The Effect of Ionic Strength and Specific Anions on Substrate Binding and Hydrolytic Activities of Na,K-ATPase

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ABSTRACT The physiological ligands for Na,K-ATPase (the Na,K-pump) are ions, and electrostatic forces, that could be revealed by their ionic strength dependence, are therefore expected to be important for their reaction with the enzyme. We found that the affinities for ADP\textsuperscript{2-}, eosin\textsuperscript{-}, \(\rho\)-nitrophenylphosphate, and \(V_{\text{max}}\) for Na,K-ATPase and K\textsuperscript{+}-activated \(\rho\)-nitrophenylphosphatase activity, were all decreased by increasing salt concentration and by specific anions. Equilibrium binding of ADP was measured at 0–0.5 M of NaCl, NaSO\textsubscript{4}, and NaNO\textsubscript{3} and in 0.1 M Na-acetate, NaSCN, and NaClO\textsubscript{4}. The apparent affinity for ADP decreased up to 30 times. At equal ionic strength, \(I\), the ranking of the salt effect was NaCl \(\approx\) NaSO\textsubscript{4} \(\approx\) Na-acetate < NaNO\textsubscript{3} < NaSCN < NaClO\textsubscript{4}. We treated the influence of NaCl and NaSO\textsubscript{4} on \(K_{\text{m}}\) for E-ADP as a “pure” ionic strength effect. It is quantitatively simulated by a model where the binding site and ADP are point charges, and where their activity coefficients are related to \(I\) by the limiting law of Debye and Hückel. The estimated net charge at the binding site of the enzyme was about +1. Eosin binding followed the same model. The NO\textsubscript{3}\textsuperscript{-} effect was compatible with competitive binding of NO\textsubscript{3}\textsuperscript{-} and ADP in addition to the general \(I\)-effect. \(K_{\text{m}}\) for E-NO\textsubscript{3} was \(\sim\)32 mM. Analysis of \(V_{\text{max}}/K_{\text{m}}\) for Na,K-ATPase and K\textsuperscript{+}-\(\rho\)-nitrophenylphosphatase activity shows that electrostatic forces are important for the binding of \(\rho\)-nitrophenylphosphate but not for the catalytic effect of ATP on the low affinity site. The net charge at the \(\rho\)-nitrophenylphosphate-binding site was also about +1. The results reported here indicate that the reversible interactions between ions and Na,K-ATPase can be grouped according to either simple Debye-Hückel behavior or to specific anion or cation interactions with the enzyme.

KEY WORDS: Debye-Hückel theory • eosin binding • K\textsuperscript{+}-phosphatase activity • protein electrostatics • nucleotide binding

INTRODUCTION

Na,K-ATPase, identical to the Na,K-pump, catalyzes an Na\textsuperscript{+} + K\textsuperscript{+}-activated hydrolysis of ATP to ADP and inorganic phosphate, P\textsubscript{i}. In plasma membranes this is manifested in the coupling of the chemical hydrolysis of ATP to the vectorial transport of Na\textsuperscript{+} out of, and K\textsuperscript{+} into the cell.

The present paper focuses on the importance of electrostatic effects for Na,K-ATPase, as manifested by the enzyme’s response to changes in the salt concentration and to the anion composition of the medium surrounding the membrane bound enzyme. Usually experiments with Na,K-ATPase are performed using chloride salts of the cations Na\textsuperscript{+}, K\textsuperscript{+} and Mg\textsuperscript{2+}, and care is generally taken to keep the ionic strength constant as it is well known that changes in this parameter affects most of the biochemical reactions of the system. Given the ionic nature of the transport ligands Na\textsuperscript{+} and K\textsuperscript{+}, the substrates Mg\textsuperscript{2+} and ATP\textsuperscript{4-} and the products ADP\textsuperscript{3-} and HPO\textsubscript{4}\textsuperscript{2-}/H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-} it is highly probable that electrostatic phenomena play a significant role in the Na,K-ATPase reaction. Generally, electrostatic effects in proteins are important for structure as well as stability and biological function, and, especially in enzymes, they play a decisive role in substrate recognition and transition-state stabilization (Warshel and Russell, 1984; Matthew, 1985; Allewell and Oberoi, 1991). An important manifestation of electrostatic interactions is the ionic strength dependence (García-Moreno E, 1994), and by analogy with a large variety of other systems (see discussion for examples of enzymes; Record et al., 1978; McLaughlin, 1989; Green and Andersen, 1991) this can be exploited to shed light on structure and reaction mechanism. Surprisingly, however, no systematic investigation of ionic strength effects on Na,K-ATPase has been reported.

With regard to the mechanism of possible specific effects of anions (other than the substrate, the products and their analogues) only little attention has been given to this subject. Recently it has been found (Post and Suzuki, 1991; Klodos et al., 1994) that the type of anion in the medium was a determinant of the steady-state value of the ratio between the two phosphorylated intermediates, \(E_{1}/E_{2}\), during turnover. It was noted that the anions ranked like the Hofmeister lyotropic series but no further interpretation of the results was presented.

The present investigation of the ionic strength and anion dependence of the reactions of Na,K-ATPase was prompted by the observation that substitution of NO\textsubscript{3}-.
for Cl\(^-\) resulted in a dramatic decrease in the affinity of Na,K-ATPase for ADP and ATP at the substrate site (Rossi and Norby, 1993). Presumably the binding of the negatively charged ADP and ATP is assisted by attraction to positive charges at or around the binding site, and the inhibitory effect of NO\(_3\)\(^-\) could be a result of a neutralization of these charges. Characterization of the ionic strength dependence and the special inhibitory effect of NO\(_3\)\(^-\) (and other anions) could be useful in evaluating the events in both substrate binding and turnover.

Here we report first a detailed characterization of anion and ionic strength dependence of the equilibrium binding of ADP (and some eosin analogues) to the high affinity substrate site of Na,K-ATPase, and secondly we describe the effects of anions on the overall Na,K-ATPase as well as the K\(^+\)-activated p-nitrophenylphosphatase (K\(^+\)-pNPase)\(^1\) reactions. It was found that the binding affinity decreased with increasing ionic strength and was further decreased by replacement of Cl\(^-\) with NO\(_3\)\(^-\), SCN\(^-\), and ClO\(_4\)\(^-\), indicative of both unspecific electrostatic interactions and specific site-binding of anions. In the absence of information about the steric structure of the binding site and the electrostatic charges of Na,K-ATPase we have evaluated the ionic strength dependence of the ADP-binding by the simplest possible model. This considers the electrostatic properties of the binding site as those of a point charge, and we found that the interaction of this point charge with the charges on ADP or eosin could be quantified by the limiting law of Debye and Hückel (1923). The charge on the high-affinity site of Na,K-ATPase was calculated to +1. The Na,K-ATPase and K\(^+\)-pNPase activities were also inhibited by increasing ionic strength and specific anions. Quantitative evaluation by conventional kinetics and transition-state theory of the K\(^+\)-pNPase results was also compatible with a charge of +1 on the pNPP binding site, whereas there seemed to be little electrostatic interaction at the low affinity ATP-binding site.

**Materials and Methods**

**Enzyme Preparations**

Two types of membrane-bound Na,K-ATPase preparation from pig kidney outer medulla were used: EI, "zonal enzyme," V\(_{\text{max}}\) for Na,K-ATPase activity = 30–32 U · (mg protein)\(^{-1}\), was prepared according to Jørgensen (1974) by selective extraction of plasma membranes with SDS (sodiumdodecylsulphate) in the presence of ATP, followed by isopycnic zonal centrifugation. EIIL, "purified enzyme," V\(_{\text{max}}\) for Na,K-ATPase = 20–24 U · (mg protein)\(^{-1}\), also was prepared using the procedures of Jørgensen (1974) but without zonal centrifugation and with the SDS-activation modifications described by Jensen et al. (1984). Both preparations were stored at −20°C in 17.6 mM imidazole, 0.625 mM EDTA, and 250 mM sucrose, titrated to pH 7.4 with HCl, with 1.3 (EI) and 4 (EIIL) mg protein · ml\(^{-1}\), which is equal to an ADP-binding site concentration of about 4.3 μM (EI) and 10 μM (EIIL). The two preparations behaved identically and their properties were equal to those published earlier: The turnover, calculated from the B\(_{\text{max}}\) of ADP-binding (e.g., see Table I) and V\(_{\text{max}}\) is close to 9,000 min\(^{-1}\), which is the same as that of the best preparations of pig kidney enzyme as well as that found for less purified preparations (Jørgensen, 1974, 1988). Also, the K\(_{\text{m}}\) for ADP-binding is 0.27 μM at basal conditions (see Table I, experiment 1A at I = 78 mM) which agrees well with the value of 0.22–0.25 μM obtained under similar conditions (Jensen et al., 1984). Furthermore, the response of the two preparations to changing ionic strength and [NO\(_3\)\(^-\)] is the same (see Fig. 5).

