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ARTICLE

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Anion interactions with Na,K-ATPase: simultaneous binding of nitrate and eosin

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Abstract Nucleotide binding affinity to Na,K-ATPase is reduced by a number of anions such as nitrate and perchlorate in comparison with affinity in the presence of chloride (all with sodium as the cation). The reduction correlates with the position of these anions in the Hofmeister series. Transient kinetic experiments using the fluorescent dye eosin—which binds to the nucleotide site of the Na,K-ATPase—show that simultaneous anion binding, exemplified with nitrate, and eosin binding is possible. The effect of nitrate on eosin binding is reflected in a decreased binding-rate constant and an increased dissociation rate constant, leading to a decreased equilibrium binding constant for eosin. Since eosin binding is analogous with nucleotide binding to Na,K-ATPase, the results suggest the simultaneous presence of nucleotide and anion binding sites.

Keywords Nucleotide binding affinity · Na,K-ATPase · Anion interactions · Simultaneous anion binding

Abbreviations E_1: the protein conformation in Na^+ · E_2: the enzyme conformation in K^+ · Eo: eosin (tetrabromofluorescein) · F: fluorescence · I: ionic strength · k_i: rate constant · K_i: equilibrium dissociation constant · $K_{i,0}$: equilibrium dissociation constant at zero ionic strength · N: nitrate · z_i: net charge · α: charge product $z_i \cdot z_j$

Introduction

Na,K-ATPase transports Na^+ and K^+ across the plasma membrane against their electrochemical gradients. The energy for this is derived from hydrolysis of ATP, which occurs via a phosphorylated intermediate (for review, see Møller et al.1996). A simplified model for the kinetic mechanism of the transport cycle involves binding 3 Na^+ (forming the E_1 conformation) and MgATP, which leads to phosphorylation (E_1P) and occlusion of Na^+. A conformational transition accompanied by release of Na^+ at the extracellular surface leads to the E_2P conformation, which upon dephosphorylation concomitant with K^+ occlusion forms the E_2 conformation with K^+ occluded. Release of occluded K^+ is accelerated by nucleotide binding, followed by a new turnover cycle (see e.g. Glynn 1985, for a review).

High affinity binding of nucleotides (ADP and ATP) occurs in the presence of Na^+ (Hegyvary and Post 1971; Nørby and Jensen 1971). It has previously been observed that the type of anion added with Na^+ affects its affinity for ADP (Rossi and Nørby 1993). Chloride and sulfate salts of Na^+ have about the same effect on nucleotide affinity in the 50–500 mM range. The dissociation constant for ADP binding ($K_{ADP} = [E]\cdot[ADP]/[E\cdot ADP]$) is about 0.5 μM at 50 mM NaCl (Nørby and Esmann 1997). Nucleotide affinity decreases at increasing ionic strength (I), probably due to shielding of the electrostatic attraction between the nucleotide and the binding site (Fedosova et al. 2002). The dependence of the dissociation constant on the ionic strength was modelled according to Debye-Hückel theory (Debye and Hückel 1923):

$$K_{ADP} = K_{ADP,0} \cdot 10^{-\alpha \cdot \sqrt{I}} \quad (1)$$

where $K_{ADP,0}$ is the equilibrium dissociation constant at zero ionic strength and $\alpha = z_{ADP} \cdot z_E$ is the product of the assumed point charges of ADP, z_{ADP}, and the binding site, z_E (see Nørby and Esmann 1997).

Addition of nitrate had a markedly greater effect on ADP affinity than that of chloride at the same concentration, interpreted as being due to nitrate binding to a specific site in a manner competitive with ADP (Scheme

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1, left), with a dissociation constant for nitrate (K_N) of about 30 mM (Nørby and Esmann 1997).

However, the equilibrium binding studies could not—due to experimental limitations—discriminate between a model with true competition (Scheme 1, left) and an extended model allowing for simultaneous binding of the nucleotide and nitrate (Scheme 1, right). The conclusion from the equilibrium binding experiments with ADP (Nørby and Esmann 1997) was that the fraction of enzyme molecules with both ADP and nitrate bound (E·N·ADP) must either be zero (Scheme 1, left) or be very small compared to the concentrations of E·N and E·ADP. A kinetic consequence of this is that if simultaneous binding of ADP and nitrate occurs, then the dissociation constant for nitrate binding to the ADP-bound form ($K_{ADP\cdot N}$) must be larger than that for binding to the unliganded enzyme (K_N) and, similarly, the dissociation constant for ADP must be larger for the nitrate-bound form ($K_{N\cdot ADP}$) than for the unliganded enzyme (K_{ADP}).

