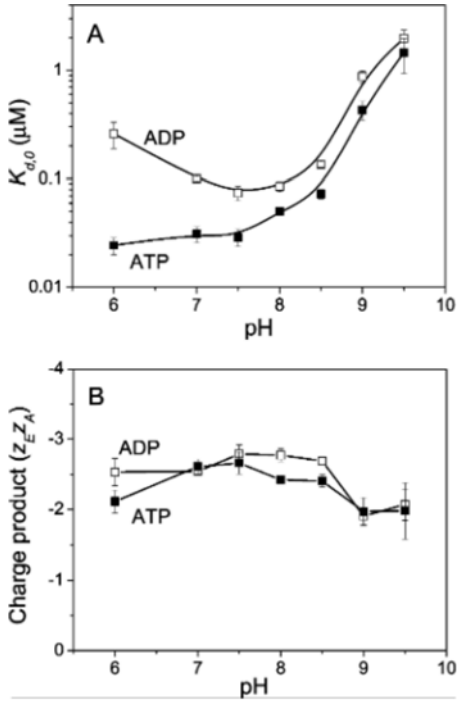

FIGURE 3: Debye−Hückel analysis of equilibrium binding of ATP and ADP. (A) pH dependence of the equilibrium binding constant at zero ionic strength ($K_{d,0}$) for ATP (■) and ADP (□). The data are extracted from Figures 1B and 2. (B) Charge product ($z_E z_A$) for ATP (■) and ADP (□), derived from the slopes of the data from Figures 1B and 2, as a function of pH.

Effect of pH on the Association and Dissociation Rate Constants for Nucleotides. The different pH sensitivities of $K_{d,0}$ for the two nucleotides initiated investigations of the association and dissociation rate constants forming the equilibrium dissociation constant. Nucleotide dissociation from Na,K-ATPase was studied using a rapid filtration technique. The dissociation rate constants for nucleotides were measured in the presence of 50 mM Na^+ in buffers in the pH range 5.0−9.5 (see examples in Figure 4A and B). Figure 4C presents k_{off} values for ATP, increasing approximately 9-fold from pH 5.0 to 9.5. In contrast to ATP, an increase in pH from pH 5.0 to 8.0 induces a 4-fold *decrease* in k_{off} for ADP (Figure 4C), and further alkalinization of the media leads to a further decrease in k_{off} for ADP. Figure 4D shows K_d for ATP and ADP at 50 mM Na^+ in the same pH range. (Note that the data at pH 6.0−9.5 are the averages of the K_d values at 50 mM Na^+, corresponding to the points at the lowest ionic strengths at each pH in Figures 1B and 2.) The pH dependence of the data in Figure 4D is similar to that for $K_{d,0}$ (Figure 3A) with values for K_d at 50 mM Na^+ about 6−8-fold higher than the values extrapolated to zero ionic strength (compare Figure 3A with Figure 4D).

The simultaneous determination of K_d and k_{off} allows for the calculation of the binding rate constant ($k_{on} = k_{off}/K_d$) for ATP and ADP (*17*). The pH dependencies of k_{on} for both nucleotides are bell-shaped, and maximum values for the association rate constants are observed between pH 7 and 8 (Figure 5). Deprotonation in the acidic part leads to a 2-fold increase in k_{on} for ATP and a similar increase in k_{on} for ADP, and deprotonation in the alkaline region causes a drastic