Protein was measured (Lowry et al., 1951) with a standard of BSA.

**Equilibrium ADP-Binding**

These measurements were performed as described previously (Norby and Jensen, 1988; Jensen, 1992). The binding assays in Cl\(^-\) were done by mixing 4 ml enzyme-stock suspension with 5.7 ml of either H\(_2\)O or NaCl of the appropriate concentration, 1.2 ml buffer (88 mM imidazole, 1,250 mM sucrose, and 3.125 mM EDTA, titrated to pH 7.4 with HCl) and 0.865 ml 150 mM EDTA/imidazole, pH 7.4. Each assay consisted of 1,300 μl of the mixture, 195–0 μl 150 mM Tris/HCl, pH = 6.3, and 5–200 μl \(^{14}\)C-ADP (e.g., 40 μM) in 150 mM Tris/HCl, pH = 6.3.

After mixing, several 75-μl aliquots from each assay were transferred to counting vials for determination of total (T) radioactivity, and 1 ml was transferred to a cold centrifuge tube and centrifuged at 0–2°C for 60 min at 100,000 g. The tubes were cautiously removed, and after transfer of the supernatant to another tube, several 75-μl aliquots of this supernatant were pipetted to counting vials to determine unbound radioactivity (F). Bound radioactive ADP (B) was determined as B = T − F.

For binding experiments in NO\(_3\)\(^-\) or SO\(_4\)\(^{2-}\), 20 ml enzyme-stock suspension in Cl\(^-\) buffer was diluted with 80 ml ice-cold ISE buffer (17.6 mM imidazole, 250 mM sucrose, 0.625 mM EDTA, titrated to pH 7.4 with HNO\(_3\) or H\(_2\)SO\(_4\)), centrifuged 60 min at 60,000 g at 2°C, and the pellet was resuspended in 35 ml of the appropriate ISE buffer corresponding to an ADP-binding capacity of ~6.5 μM in the suspension. 4 ml of this suspension was mixed on ice with 5.7 ml of either H\(_2\)O, NaNO\(_3\), or Na\(_2\)SO\(_4\), pH 7.65–7.82 (SO\(_4\)\(^{2-}\)). Each assay consisted of 1,300 μl of this mixture, 195–0 μl 150 mM Tris/HNO\(_3\) or 100 mM Tris/H\(_2\)SO\(_4\) buffer, pH = 6.3, and 5–200 μl \(^{14}\)C-ADP (e.g., 40 μM) in 150 mM Tris/HNO\(_3\) or 100 mM Tris/H\(_2\)SO\(_4\) buffer. Determination of bound and free ADP was done as described for the experiments in Cl\(^-\). The binding experiments were performed at 0–2°C and the pH was 7.66–7.71 (Cl\(^-\) experiments), 7.69–7.74 (NO\(_3\)\(^-\)), and 7.65–7.82 (SO\(_4\)\(^{2-}\)).

**Calculation of Ionic Strength, I, for the ADP-Binding Experiments**

The ionic strength is I = \(1/2 \sum z_i \epsilon_i^2\), where \(\epsilon_i\) and \(z_i\) are the concentration and valence of the ith ion in the solution (Robinson and Stokes, 1970). To calculate I for the ADP-binding assay solutions, it is necessary to have appropriate pK\(_r\)-values for imidazoleH\(^+\), H\(_2\)EDTA\(^2-\), and TrisH\(^+\). The pK\(_r\)-values depend on the ionic strength of the solution, but since the buffers are only

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\(^1\)Abbreviations used in this paper: CDTA, trans-1,2-cyclohexylenedinitrilotetraacetic acid; K\(^+\)-pNPase, K\(^+\)-activated p-nitrophenylphosphatase; pNPP, p-nitrophenylphosphate.
dominating at relatively low ionic strength (before the addition of Na+ salts), we have chosen constants corresponding to I around 0.1 M. Thus, the following pKₐ values should be acceptable approximation: imidazoleH⁺, pKₐ = 7.5 (Perrin, 1965); H₂EDTA²⁻, pKₐ = 6.5 (Kortüm et al., 1961); TrisH⁺, pKₐ = 8.8 (Perrin, 1965). From the pH = 7.7 and the total concentrations of these three compounds, the approximate concentrations of the different charged species can be calculated: Common to all experiments: [imidazoleH⁺] ≈ 32.6 mM, [HEDTA²⁻] ≈ 9.4 mM, [H₂EDTA⁴⁻] ≈ 0.6 mM. In Cl⁻ and NO₃⁻ experiments: [TrisH⁺] = 18.5 mM, and in SO₄²⁻ assays: [TrisH⁺] = 12.3 mM. This gives a "basal" ionic strength = 78 mM in the binding experiments, and the concentration of Cl⁻, NO₃⁻, and SO₄²⁻ is 19, 19, and 6 mM, respectively.

**Na,K-ATPase Activity**

This activity was measured at 37°C at pH = 7.4 in 30 mM histidine/HCl buffer, with 10 pmol enzyme · ml⁻¹, 3 mM ATP, and 4 mM MgCl₂ (if not otherwise indicated), and the concentration and type of Na+ and K+ salts indicated in the figures. The basal ionic strength was 42 mM. The assay was started by addition of enzyme, stopped after 3 min with ice-cold TCA (trichloroacetic acid; final concentration 5%), and the amount of liberated phosphate was determined (Esmann, 1988). Blanks contained 1 mM ouabain, which was added before the enzyme.

**K⁺-dependent ρ-Nitrophenylphosphatase Activity**

This catalytic activity was measured at 37°C and pH = 7.4 in 30 mM histidine/HCl buffer with 10 pmol enzyme · ml⁻¹, 10 mM Na₂pNPP (if not otherwise indicated), 20 mM MgCl₂ and the concentration and type of K⁺ salts indicated in the figures. The basal ionic strength was 90 mM (including 10 mM Na₂pNPP). Reaction time was 3 min; ice-cold TCA (final concentration 5%) was added, and the reaction product ρ-nitrophenol determined as described (Esmann, 1988). The blank was a reagent-blank without enzyme.

**Fluorescence Measurement of Eosin- and 6- Carboxy eosin Binding**

The fluorescence of eosin or 6-carboxy eosin was measured at 20°C at pH = 7.0 with a Perkin-Elmer MFP 44A spectrofluorometer with two monochromators (Perkin-Elmer Cetus Instruments, Emeryville, CA) (Esmann, 1992). The excitation was at 530 nm, and the emission was monitored at 560 nm, both slits being 10 nm. The cuvette contained 0.2 nmol enzyme · ml⁻¹, 10 mM histidine/HCl buffer, 1 mM CDTA (trans,1,2-cyclohexylenedinitrilotetraacetic acid)/2- amino-2-methyl-1,3-propanediol, 20 mM NaCl, 0.5 mM eosin or 6-carboxy eosin and the Na⁺ salts indicated in Fig. 11. The basal ionic strength was 27 mM.