The purpose of the present study is to employ transient kinetic fluorescence methods to distinguish between the two models of Scheme 1, to characterise the nitrate binding process and to elucidate the consequences of nitrate binding for the nucleotide site.

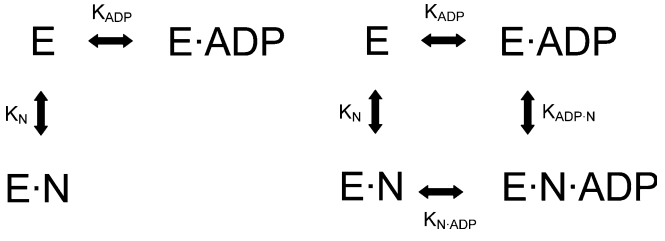
The fluorescent dye eosin is used as an analogue of ADP. Eosin binds with high affinity to Na,K-ATPase in the presence of Na^+ (Skou and Esmann 1981) and is competitive with ADP, suggesting that the binding site for eosin is identical to at least part of the nucleotide site (Esmann 1992; Esmann and Fedosova 1997). The five-fold increase in fluorescence yield upon binding to the enzyme makes it very suitable for fluorescence experiments.

Binding of eosin to Na,K-ATPase in the presence of Na^+ obeys the kinetics of a bimolecular reaction (Esmann 1992), and a perturbation of the binding equilibrium can be described by an observed rate constant for chemical relaxation (Ruf and Grell 1981):

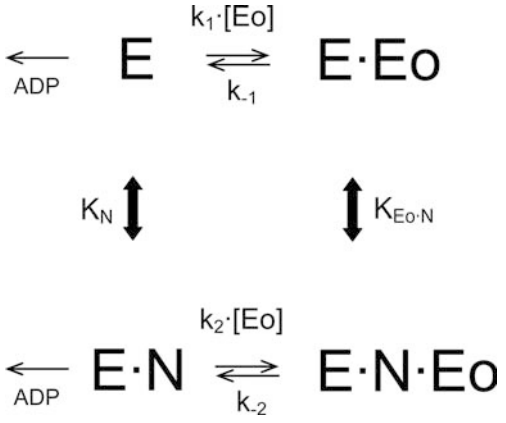
$$k_{obs} = k_1 \cdot [Eo] + k_{-1} \quad (2)$$

where k_1 represents the binding rate constant and k_{-1} the dissociation rate constant for eosin (top line in Scheme 2), giving an equilibrium dissociation constant for eosin of $K_{Eo} = k_{-1}/k_1$.

The rate constant for eosin dissociation can be determined from a stopped-flow experiment where ADP



Scheme 1 Possible interactions between Na,K-ATPase, nitrate (*N*) and ADP. The *left model* is for competitive interaction between nitrate and ADP, and the *right model* allows for simultaneous binding of nitrate and ADP



Scheme 2 A model for interactions between Na,K-ATPase, eosin (Eo), nitrate (N) and ADP. The stopped-flow experiments are designed to follow the decrease in fluorescence when Na,K-ATPase—in equilibrium with eosin and nitrate—is mixed with a high concentration of ADP, which leads to a complete displacement of eosin from the enzyme (indicated by the irreversibility of the ADP-binding reactions towards the left). All the enzyme forms are of the E_1-form with Na^+ bound, the subscript is omitted for clarity

is added at a high concentration to a mixture of Na,K-ATPase, eosin and Na-salt, since ADP will displace eosin from the enzyme (with a high ADP concentration effectively decreasing [Eo] to zero), so $k_{obs} = k_{-1}$ (Esmann 1992).

The idea is the following: if eosin binding occurs only to the enzyme with no nitrate bound (similar to the model for ADP binding shown in Scheme 1, left), then the dissociation rate constant for eosin (k_{-1}) should be the same irrespective of the amount of nitrate added (Scheme 2). If, on the other hand, nitrate and eosin bind at the same time, the observed dissociation rate constant for eosin could be affected by nitrate binding.