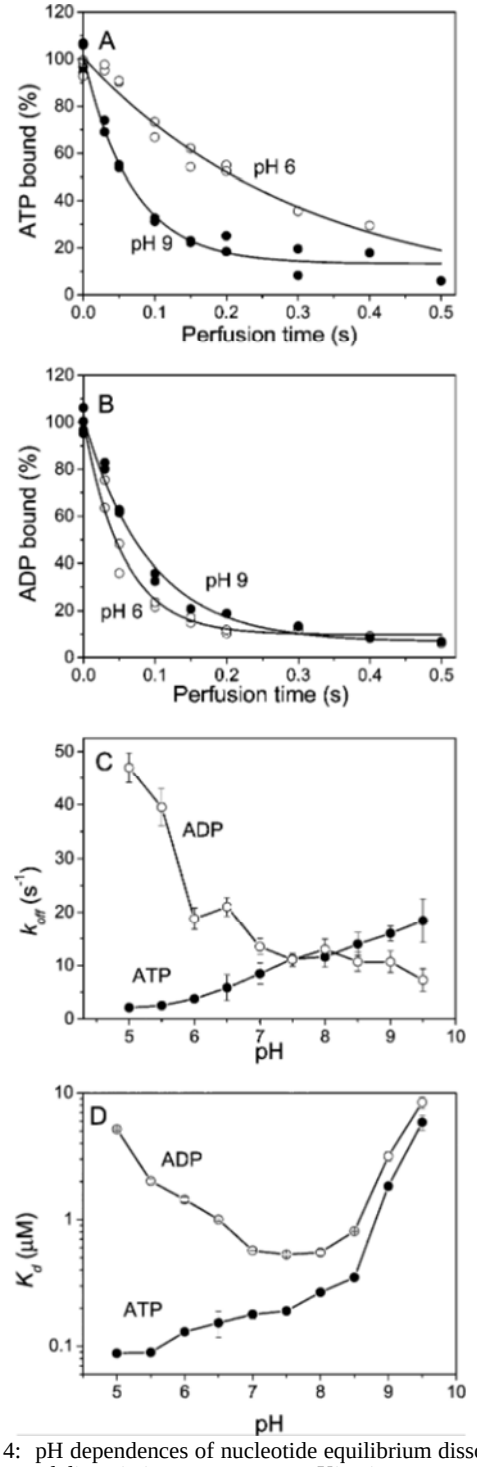

FIGURE 4: pH dependences of nucleotide equilibrium dissociation constants and dissociation rate constants. Kinetic measurements of the rates of dissociation of (A) enzyme−ATP or (B) enzyme−ADP complexes at pH 6.0 (○) and pH 9.0 (●). The amount of bound nucleotides is expressed as percent of their initial amount reached upon equilibration of the enzyme with 1 μM nucleotide. The curves are single-exponential fits of the experimental data. See Experimental Procedures for details. (C) pH effect on the dissociation rate constants for ATP (●) and ADP (○) at 50 mM Na^+. (D) pH effect on the equilibrium dissociation constants (K_d) for ATP (●) and ADP (○) at 50 mM Na^+. The data points represent the average of at least three experiments ± SE. The lines are drawn to guide the eye.

decline in k_{on}, precluding association reactions at pH > 9.5 for both nucleotides. The described changes in k_{on} were interpreted in terms of two independent deprotonation reactions.

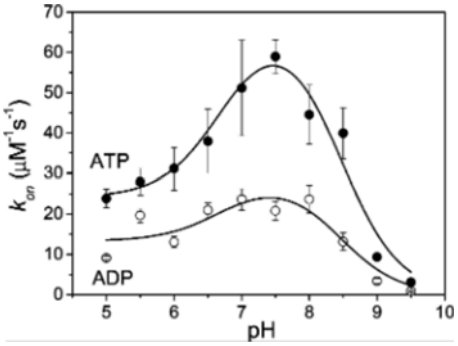

FIGURE 5: Association rate constants for ATP (●) and ADP (○) as a function of pH. The values are calculated from the data shown in Figure 4C and D, $k_{on} = k_{off}/K_d$. The full lines represent the best fit to eq 2. The parameters obtained for ATP are as follows: $k_1 = 24 \pm 4.2\ \mu M^{-1}\cdot s^{-1}$, $k_2 = 70 \pm 14\ \mu M^{-1}\cdot s^{-1}$, k_3 □0 $\mu M^{-1}\cdot s^{-1}$; pK_1 = 6.7; pK_2 = 8.4. Fitting of ADP data was performed with fixed pK values (pK_1 = 6.7; pK_2 = 8.4) and resulted in $k_1 = 13 \pm 2.0\ \mu M^{-1}\cdot s^{-1}$, $k_2 = 29 \pm 3.9\ \mu M^{-1}\cdot s^{-1}$, and k_3 □ 0 $\mu M^{-1}\cdot s^{-1}$.