**Reagents and Processing of Data**

The reagents were of analytical grade. The various Na⁺ and K⁺ salts, H₂EDTA (titrplex II), CDTA, Na₂pNPP, and histidine were from Merck (Darmstadt, Germany); 2-amino-2-methyl-1,3-propanediol was from Fluka Chemie AG (Buchs, Switzerland); Tris-base and imidazole were from Sigma Chemical Co. (St. Louis, MO); sucrose was from British Drug House (Poole, UK); Na₂ATP was from Boehringer Mannheim Biochemicals (Indianapolis, IN); ouabain was from Serva Biochemicals (Parsum, NJ); eosin was from Hopkin and Williams (Chadwell Heath, UK), and 6-carboxy eosin was custom-synthesized by Molecular Probes, Inc. (Eugene, OR). I⁴-ADP from NEN (Boston, MA) was chromatographed on DEAE Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) and converted to Tris salt in 150 mM Tris/HCl or HNO₃ buffer or 100 mM Tris/H₂SO₄ buffer, pH 6.3 as described (Nørby and Jensen, 1971). The I⁴-ADP concentration was 40 μM and the specific activity was about 1.4 × 10⁶ Bq · μmol⁻¹.

Curvfitting and presentation of data was done with the Origin software, Microcal, Amherst, MA.

**Theory**

The influence of ionic strength, I, on the equilibrium dissociation constant. The dissociation of ADP from EADP (E is Na,K-ATPase)

\[
\text{EADP} \rightleftharpoons \text{E} + \text{ADP}
\]

...is characterized by the measured (apparent) equilibrium constant, Kₐₐ₃ₐ:

\[
K_{diss} = \frac{[E][ADP]}{[EADP]},
\]

(1)
calculated using the concentrations of unbound ADP, [ADP], bound ADP, [EADP], and nonoccupied enzyme binding sites, [E], in moles · liter⁻¹.

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**Table 1**

<table>
<thead>
<tr>
<th>Exp. [Cl⁻]</th>
<th>Bmax</th>
<th>Kdiss</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>A1 1</td>
<td>19</td>
<td>78</td>
</tr>
<tr>
<td>A2 1</td>
<td>176</td>
<td>1.20 ± 0.01</td>
</tr>
<tr>
<td>A3 6</td>
<td>278</td>
<td>1.20 ± 0.02</td>
</tr>
<tr>
<td>A4 6</td>
<td>378</td>
<td>1.13 ± 0.11</td>
</tr>
<tr>
<td>A5 543</td>
<td>453</td>
<td>1.29 ± 0.09</td>
</tr>
<tr>
<td>A6 6</td>
<td>453</td>
<td>1.29 ± 0.09</td>
</tr>
<tr>
<td>A7 519</td>
<td>578</td>
<td>0.98 ± 0.09</td>
</tr>
</tbody>
</table>

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**Table 2**

<table>
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<th>Exp. [SO₄²⁻]</th>
<th>Bmax</th>
<th>Kdiss</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>B1 1</td>
<td>6</td>
<td>78</td>
</tr>
<tr>
<td>B2 6</td>
<td>78</td>
<td>1.73 ± 0.02</td>
</tr>
<tr>
<td>B3 36</td>
<td>168</td>
<td>1.71 ± 0.02</td>
</tr>
<tr>
<td>B4 66</td>
<td>258</td>
<td>1.77 ± 0.03</td>
</tr>
<tr>
<td>B5 96</td>
<td>348</td>
<td>1.72 ± 0.06</td>
</tr>
<tr>
<td>B6 126</td>
<td>438</td>
<td>1.96 ± 0.10</td>
</tr>
<tr>
<td>B7 156</td>
<td>528</td>
<td>1.77 ± 0.08</td>
</tr>
</tbody>
</table>

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**Table 3**

<table>
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<tr>
<th>Exp. [NO₃⁻]</th>
<th>Bmax</th>
<th>Kdiss</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>C1</td>
<td>19</td>
<td>78</td>
</tr>
<tr>
<td>C2</td>
<td>24</td>
<td>83</td>
</tr>
<tr>
<td>C3</td>
<td>49</td>
<td>108</td>
</tr>
<tr>
<td>C4</td>
<td>79</td>
<td>138</td>
</tr>
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<td>C8</td>
<td>169</td>
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Values obtained by nonlinear regression are ± SE. Bmax and Kdiss (the latter calculated by nonlinear regression using a common Bmax in each series) were obtained from the data in Fig. 1, to which the numbers of the experiments also refer. The ionic strength before addition of the Na⁺ salts (experiments A1, B1, B2, and C1) was calculated as described under Materials and Methods.
According to experiment (and theory, see below), $K_{\text{diss}}$ may vary with the ionic strength, $I$, and this dependence can be evaluated by the introduction of the relation (Tanford, 1961; Robinson and Stokes, 1970)

$$a_i = c_i \cdot \gamma_i,$$

(2)

where $a_i$ is the $i$-independent activity, $c_i$ the molar concentration, and $\gamma_i$ the molar activity coefficient. By combining Eqs. 1 and 2 we get

$$K_{\text{diss}} = \frac{a_{E_{\text{ADP}}} \cdot a_{\text{ADP}}}{a_{\text{ADP}}} \cdot \frac{\gamma_{E_{\text{ADP}}}}{\gamma_{\text{ADP}}},$$

(3)

or

$$K_{\text{diss}} = K_0 \cdot \frac{\gamma_{E_{\text{ADP}}}}{\gamma_{\text{ADP}}},$$

(4)

Per definition (Johnson, 1960; Kirkwood and Oppenheim, 1961), the thermodynamic equilibrium constant, $K_0$, is thus independent of the ionic strength, and any influence of $I$ on $K_{\text{diss}}$ stems from the dependence of the ratio of activity coefficients on $I$.

Debye and Hückel (1923) derived a relationship between the individual activity coefficients for an ion, $\gamma_i$, the ionic strength, $I$, the ionic charge, $z_i$, and a number of physical constants and parameters, here condensed in the terms $A$ and $B$ (for details and assumptions in the derivation see Tanford, 1961, and Robinson and Stokes, 1970).

$$\log \gamma_i = -\frac{A \cdot z_i^2 \cdot e^{-I}}{1 + B \cdot a \cdot e^{-I}},$$

(5)

where $a$ is the distance (in cm) of closest approach of the ions. In water at 25°C, $a$ is 0.508 (1 m-1)1/2 and $B = 0.329 \times 10^8$ cm-1 (1 m-1)1/2. $A$ and $B$ are quite independent of temperature.

An expression describing the effect of ionic strength on the dissociation constant is now obtained by insertion of Eq. 6 into the logarithmically transformed Eq. 4

$$\log \frac{K_{\text{diss}}}{K_0} = -A \cdot (z_{E_{\text{ADP}}}^2 - z_{E_{\text{ADP}}}^2 - z_{\text{ADP}}^2) \cdot e^{-I},$$

(7)

and if we furthermore assume $z_{E_{\text{ADP}}} = z_{E_{\text{ADP}}} + z_{\text{ADP}}$ and set $A = 0.5 (1 \cdot \text{m}^{-1})^{1/2}$, see above, Eq. 7 reduces to

$$\log \frac{K_{\text{diss}}}{K_0} = -z_{E_{\text{ADP}}} \cdot z_{\text{ADP}} \cdot e^{-I}.$$

(8)

A similar equation is used to describe cosin-binding to Na,K-ATPase.

The effect of ionic strength on enzyme reactions. We have not found any general description or evaluation of this subject in the literature, although special features like substrate- and ligand-binding (including protons) as influenced by electrolyte concentration have been measured and treated theoretically (see discussion).

In the present work we have determined $V_{\text{max}}$ and $K_m$ ($K_0$) for ATP or pNPP for the Na,K-ATPase and K-`pNPPase activity in media of different ionic strength. To evaluate the observed changes in $V_{\text{max}}$ and $K_m$ we develop a simple model based on conventional enzyme kinetics (Cleland, 1963; Segel, 1975; Palmer, 1991) and transition-state theory for chemical and enzymatic reactions (Kraut, 1988). Kinetics of single-substrate, S, enzyme catalyzed reactions (or multiple substrate reactions where all but one substrate are saturating) follow the Michaelis-Menten equation

$$v_0 = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]}$$

(9)

where $v_0$ is the initial rate (mol · liter-1 · s-1) of formation of product, $V_{\text{max}}$ is $v_0$ for $[S] \rightarrow \infty$, and $K_m$ is a constant. Both $V_{\text{max}}$ and $K_m$ can be determined from measurements of $v_0$ as a function of $[S]$, e.g., Figs. 7 and 9.