The experiments presented here show that the observed dissociation rate constant for eosin is indeed larger in the presence of nitrate than in the presence of chloride over the full range of concentrations used (20–400 mM). The data can be fitted according to Scheme 2 with a dissociation rate constant k_{-2} which is about two-fold larger than the dissociation rate constant k_{-1} of about 11 s^{-1} in 30 mM NaCl. This implies a reduced affinity for eosin to the E·N form, in agreement with the observations on ADP binding. Analysis of the eosin dependence of the rate of binding shows that nitrate lowers the binding rate constant two-fold. The results demonstrate simultaneous binding of nitrate and eosin, and thus possibly of nitrate and nucleotide to the Na,K-ATPase.

Materials and methods

Enzyme

Na,K-ATPase was prepared from pig kidney outer medulla using the SDS-procedure of Jørgensen (1974) as

modified by Klodos et al. (2002). The specific activity of the membranous preparation was 1,500 μmol ATP hydrolysed per mg protein per hour (Esmann 1988).

Equilibrium fluorescence experiments

Eosin-fluorescence was monitored at 20 °C with an excitation wavelength of 530 nm (0.4 nm bandpass) and emission above 550 nm with a cut-off filter using a Spex Fluorolog fluorometer. Na,K-ATPase (0.05 mg/ml) in 10 mM histidine (pH 7.4), 1 mM CDTA and 0.5 μM eosin was mixed with NaCl or $NaNO_3$ and ADP as shown in Fig. 1.

Stopped-flow fluorescence experiments

Stopped-flow fluorescence experiments were performed on two different instruments. For the experiments shown in Figs. 2, 3, and 4, the fluorescence of eosin was monitored with an excitation wavelength of 530 nm (10-nm bandpass) and emission above 550 nm with a cut-off filter using a Bio-logics SFM-2 stopped-flow apparatus (Bio-logic, Claix, France). Both syringes (A and B) contained 10 mM histidine (pH 7.4), 1 mM CDTA and NaCl or $NaNO_3$ as indicated in the legends. In addition, syringe A contained 0.1 mg/ml Na,K-ATPase membranes and 0.5 μM eosin, whereas syringe B contained 200 μM ADP and 0.5 μM eosin. For the binding experiments shown in Fig. 5, a DX 17MV stopped-flow spectrofluorometer (Applied Photophysics) was used with an excitation wavelength of 530 nm, slits 10 nm and a 550-nm cut-off filter on the emission side. Here,

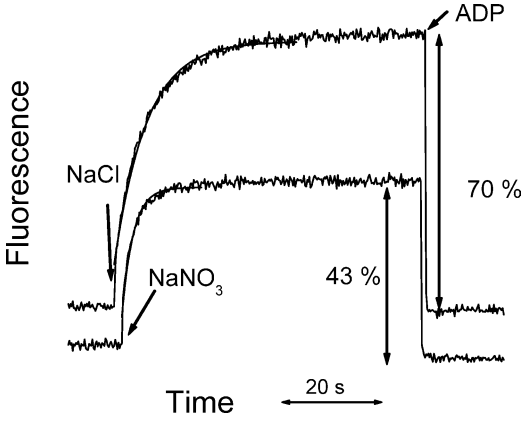


Fig. 1 Eosin fluorescence changes upon addition of Na-salt and ADP. Na,K-ATPase (0.05 mg/ml) in 10 mM histidine (pH 7.4), 1 mM CDTA and 0.5 μM eosin was mixed with NaCl (final concentration 50 mM, *upper tracing*) or with $NaNO_3$ (final concentration 50 mM, *lower tracing*). The fluorescence increase of about 70% is fully reversible by the addition of 100 μM ADP, as indicated. The amplitude of the fluorescence change with 50 mM $NaNO_3$ is about 43%. The *unbroken lines* through the initial fluorescence increases are single-exponential curve fits with rate constants of 0.11 s^{-1} for NaCl and 0.32 s^{-1} for $NaNO_3$ data, respectively. Temperature, 20 °C