The deprotonation reactions are represented by the equilibrium constants K_1 and K_2 ($K_1 = [EH^-][H^+]/[EHH]$, $K_2 = [E^{2-}][H^+]/[EH^-]$). The binding reaction is described by the following overall scheme.

$$\begin{array}{l} EHH + S \xrightarrow{k_1} EHH\cdot S \\ \updownarrow \\ EH^- + S \xrightarrow{k_2} EH^-\cdot S \\ \updownarrow \\ E^{2-} + S \xrightarrow{k_3} E^{2-}\cdot S \end{array}$$

Because $K_1 \gg K_2$, the dependencies of the observed k_{on} on pH for both nucleotides (Figure 5) were fitted to the following equation derived from Michaelis pH function theory (*18*):

$$k_{on} = \frac{k_1}{1 + 10^{pH-pK_1}} + \frac{k_2}{1 + 10^{pH-pK_2} + 10^{pK_1-pH}} + \frac{k_3}{1 + 10^{pK_2-pH}} \quad (2)$$

The best fit for ATP was obtained with the following parameters: pK_1 = 6.7, pK_2 = 8.4, $k_1 = 24\ \mu M^{-1}\cdot s^{-1}$, $k_2 = 70\ \mu M^{-1}\cdot s^{-1}$, and $k_3 = 0\ \mu M^{-1}\cdot s^{-1}$. Data from ADP experiments, although more scattered, were fitted using the two pK values determined from the ATP experiments (pK_1 = 6.7; pK_2 = 8.4). The ADP association reaction then was fitted by the following set of parameters: k_1= 13 $\mu M^{-1}\cdot s^{-1}$, $k_2 = 30\ \mu M^{-1}\cdot s^{-1}$, and $k_3 = 0\ \mu M^{-1}\cdot s^{-1}$.

DISCUSSION

The aim of the present investigation was to reveal pK values of the charged groups of the enzyme essential for nucleotide binding to Na,K-ATPase. Their importance may have different origins. For the binding reaction, their charge might be involved either in the electrostatic attraction of the charged nucleotide or in the proper organization of the (empty) ligand binding site, and these effects will thus be reflected in the pH dependence of k_{on}. For the dissociation reaction, the charges might be in direct contact or ensure

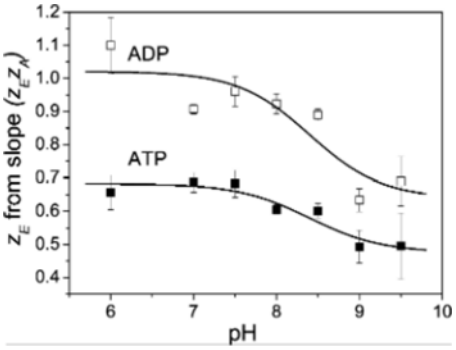

FIGURE 6: pH dependence of the charge on the enzyme implicated in nucleotide binding. The charge of the enzyme (z_E) is calculated from the charge product $z_E z_A$ (cf. Figure 3B) under the assumption that the nucleotides contribute to the charge product with their full charge at a given pH. The z_E values derived from ATP experiments are shown as filled squares and that from ADP-experiments with open squares. The full lines represent least-square fits of a deprotonation reaction with a fixed pK = 8.4, and the enzyme charge decreases from 0.68 (±0.02) to 0.48 (±0.02) for the ATP data and from 1.02 (±0.04) to 0.64 (±0.06) for the ADP data.

the proper organization of the interacting species in the bound state, thus affecting k_{off}. In order to estimate the contribution of these factors, we have studied and compared the ionic strength effects on enzyme affinity for two nucleotides at different pH values with direct pH effects on association and dissociation rate constants.