We shall use a simple transition-state model as basis for the evaluation of the effect of ionic strength on the enzyme reactions:

$$E + S \rightleftharpoons (ES) \rightleftharpoons ES \rightleftharpoons (EP) \rightarrow E + P,$$

(10)

where $E$ is the enzyme, $S$ = substrate, $P$ = product, ES is the (stable) enzyme-substrate complex and (ES) and (EP) are transition-state complexes. Since $[P] = 0$, reaction 4 is considered irreversible. The total concentration of enzyme is


(11)

In transition-state theory (e.g., Segel, 1975; Kreevoy and Truhlar, 1986) the transition state is an activated complex through which the reactants must pass before proceeding to form products. Transition-state theory is based on two assumptions: equilibrium between the transition-state and the reactants, and the “dynamic bottleneck assumption” meaning that the reaction rate, $v_0$, is controlled by decomposition of the activated transition-state complex (Segel, 1975; Kreevoy and Truhlar, 1986; Kraut, 1988).

In accordance with Eq. 10 we have

$$v_0 = k_4 \cdot [(EP)]$$

(12)

where $k_4$ is independent of $I$, whereas [(EP)] need not be. Expressed in terms of the conventional (possibly independent) equilibrium constants corresponding to Eq. 10

$$v_0 = \frac{k_1K_1K_3K_4[E]_t}{1 + K_1 + K_2 + K_3K_4K_5}$$

(13)
from which, when \([S] \to \infty\)

\[
V_{\text{max}} = \frac{k_1 K_1 K_2 K_3 [E_{\text{ad}}]}{K_1 + K_2 K_3 + K_1 K_2 K_3},
\]  

(14)

and \(K_m\), see also Eq. 9,

\[
K_m = \frac{1}{K_1 + K_2 K_3 + K_1 K_2 K_3}.
\]  

(15)

As seen from Eqs. 14 and 15 and as pointed out by Plesner (1986), \(V_{\text{max}}\) and \(K_m\) in general are complicated functions containing all the rate constants in the reaction mechanism, whereas the ratio, \(V_{\text{max}} / K_m\), between these parameters is simpler. . . and contain only rate constants characterizing a) intermediates directly interacting with the appropriate substrate, b) the complex of the enzyme with the substrate in question or c) intermediates reversibly connected to them, if any . . .” When we form this ratio from Eqs. 14 and 15, we get

\[
V_{\text{max}} / K_m = k_4 \cdot K_1 K_2 K_3 [E_{\text{ad}}].
\]  

(16)

Now, since

\[
K_1 K_2 K_3 = \frac{([E_{\text{p}}])}{[E] \cdot [S]} = \frac{a}{[E_{\text{p}}]} \cdot \frac{\gamma_E \cdot \gamma_S}{\gamma_{(E_{\text{p}})}},
\]  

(17)

we obtain

\[
V_{\text{max}} / K_m = k_4 \cdot \frac{a}{[E_{\text{p}}]} \cdot [E_{\text{ad}}] \cdot \frac{\gamma_E \cdot \gamma_S}{\gamma_{(E_{\text{p}})}},
\]  

(18)

which converts to

\[
V_{\text{max}} / K_m = (V_{\text{max}} / K_m)_{0} \cdot \frac{\gamma_E \cdot \gamma_S}{\gamma_{(E_{\text{p}})}},
\]  

(19)

where \((V_{\text{max}} / K_m)_0\) is the “thermodynamic ratio,” independent of \(I\).

The calculated values for \((V_{\text{max}} / K_m)_0\) are evaluated using the same mathematical procedure as outlined for the dissociation constant \(K_{\text{diss}}\) (Eqs. 6–8). We assume that

\[
z_{(E_{\text{p}})} = z_E + z_S,
\]  

(20)

and apply the limiting law of Debye and Hückel, to get

\[
\log \left(\frac{V_{\text{max}} / K_m}{V_{\text{max}} / K_m}_0\right) = z_E \cdot z_S \cdot \sqrt{I}.
\]  

(21)

This means that, like the equilibrium measurements (Eq. 8), the kinetic measurements may be used for estimation of the charge at the site where the substrate (or ligand) interacts with the enzyme.

**RESULTS**

**Equilibrium Binding of ADP: The Effect of Ionic Strength and Type of Anion on the Dissociation Constant, \(K_{\text{diss}}\)**

The equilibrium binding of ADP to pig kidney Na,K-ATPase was characterized in three series of experiments where the ionic strength, \(I\), was varied by addition of NaCl, Na\(_2\)SO\(_4\), or NaNO\(_3\). The binding isotherms are shown in Fig. 1, where ADP-binding to the enzyme, \(E\), is described by a model in which \(E\) has only one ADP-binding site. The anion concentrations, the ionic strength, and the values for \(B_{\text{max}}\) and \(K_{\text{diss}}\) derived from Fig. 1 are given in Table I, which shows that the binding capacity, \(B_{\text{max}} (= [E\text{ADP}]_{\text{max}})\), is independent of \(I\). The only parameter that varies between the curves is the dissociation constant, \(K_{\text{diss}}\), for EADP. For all three salts \(K_{\text{diss}}\) increases dramatically with increasing \(I\).

A few ADP-binding experiments, in which 100 mM of some other Na\(^+\) salts were added to the basal medium, are shown as Scatchard plots in Fig. 2. Compared to Cl\(^-\), NO\(_3^-\) decreases the binding affinity and the decrease is even more pronounced with SCN\(^-\) and ClO\(_4^-\) whereas 100 mM Na-acetate has the same effect on \(K_{\text{diss}}\) as 100 mM NaCl (Fig. 2). For further illustration, the complete isotherm for 100 mM acetate is shown in Fig. 1A.

The relationship between \(K_{\text{diss}}\) for EADP and the ionic strength is illustrated in Table I and Fig. 3. In Cl\(^-\) and SO\(_4^{2-}\)-media, \(K_{\text{diss}}\) at a given ionic strength is about the same. It increases from 0.3 to 7 \(\mu\)M when \(I\) is increased from 0.075 to 0.52 M, whereas in NaNO\(_3\) the same rise in \(K_{\text{diss}}\) requires only addition of ~150 mM NaNO\(_3\). Since no salt has less effect on \(K_{\text{diss}}\) than NaCl, Na\(_2\)SO\(_4\), or Na-acetate when \(I\) is considered the independent variable, we ascribe the influence of these salts on \(K_{\text{diss}}\) to a “pure” ionic strength effect. Consequently, the response in the other salt media is considered a result of an ionic strength effect plus a specific interaction of the particular anion (in the binding experiments the cation is always Na\(^+\)) with the enzyme protein.

**Influence of \(I\) on \(K_{\text{diss}}\) of EADP**

The dissociation constants for EADP in Table I are apparent dissociation constants calculated using the concentrations of the species involved, cf. Eq. 1 in the Theory section. In that section we have evaluated the possible effects of ionic strength on \(K_{\text{diss}}\) using the Debye-Hückel theory for interaction between ions in solution (Eqs. 1–8). As mentioned above, we regard the effects of [NaCl] and [Na\(_2\)SO\(_4\)] on \(K_{\text{diss}}\) to be due solely to a change in \(I\), and in Fig. 3 we have explored this relationship by plotting log \(K_{\text{diss}}\) for the NaCl and Na\(_2\)SO\(_4\) experiments as a function of \(I\). It appears that straight lines fit the data perfectly. Interpretation of these data according to Eq. 8 determines the thermodynamic equilibrium constant \(K_0\) to be between 3 and 7 \(\times 10^{-5}\) M. The slope of the lines has the value of 2.72 and 3.25 (both with a SE of \(\pm 0.07\)), which is equal to \(-z_E \cdot z_{\text{ADP}}\).

Since \(z_{\text{ADP}}\) is \(-3\) this leads to a value for the charge of E (the enzyme site), \(z_E = 0.91\) (SO\(_4^{2-}\) data) or +1.08 (Cl\(^-\) data), both with SE = \(+0.02\).

**The Effect of NO\(_3^-\) on ADP-binding**

Assuming that the influence of the ionic strength per se is described by the NaCl- and Na\(_2\)SO\(_4\)-experiments, we have analyzed the more pronounced effect of NaNO\(_3\).
on $K_{\text{diss}}$ (Table I) using the simple model shown in Fig. 4. If the simultaneous occupancy with $\text{NO}_3^-$ and ADP is prohibited, i.e., if $\text{ENO}_3\text{ADP}$ does not exist, the model corresponds to competitive inhibition of ADP-binding by $\text{NO}_3^-$. $K_{\text{A}}$, the dissociation constant for $E\text{ADP}$ in the absence of $\text{NO}_3^-$, varies with $I$. This variation contributes to the variation of $K_{\text{diss}}$ in the presence of $\text{NO}_3^-$, and we therefore use the ratio $K_{\text{diss}}/K_{\text{A}}$, where $K_{\text{diss}}$ and $K_{\text{A}}$ are measured at the same ionic strength, to illustrate the specific effect of $\text{NO}_3^-$. Fig. 5 is a plot of $K_{\text{diss}}/K_{\text{A}}$ for two series of experiments: one in which the data were calculated (closed symbols) from Table I and where $I$ varies from about 78 to 228 mM, and another (open circles) where the ionic strength was kept constant at 178 mM (binding isotherms not shown).

The data in Fig. 5 fall on a straight line with a slope of 0.031 mM$^{-1}$. For these data to be described by the same model in Fig. 4, $K_{\text{AN}}$ must be much larger than the highest value of $[\text{NO}_3^-]$ used in these experiments, i.e., $K_{\text{AN}} \gg 150$ mM. In this case we can obtain a value for the dissociation constant of $E\text{NO}_3$, $K_{\text{N}}$, which is the reciprocal slope: $K_{\text{N}} \approx 32$ mM. Using this value for $K_{\text{N}}$ we can estimate a value for $K_{\text{AN}}$ (at $I = 0.178$ M) from the relation $K_{\text{N}} \cdot K_{\text{NA}} = K_{\text{A}} \cdot K_{\text{AN}}$ by insertion of $K_{\text{A}} = 6 \times 10^{-7}$ M (Table I) and $K_{\text{NA}} = 10^{-4}$ M (this is a minimum estimate of the $E\text{ADP}$ dissociation constant made at high $[\text{NO}_3^-]$; Rossi and Nørby, 1993). The result is that $K_{\text{AN}} \approx 5$ M. These considerations do not exclude simultaneous binding of $\text{NO}_3^-$ and ADP to the enzyme, but they strongly suggest that the affinity of EADP for $\text{NO}_3^-$ is manyfold smaller than the affinity of E for $\text{NO}_3^-$, indicating that $\text{NO}_3^-$-binding is close to being competitive.

The Influence of Different Anions and of the Ionic Strength on the Na,K-ATPase Activity and the K$^+$-pNPPase Activity of Na,K-ATPase

Na,K-ATPase activity. The effect of anions and salt concentration on the rate of ATP-hydrolysis was characterized in several series of experiments. In one, the results of which are given in Fig. 6 with closed symbols, different Na$^+$ salts were added to a medium with constant [KCl] = 20 mM, 3 mM ATP and 4 mM MgCl$_2$. With all salts there is an increase in the activity at low salt concentration (due to the activation by Na$^+$) followed by a
Nørby and Esmann

...rate of hydrolysis with increasing salt concentration. With NaNO₃ and NaClO₄ the inhibition is more pronounced than with NaCl, as was the case when ADP-binding was measured. The Na₂SO₄-curve follows the NaCl curve when [Na] is the independent variable, whereas when the activity is plotted versus I (not shown), the inhibitory effect of Na₂SO₄ is 10–30% less than that of NaCl. Experiments with Na-acetate up to 800 mM (not shown) gave the same results as with NaCl.

Figure 2. Normalized Scatchard plots of ADP-binding to Na,K-ATPase in media with 19 mM Cl⁻ + 100 mM Na⁺ salts of the anions indicated. The ionic strength, I, was 178 mM in all experiments. The complete isotherms for Cl⁻ and CH₃COO⁻ (acetate) are given in Fig. 1A. In these experiments the same enzyme concentration was used throughout. The calculated values for K₉₃ (μM) ± SE are: 0.640 ± 0.007 (Cl⁻); 0.635 ± 0.004 (acetate); 3.24 ± 0.05 (NO₃⁻); 18.3 ± 0.2 (SCN⁻); 38.6 ± 0.70 (ClO₄⁻).

Figure 3. The values for K₉₃ for the experiments in Cl⁻ (■) or SO₄²⁻ (▲), at varying ionic strength, I, are taken from Table I, and here plotted as log(K₉₃/μM) versus I in order to investigate the Debye-Hückel relation (see Theory, Eq. 8 and the text). The lines were calculated by nonweighted, linear regression. The ordinate intercept corresponds to the thermodynamic equilibrium constant, K₀ = 0.070 ± 0.005 μM (in SO₄²⁻) and 0.031 ± 0.003 μM (in Cl⁻). The slope ± SE of the lines is 2.72 ± 0.07 M⁻¹/² (in SO₄²⁻) and 3.25 ± 0.07 M⁻¹/² (in Cl⁻).

Figure 4. A simple model for the effect of NO₃⁻ on ADP-binding. Kₙ and K₉₃ are the apparent EADP-dissociation constants in the absence and presence of NO₃⁻.

\[
\frac{K_{\text{diss}}}{K_A} = \frac{([E] + [E \cdot \text{NO}_3^-]) [\text{ADP}]}{[E \cdot \text{ADP}] + [E \cdot \text{NO}_3^-] \cdot \text{ADP}}
\]

If Kₙ ≫ [NO₃⁻], then: K₉₃/Kₙ = 1 + [NO₃⁻]/Kₙ.

Figure 5. The effect of NO₃⁻ on the binding of ADP, analyzed according to the model in Fig. 4. Kₙ and K₉₃ are the apparent EADP-dissociation constants in the absence and presence of NO₃⁻, respectively. Two series of data are shown: (●) are data taken from Table I, where K₉₃ in NO₃⁻ is divided by a corresponding (same I) Kₙ-value in Cl⁻ medium. In these experiments I varies from 78 to 228 mM. The open circles (○) are from a set of experiments (not shown, see text) where I was constant = 178 mM, where [Cl⁻] + [NO₃⁻] = 119 mM, and where [NO₃⁻] was varied from 0 to 100 mM. The line is drawn through (0.1) with a slope ± SE of 0.0399 ± 0.0006 M⁻¹. From the analysis in Fig. 4, this corresponds to a dissociation constant, Kₙ, for ENO₃ equal to ≈32 mM.
The reversibility of the salt effect was demonstrated by first suspending the enzyme in 2 M NaCl or 0.5 M NaNO₃ and thereafter removing the salt by centrifugation. After resuspension of the pellet in the normal storage medium, the Na,K-ATPase activity was the same as before the salt treatment.

A second series of Na,K-ATPase measurements addressed the problem of whether increasing the concentration of Na⁺ (relative to K⁺) is a decisive factor in the decline in activity. Hypothetically this could occur because of competition between Na⁺ and K⁺ for the activating, extracellular K⁺ site. We therefore varied the salt concentration like in the first series, but kept the ratio [NaCl]/[KCl] = 8 (the ratio at optimal activity, i.e., 160 mM NaCl/20 mM KCl). From Fig. 6 (open symbols) it is seen that at any salt concentration (Cl⁻ salts) above I = 0.2 M, the Na,K-ATPase activity is the same whether the [NaCl]/[KCl] ratio is constant = 8 or whether it increases from 8 at optimal activity to 40 at 0.8 M salt. It therefore appears that specific, competitive inhibition by Na⁺ can be ruled out in the experiments of Figs. 6 and 7.