Data Analysis According to the Debye−Hückel Model. The linearity of the dependencies of logK_d for the nucleotides (ATP and ADP) on $\sqrt{I}$ observed at all pH tested (Figures 1B and 2) suggested the involvement of long-range electrostatic forces in the association process, according to the Debye−Hückel model. An analysis revealed a pattern identical for both nucleotides: a considerable increase in the equilibrium dissociation constants $K_{d,0}$ accompanied by a modest decrease in the numeric value of the charge product at pH values above 8.0 (Figure 3A and B). These changes appeared at the alkaline range of pH, where the ligands are already fully deprotonated, and their charges are constant ($z_{ADP} = -3$; $z_{ATP} = -4$) (*19*). The decrease of the numeric value of the charge product, that is, a decline in the ionic strength sensitivity of the association reaction, reflects a decrease in the positive charge of the binding site of the protein, probably because of the deprotonation of an amino acid residue with a pK above 8.0. The presence of this positive charge accelerates the association reaction (which has a maximum at pH 7 to 8; see below) and steers the negatively charged ligands into the binding site.

Another conclusion brought up by ionic strength experiments concerns the pH dependence of the charges of the interacting species. Deprotonation of ADP or ATP (i.e., the increase in their negative charges from pH 6 to 7) did not cause consistent changes in the values of the estimated charge product $z_E z_A$ in the range pH 6 to 7. For ATP, an increase of the numeric value was observed, and for ADP, the product remained constant. The pH dependences of the charge on the enzyme can be calculated under the assumption that the nucleotides contribute to the charge product with their total charges (Figure 6). The pH dependencies can be satisfactorily fitted using a pK of 8.4 (obtained separately from association rate constants; see Figure 5). The magnitude of the change in charge with pH is modest, about 0.2 charge units for ATP and 0.4 for ADP. At all pH values, z_E calculated from ADP experiments is larger than that from ATP data. This suggests that ADP experiences a larger enzyme charge during the association reaction than ATP does. We have suggested earlier that for the association reaction the effective charge of the nucleotide is lower than its total charge (*10*). The difference between z_E calculated from ADP and that from ATP data (Figure 6) points in the same direction.

pH Dependence of Association Rate Constants. The pH dependencies for $K_{d,0}$ in the acidic range differed considerably for ATP and ADP (Figure 3B) and required further investigations of association and dissociation processes. We have therefore measured both equilibrium dissociation constants (K_d) and dissociation rate constants (k_{off}) as functions of pH for both nucleotides (Figure 4) and used the results for the calculation of the association rate constants in a large pH range (Figure 5) (the ionic strength was kept at about 0.08 M). Thus, we separated the association reaction, influenced by electrostatic steering and conformational compatibility of the interacting species, from the dissociation reaction of the enzyme−ligand complex. As expected, binding reactions for both nucleotides showed the same pH dependence. They revealed pK values of two groups affecting interactions. They both were ascribed to the protein because (1) deprotonation of the first group in the acidic part has no common effect on the charge products for the interaction between nucleotides and Na,K-ATPase (Figure 3B); therefore, acceleration of the association reaction is not due to a direct electrostatic attraction but rather due to structural rearrangements or a better fit of the interacting species. Because the nucleotide conformation is hardly affected by deprotonation of the terminal phosphate group (occurring at pH 5−7.5), the position of the essential group was tentatively assigned to the active site of the protein. (2) The second essential group with a pK in the alkaline region undoubtedly belongs to the protein because the nucleotides are fully deprotonated in this pH range. One of the pK values (pK = 8.4) might be related to the charge involved in the long-range attraction, as revealed by a Debye−Hückel analysis of the ionic strength dependencies for the charge products (Figure 3B). A candidate for this effect could be Cys421, which for Na,K-ATPase is located close to the nucleotide site. In the structure of the crystallized N-domain (*4*), this residue is adjacent to Lys501, which in analogy with Ca-ATPase is thought to be part of the nucleotide binding domain, and in the NMR structure of the N-domain (*3*) with ATP bound (pdb entry 1MO8), Cys421 is in direct contact with the bound nucleotide. In addition, this group may, similar to the other group (pK = 6.7), be important for the proper conformational organization of the enzyme binding site.