To explore the possibility that a drastic increase in $K_m$ for ATP with increasing salt concentration could contribute to the observed fall in activity, $K_m$ for ATP for the Na,K-ATPase activity was measured with 20 mM KCl, [Mg₅₀] = [ATP] + 1 mM and 0.16–0.75 M NaCl (Fig. 7). As seen from the inset on Fig. 7 there is a slight decrease in $K_m$ with increasing I, so lack of ATP cannot contribute to the fall in activity (Fig. 6, and $V_{max}$ on the inset of Fig. 7). Similar experiments in 0.2 M NaClO₄ (not shown) likewise revealed no effect on activity of an increase in [ATP] from 3 to 10 mM.

According to the model in the Theory section regarding the effect of ionic strength on enzyme kinetics, it might be possible, from the slope of a plot of log($V_{max}/K_m$) versus $\sqrt{I}$ (Eq. 21), to estimate the charge of the low-affinity ATP-binding site ($z_q$). Such a plot, using the values from the inset of Fig. 7, is shown on Fig. 10. The value of the slope, which is equal to $2z_qK_{ATP}$, is determined by linear regression to $-0.50 \pm 0.05$. Since $z_qK_{ATP}$ is $-4$, $z_q$ becomes $+0.13 \pm 0.01$.

$K^+$-activated pNPPase activity. Fig. 8 shows the value for this activity as a function of the concentration of different K⁺ salts. After the initial activation by K⁺, further addition of salt in all cases leads to a lower pNPPase activity. In contrast to what is seen with ADP binding and Na,K-ATPase activity, NO₃⁻ does not have any special effect. The similarity between the effect of KCl and KNO₃ is also found when the activity is measured as a function of I at a higher [pNPP] = 25 mM (not shown). When the SO₄²⁻ data are related to the ionic strength (rather than to [K⁺]), the apparent special effect of SO₄²⁻ also disappears.

Under the conditions of the assay for the data of Fig. 8 ([pNPP] = 10 mM) the enzyme is only 70–80% saturated with substrate when the salt concentration is 0.05 M or lower, since $K_m$ for pNPP is 2–3 mM at low ionic strength (Skou, 1974; Campos et al., 1988; Fig. 9 in this paper). To investigate whether the inhibition of the K⁺-pNPPase activity at high ionic strength could result from an increase in $K_m$ for pNPP (substrate desaturat-
function), we measured the pNPPase activity as a function of [pNPP] in 0.05–0.6 M KCl. It was found (Fig. 9, inset) that $K_m$ increased up to 10–12 mM with increasing [KCl]. The consequence is, that in 0.5 M KCl, $V_{max}$ would be twice the activity measured with 10 mM pNPP (Fig. 8). Substrate desaturation therefore contributes significantly to the decrease in activity with increasing [KCl]. The special ClO$_4^-$ effect (relative to that of Cl$^-$) is not an effect on $K_m$ in an experiment (not shown) similar to that in Fig. 9, with varying [pNPP] and with 0.125 M Cl$^-$ or 0.125 M ClO$_4^-$: we found $K_{m,5}$ to be similar, namely about 4.5 mM in 0.125 M Cl$^-$ and 5.5 mM in 0.125 M ClO$_4^-$.

Like for the Na,K-ATPase activity, the effect of I on the K$^+$-pNPPase activity was quantified using the ratio $V_{max}/K_m$ as outlined in the Theory section (Eq. 21). For the experiments with KCl, the set of 7 directly determined $K_m$ and $V_{max}$ values shown on the inset of Fig. 9 was used. In the case of the K$_2$SO$_4$ experiments, the $K_m$ values of Fig. 9 and the $V_j$ data from Fig. 8 were used to calculate $V_{max}$ (Eq. 9). On Fig. 10, log($V_{max}/K_m$) for K$^+$-pNPPase is plotted as a function of $I$. According to Eq. 21 the slope of such a plot is the product of the charges at the binding site and of the substrate,  $z_{pNPP}$. The values for the slope ± SE are $-1.68 ± 0.06$ (in SO$_4^{2-}$) and $-2.11 ± 0.08$ (in Cl$^-$) which, since $z_{pNPP} = -2$, estimates $z_2$ to $+0.84 ± 0.03$ (in SO$_4^{2-}$) and $+1.06 ± 0.04$ (Cl$^-$), i.e., the same values as obtained in the ADP-binding experiments.

![Figure 8. K$^+$-p-nitrophenylphosphatase (K$^+$-pNPPase) activity of Na,K-ATPase measured with 10 mM Na$_2$pNPP, 20 mM MgCl$_2$, and the concentrations of KCl (●), KNO$_3$ (●), K$_2$SO$_4$ (●), and KClO$_4$ (○) shown. The temperature was 37°C, pH was 7.4, and activity = 100 corresponds to 5 U·mg$^{-1}$. The data are plotted versus the K$^+$-concentration. The ionic strength, $I$, before addition of K$^+$ salts was 0.090 M, see materials and methods. In each particular experiment with Cl$^-$, NO$_3^-$, or ClO$_4^-$, $I$ (M) is thus $[K^+] + 0.09$. For the SO$_4^{2-}$-data, $I = 1.5·[K^+] + 0.09$. Data are average of three to four experiments and the relative SE was <66%.](image)

![Figure 9. The K$^+$-pNPPase activity as a function of [Na$_2$pNPP] at, from top to bottom, 0.05 M (●), 0.125 M (●), 0.2 M (●), 0.3 M (●), or 0.4 M KCl (●). Conditions were otherwise as in Fig. 8 and activity = 100 corresponds to 5 U·mg$^{-1}$. Data are average of two to four experiments, and the error bars give the SE. Since [Na$^+$] increases with [Na$_2$pNPP] the inhibition seen at high [Na$_2$pNPP] for the experiment at 0.05 M KCl is expected (Skou, 1974). A simple model taking this Na$^+$/K$^+$ competition into account:](image)
The present work concerns the influence of various salts on equilibrium binding of ligands (ADP, eosin and 6-carboxyeosin) to Na,K-ATPase as well as on two hydrolytic activities of the enzyme, Na,K-ATPase activity and the K⁺-pNPPase activity. We observed a dramatic decrease in affinity for the ligands as well as an inhibition of both of the enzymatic activities with increasing salt concentration. In a theoretical paper Record et al. (1978) have listed five potential origins for such salt effects: (1) differential cation binding; (2) differential anion binding; (3) differential hydration (at high electrolyte concentration); (4) differential screening (Debye-Hückel) effects of electrolyte on the macromolecular charges, reflected in a variation of the macromolecular activity coefficient; (5) effects of electrolyte on the activity coefficient of the ligand. Of these items, No. 3 will not be discussed in any detail since we have no relevant results in this work. The concept of “Hofmeister series” of anions will be briefly discussed later.

The salts used in this work are Na⁺ salts or K⁺ salts. These cations are the natural activators of the enzyme, and they influence the nucleotide binding profoundly. The enzyme binds ATP, ADP and eosin with only low affinity at very low ionic strength (no Na⁺ or “Na⁺-like” buffers), or in the presence of K⁺ salts (Skou and Esman, 1981; Glynn and Richards, 1982; Jensen and Ottolenghi, 1983b). When the ionic strength, I, is increased somewhat either by buffers or Na⁺ salts, the enzyme system changes to a Na⁺-form (E₁-form) that displays a high affinity (e.g., $K_{\text{dis}} < 1 \mu M$ at $I < 0.1$ M) for ATP, ADP and the eosins (Nørby and Jensen, 1971;
Hegyvary and Post, 1971; Jørgensen, 1975; Skou and Esman, 1981). This effect of increasing \( I \), as well as the specific effects of \( Na^+ \) and \( K^+ \), all saturate at relatively low salt concentrations. There is no experimental evidence that increasing the \( Na^+ \) concentration changes the enzyme from a high-affinity nucleotide-binding \( E_1^- \) form to a low-affinity form \( (E_2^-) \). Likewise, results with the pNPPase reaction at higher \( K^+ \) concentrations do not reflect a conformational change from the \( E_2^- \) form to the \( E_2^- \) form. Thus, as will be substantiated below, we believe that can rule out differential cation binding (item No. 1 of the list above) as a cause for the observed inhibitions by increasing salt concentration.

It is possible that effects of increasing [salt] are mediated by differential anion binding (item No. 2 of Record et al.’s list). In this regard our experiments seem to fall into two groups. At equal ionic strength, the results with \( Cl^- \), \( SO_4^{2-} \) and acetate are comparable in that they show the smallest inhibition of ADP- and eosin-binding (Figs. 1, A and B, 2, and 11), Na,K-ATPase activity (Fig. 6) and pNPPase activity (Fig. 8), whereas the \( NO_3^- \), \( SCN^- \), and \( ClO_4^- \) salts show progressively larger effects, the lack of a special inhibition of \( K^+ \)-pNPPase by \( NO_3^- \) being an exception. We therefore hypothesize that the observations with \( Cl^- \), \( SO_4^{2-} \) and acetate salts are related to electrostatic events alone, and that the more pronounced responses obtained with the other anions reflect additional specific anion-binding, and perhaps other properties of strong electrolytes.

Ionic Strength, \( I \), and Equilibrium, High-Affinity Binding of ADP, Eosin, and 6-Carboxyeosin to the Substrate Site of Na,K-ATPase

Increasing the concentrations of NaCl, \( Na_2SO_4 \), and \( Na\)-acetate led to a marked fall in the affinity of Na,K-ATPase for ADP, eosin and 6-carboxyeosin (Figs. 1, A and B, 3, 11 and Table 1). Comparison of the results for ADP (charge \(-3\)) and eosin (charge \(-2\)) following the procedure outlined in the Theory section, Eqs. 1–8, shows that the larger the charge on the binding ligand, the more pronounced is the effect of the salt concentration on binding. This supports the theory that electrostatic interactions are important in this process.

There are few published data that allow a judgement of the influence of \( I \) on the binding of ligands to the substrate site of Na,K-ATPase, but they all show the same qualitative trend as here. Norby and Jensen (1974) reported that increasing \( I \) from 0.08 to 0.12 M with Tris-buffer increased \( K_{\text{diss}} \) for EATP by a factor of 1.5, and the same can be deduced from comparison of the results in Ottolenghi and Jensen (1983) and Jensen and Ottolenghi (1983a) where \( I \) was changed with \( NaCl \). A very pronounced inhibition of eosin binding by increased concentrations of choline-Cl or NaCl up to 0.45 M was demonstrated by Skou and Esman (1980, 1981). Like our experiments with NaCl and \( Na_2SO_4 \), where \([Na^+]\) in the latter is only two-thirds of that in NaCl at equal ionic strength, these studies support that the important parameter is the ionic strength rather than the type of cation used.

The high-affinity ATP-binding site is located in the large cytoplasmic loop of the \( \alpha \)-subunit, presumably in a polar milieu, and several charged amino acid residues (see reviews by Kaplan, 1991; Lingrel and Kuntzweiler, 1994) have been implicated as part of this site. The picture in Kaplan (1991) for example shows 7 lysines (2 of which may not be essential for ATP-interactions [Lingrel and Kuntzweiler, 1994]), 1 arginine, and 3 aspartates. The importance of a net positive charge for substrate binding was already suggested by the pH profile of \( K_{\text{diss}} \) for EATP (Hegyvary and Post, 1971): \( K_{\text{diss}} \) was low and constant for \( pH = 5 \) to 7, but it increased sharply when \( pH \) was raised from 7.5 to 9. This must reflect the removal of a positive charge or the creation of a negative charge. The importance of electrostatic interaction for ATP-binding is likewise illustrated by the finding that substitution of Lys480 to Arg or Ala had little effect on \( K_{\text{diss}} \) but in the mutant Lys480Glu the affinity was reduced \(~10\)-fold [Wang and Farley, 1992]. The negatively charged Asp369 is a crucial residue at the catalytic site of the ATPase, since this is the residue phosphorylated by the \( \gamma \)-phosphate of ATP during turnover. During binding of the negatively charged ATP this residue must exert a repulsive force, and this is convincingly demonstrated in a recent work (Pedersen et al., 1996) where substitution of Asp369 with uncharged Asn or Ala resulted in a 20–30-fold increase in the affinity for ATP at the high-affinity site. At the same time the catalytic activity was completely lost.

In the evaluation of the effect of ionic strength on ADP and eosin binding we model the substrate site as a point charge and use the Debye-Hückel limiting law for the relation between the activity coefficient and ionic strength (Eq. 6). The validity of these assumptions is discussed in the next section. The analysis (Figs. 3 and 11) leads to the estimate that the net charge of the high-affinity binding site (the substrate site of Na,K-ATPase) is \(+0.9\) to \(+1.1\) (ADP-binding experiments) and about \(+0.7\) (eosin and carboxyeosin experiments).

The Applicability of the “Point Charge” Assumption for the Binding Site and the Limiting Law of Debye-Hückel to Evaluate the Ionic Strength Effects

There are two main principles in the derivation of models for the electrostatics of proteins: the macroscopic, continuum model, and the microscopic, all atoms model (Warshel and Russell, 1984; Matthew, 1985; Allewell and Oberoi, 1991; Nakamura, 1996). The microscopic model depend on x-ray structural information. Unfortunately no x-ray data are available for Na,K-
ATPase, and we have therefore used the macroscopic approach.

In both types of model it is essential to know the roles of the different charges on the protein. For the macroscopic model, the two extremes are: does the charge on the whole protein or the charge only in the microenvironment where the reaction takes place (the binding site or cleft) determine the direction and magnitude of the salt effects? The latter view, which we here call the “point charge assumption for the binding site,” has considerable support in previous studies in which it was demonstrated that the local charges at the site of reaction are much more important than distant charged groups, the effect of which furthermore dramatically decrease with increasing I (Alberty and Hammes, 1958; Hammes and Alberty, 1959; Snyder et al., 1981; Loewenthal et al., 1993). The selective importance of local electrostatic attraction is also underscored for superoxide dismutase by Getzoff et al. (1992). The net charge of this enzyme is negative like that of the substrate O$_2^-$ but site directed mutagenesis and ionic strength effects convincingly demonstrate that it is the charge at the substrate site that decides substrate recognition and attraction.

In the analysis of the electrostatic interaction between the charge of the substrate site and the charge of the substrate, we have applied the limiting law of Debye-Hückel (Eq. 6). Theoretically this equation is only valid in very dilute solutions and the good correlations between our data and Eqs. 8 and 21 derived on the basis of the limiting law (see Figs. 3 and 10) are therefore perhaps surprising. A survey of the literature reveals, however, that this finding is by no means unique. One prominent example is the usefulness of the limiting law of Debye-Hückel in relating the calculated effective dielectric constant, D$_{eff}$, in the active site cleft of subtilisin to the ionic strength of the bulk medium (Russell et al., 1987). D$_{eff}$ was calculated by assuming two point-charges separated by a certain distance, and the simple relationship ln D$_{eff}$ = ln D$_{water}$ + $\alpha \cdot I$ was found to be valid over the entire range of [KCl] from 0.1 to 1 M.

Studies of the effects of ionic strength on enzyme reactions have often concentrated on the rate of binding of the substrate. The I-dependence of this reaction can also be found to follow the law of Debye and Hückel (Eq. 5) or the limiting law (Eq. 6), far beyond the upper limit of their theoretical validity (the Cu,Zn superoxide dismutase reaction, see Argese et al. [1987], and Getzoff et al. [1992], binding of ATP to myosin subfragment 1, Johnson and Taylor [1978], binding of butyrylthiocholine to butyrylcholinesterase, Masson et al. [1996], hydrogen exchange of various peptide groups in bovine trypsin inhibitor, Christoffersen et al. [1996]). Likewise at NaCl concentrations up to 1 M, Snyder et al. (1981) found that the ionic strength dependence of the bimolecular rate constant for reaction of negative disulfide substrates with cysteines in naturally occurring proteins fits with that described by the Debye-Hückel law.