There is an obvious difference in the calculated association rate constants for ATP and ADP, with k_{on} values for ATP approximately 2-fold higher than those for ADP (Figure 5). The collisions between Na,K-ATPase and ATP are more productive than those with ADP. A straightforward explanation could be that the larger negative charge of the ATP molecule promotes its interaction with the positively charged binding site. However, the calculated charge on the enzyme experienced by ATP is lower than that for ADP (cf. Figure

6), canceling out this effect. At present, we have no explanation for this difference in k_{on}.

pH Dependence of Dissociation Rate Constants. The information obtained from the pH dependencies for the dissociation rate constants is more difficult to interpret. The protonation states of the related groups influence the short-range interactions within the enzyme−ligand complex both directly (electrostatic attraction and repulsion) and indirectly (structural reorganization or induced fit), and at present, we cannot separate these effects. In the interpretation of these data, we assume that the main difference between ADP and ATP interactions with the enzyme is in the number of phosphate groups and hence the magnitude and distribution of the negative charge; the adenine and ribose groups are invariant between the two.

Measurements of the dissociation rate constants for the Na,K-ATPase−nucleotide complexes revealed a striking and most unexpected difference between ATP and ADP. At slightly alkaline pH □8, the dissociation rate constants were similar for both nucleotides, that is, preformed complexes between the enzyme and ATP (or ADP) were equally stable, and outside this range, changes in pH resulted in opposite effects. Thus, increase in pH from 5.0 to 7.5 induced a 3-fold decrease in k_{off} for ADP (Figure 4C), and further alkalinization of the media had little influence on k_{off} for this ligand. Because the interacting species (ADP and the binding site) have opposite charges, we suggest that the observed stabilization of the complex is due to an increase in the negative charge on ADP. The fact that at acidic pH the dissociation rate constant of the more negatively charged ATP is even slower than that of ADP corroborates this idea (Figure 4C). The increase of the total charge of ATP (from −3 at pH 5 to −4.0 at pH 8), however, has an opposite effect on dissociation, and we observe a clear *increase* in k_{off}. At the alkaline pH, the dissociation rate constant k_{off} for ATP surpasses that of ADP, that is, the ATP complex is destabilized by an increase in pH, whereas the ADP complex remains unaffected. We ascribe the last finding to the difference in the charge distribution between ATP and ADP due to the presence of the γ-phosphate. It was shown that the local charge of the γ-phosphate, being in close proximity to Asp369, plays a particular role in increasing electrostatic repulsion (*8*). The importance of this force for the catalytic cycle of Ca-ATPase was recently discussed (*20*). It is reasonable to suggest the existence of the positively charged group(s) in the surroundings of Asp369 aimed to diminish this electrostatic repulsion from γ-phosphate. In this case, increasing pH could lead to a titration of these groups (e.g., deprotonation of a suitably located lysine), which would intensify repulsion and specifically destabilize the complex with ATP at high pH.

Conclusions. Investigation of the electrostatic component of the interactions between nucleotides and Na,K-ATPase revealed that the binding process depends on amino acid residues with $pK_1 = 6.7$ and $pK_2 = 8.4$. Their protonation states are critical for the structural organization of the Na,K-ATPase binding site. Moreover, the charge of the residue with a slightly basic p*K* (a potential candidate could be Cys421) participates in the long-range electrostatic attraction of the nucleotides. In the bound state, the nucleotide establishes multiple contacts with the protein. Comparison of the pH dependencies for the dissociation rate constants of ADP and ATP from Na,K-ATPase suggests two effects: (1) at acidic pH, the total negative charge of the nucleotide determines the tightness of its binding, the larger charge correlates with lower dissociation rate constant; and (2) the contribution of the terminal phosphate group to the short-range interactions appears to be important for nucleotide dissociation from the site.

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