**Ionic Strength and the Kinetics of the Na,K-ATPase and K$^+$-pNPPase Activity**

The maximal rate of hydrolysis of ATP as well as pNPP, i.e., the rate at saturating substrate and activating ligand concentrations, decreased with increasing ionic strength. For the Na,K-ATPase activity the decrease was roughly the same whether Cl$^-$, SO$_4^{2-}$ or acetate$^-$ was the anion (Fig. 6), and the relationship between V$_{max}$ and I was also the same whether I was increased with NaCl ([KCl] fixed at 20 mM) or with NaCl + KCl in a constant ratio [NaCl]/[KCl] = 8 (Fig. 6). This supports the hypothesis that it is an ionic strength effect rather than a competitive binding of Na$^+$ to activating K$^+$-sites. For K$^+$-pNPPase the I-dependence of v$_0$ (with 10 mM pNPP) was the same in KCl, K$_2$SO$_4$ and KNO$_3$ (Fig. 8), emphasizing the importance of I rather than specific anions also here. According to the transition-state model in the Theory section (Eq. 14), there are no simple explanations for the dependence of V$_{max}$ on ionic strength.

We did, however, exploit the kinetic measurements using the expression V$_{max}$/K$_m$ that characterizes the reaction of the enzyme with the substrate (Theory, Eqs. 18–21). The ionic strength dependence of this ratio (Fig. 10) revealed that there is a significant electrostatic interaction involved in the binding of pNPP$^{2-}$ to the substrate site. The net charge at this enzyme site for pNPP was calculated to be between +0.8 and +1.1, i.e., close to the values found for the high-affinity ADP- and eosin-binding site. In contrast, the low affinity binding of ATP$^{1-}$, which has a decisive rate-determining role in the Na,K-ATPase activity, was much less I dependent (the estimated net charge at the enzyme site was +0.13). If the low-affinity ATP binding is in the same topological region as the high-affinity binding, then this region must undergo a drastic electrostatic rearrangement (e.g., a deprotonation), when it changes from the high-affinity to the low-affinity conformation. Likewise, we may suggest, as did also Beaugé et al. (1984), that the pNPP-binding site is different from the low-affinity ATP site. The enzyme is known to be phosphorylated by ATP at Asp369 when in the high affinity form, and it is also phosphorylated by pNPP when Na$^+$ is present, presumably at the same Asp369-residue (Yamazaki et al., 1994). If pNPP binds at the same place during both the Na$^+$,K$^+$-pNPPase and K$^+$-pNPPase reaction, this is likely to be at the high affinity ATP binding site in the same effective “electrostatic conformation.” The difference in NO$_3^-$-sensitivity between the equilibrium binding of ADP and pNPP in the pNPPase
reaction could be due to the fact that ADP has 3 but pNPP only 2 negative charges.

Our results are comparable to the few observations on the effect of ionic strength on the hydrolytic activities of Na,K-ATPase that have been published. In the absence of K\(^+\), the Na\(^+\)-ATPase activity was clearly inhibited by both [NaCl] > 0.2 M and by Tris-Cl (Nørby et al., 1983), whereas if I was kept constant with Tris-Cl + NaCl, only activation by Na\(^+\) was seen. Likewise, in experiments with a constant ratio of [Na\(^+\)]/[K\(^+\)] = 6.5, the Na,K-ATPase activity increased with the (Na\(^+\)+K\(^+\)) salt concentration when I was kept constant with Tris-Cl, but without Tris-Cl there was inhibition above 0.2 M salt (Skou, 1979). There is thus an inhibitory effect on the Na,K-ATPase activity of NaCl, NaCl + KCl, or Tris-Cl (provided that there is a certain minimum concentration of the activating ions). The pNPPase activity was inhibited by increasing ionic strength, irrespective whether I was increased by KCl (Koyal et al., 1971; Skou, 1974) or choline-Cl (Skou, 1974). All these observations again support our assumption that we are dealing with effects of ionic strength and not of specific cations.

**The Effect of Special Anions on the Properties of the Na,K-pump**

Among the anions examined in this work, NO\(_3^–\) and ClO\(_4^–\) have special inhibitory effects on equilibrium binding of ADP (Table I, Fig. 2), eosin and carboxy-eosin (Fig. 11), and on Na,K-ATPase activity under \(V_{\text{max}}\) conditions (Fig. 6), whereas only ClO\(_4^–\) showed specific inhibition of the K\(^+\)-pNPPase activity (Fig. 8). ADP-binding is also specifically inhibited by SCN\(^–\) (Fig. 2).

The effect of NO\(_3^–\) on ADP binding is described in detail (Figs. 1 C, and 5, Table I) and in the concentration range studied, it is found to be compatible with a simple model (Fig. 4) involving competitive binding between ADP and NO\(_3^–\) with a dissociation constant for E-NO\(_3\) of 32 mM (Fig. 5). According to this model, NO\(_3^–\) may also bind to EADP but with a very low affinity, \(K_{\text{diss}}\) for EADP-NO\(_3\) probably being larger than 5 M. We do not know whether SCN\(^–\) and ClO\(_4^–\) act on ADP and eosin binding by a similar mechanism. Since we have obtained evidence for the binding of NO\(_3^–\) to the substrate site of Na,K-ATPase, it is relevant to mention that inhibition of creatine kinase by NO\(_3^–\) is proposed to be caused by a formation of a dead-end complex creatine-enzyme-MgADP-NO\(_3\) (review by Watts, 1973). \(K_{\text{diss}}\) for NO\(_3^–\) is <1 mM. Further molecular details are not known, but it seems that the NO\(_3^–\) binding observed in this work is different (\(K_{\text{diss}} = 32\) mM for ENO\(_3\) and > 5 M for EADP-NO\(_3\)) also because it is competitive to ADP-binding and not synergistic. It is also of particular interest that some H\(^–\)-translocating Mg-ATPases from tissues of higher plants are inhibited by NO\(_3^–\) with a \(K_{\text{diss}}\) of 10–50 mM, i.e., comparable to the \(K_{\text{diss}}\) found here (Sze, 1985; Jacoby, 1987; Blumwald et al., 1987). The mechanism for inhibition is not known. There are several papers dealing with anion inhibition of ATPases from other tissues, but a discussion of those is beyond the scope of this article. We should point out though, that in the light of the results presented here and the findings just discussed, the statement that inhibition by “KNO\(_3\) and KSCN . . . appear to be more specific for the V-than the P- and F-type ATPases” (Pedersen and Carafoli, 1987) may need revision.

None of the anions investigated in this work seem to interfere with the low-affinity ATP-binding site involved in Na,K-ATPase activity. Perhaps this reflects that electrostatic interactions are not especially important at this site (see above). The lack of specific interference of NO\(_3^–\) with the K\(^+\)-pNPPase activity shows that at least one anion sensitive step is different between the two hydrolytic processes of the enzyme.

Regarding \(V_{\text{max}}\) for Na,K-ATPase activity (Figs. 6 and 7) and K\(^+\)-pNPPase activity (Figs. 8 and 9), we have no explanation for either the special effect of the anions or for the effect of increasing \(I\), but these results show that other steps in the reaction mechanism than the binding of substrate and ligands are sensitive to anions and ionic strength. According to recent studies by Post and Suzuki (1991) and by Klodos and colleagues (Klodos and Forbush, 1991; Klodos, 1991; Klodos et al., 1994) both anions and salt concentration as such have profound influence on the formation and dephosphorylation of the phosphorylated intermediates, EP, in the Na\(^+\)-ATPase reaction. The model proposed for the results of changing salt (NaCl) concentrations does not use the concept of ionic strength effects on the protein but rather a “control of enzyme conformation by changes in separate unmixed phases of the lipid of the membrane” (Klodos et al., 1994). The effect of the anions was ranked according to their ability to influence the ratio between ADP and K\(^+\)-sensitive phosphoenzyme, E\(_1\)P/E\(_2\)P, and the rate of dephosphorylation of EP, and it was found that the ranking conformed with the Hofmeister series for anions (see below and the review by Collins and Washabaugh, 1985). This suggested that water structure could be an important parameter in these enzymatic phenomena (although it has been claimed [p. 277 in Edsall and Wyman, 1958] “that the significance of the series is quite unrelated to colloidal chemistry in any special sense. It is rather at general function of the size and hydration of ions”).

The effects of anions reported in the present work do not rank systematically in the Hofmeister series, although this does not exclude that the lyotropic properties could be influential especially perhaps at higher salt concentrations (Leberman, 1991; Parsegian, 1995;
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