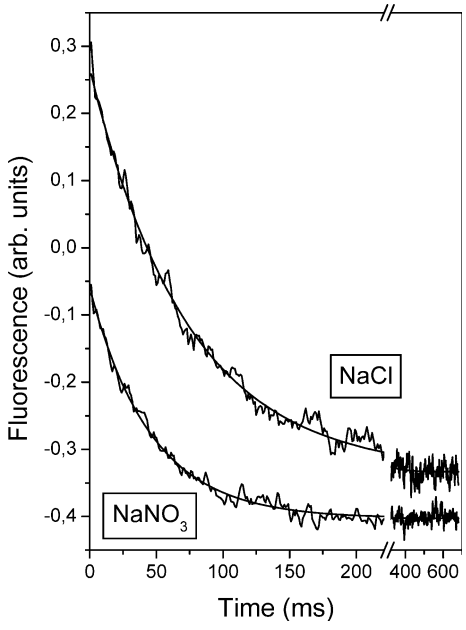


Fig. 2 Stopped-flow measurements of the decrease in eosin fluorescence induced by addition of ADP. Na,K-ATPase (0.1 mg/ml) in 50 mM Na-salt, 10 mM histidine (pH 7.4), 1 mM CDTA and 0.5 μM eosin was mixed with ADP in the same buffer to a final ADP concentration of 100 μM. The *upper tracing* (NaCl) is fitted by a single exponential function with a rate constant of 13 s^{-1}. The *lower tracing* ($NaNO_3$) has a rate constant of 22 s^{-1}. The amplitude of the fluorescence change with 50 mM $NaNO_3$ is about half that with NaCl (cf. also Fig. 3). Temperature, 4 °C

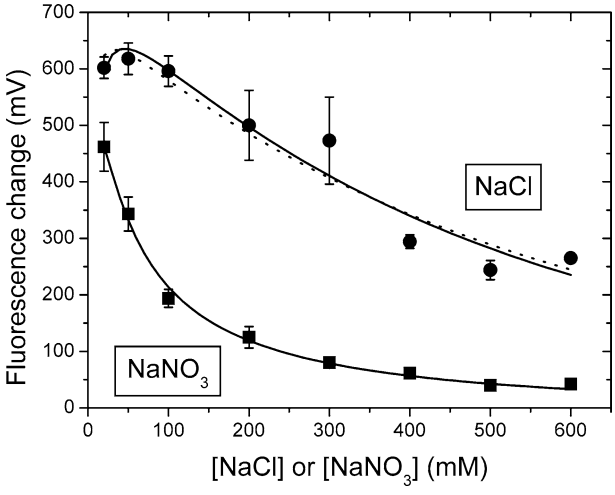


Fig. 3 Eosin fluorescence decrease induced by the addition of ADP as a function of the salt concentration. Na,K-ATPase (0.1 mg/ml) in Na-salt, 10 mM histidine (pH 7.4), 1 mM CDTA and 0.5 μM eosin was mixed with ADP in the same buffer to a final ADP concentration of 100 μM, and the amplitude of the fluorescence decrease is given as a function of the NaCl concentration (*filled circles*) and the $NaNO_3$ concentration (*filled squares*). The *unbroken lines* are fitted according to Eq. (7) with $K_{Eo,0} = 0.1$ μM, $\alpha 1 = -1.6$, $K_{Na} = 6.3$ mM, $\Delta F_{max} = 1033$ mV, $K_N = 12$ mM and $K_{N\cdot Eo,0} = 1.23$ μM. The *dotted line* represents a fit of the chloride data with $K_{Eo,0} = 0.1$ μM, $\alpha 1 = -1.5$, $K_{Na} = 3$ mM and $\Delta F_{max} = 950$ mV. Temperature, 4 °C

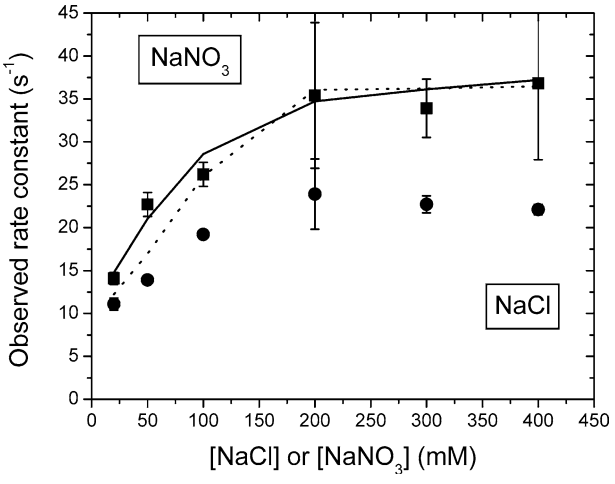


Fig. 4 Observed rate constants of the decrease in eosin fluorescence after addition of ADP. Na,K-ATPase (0.1 mg/ml) in Na-salt, 10 mM histidine (pH 7.4), 1 mM CDTA and 0.5 μM eosin was mixed with ADP in the same buffer to a final ADP concentration of 100 μM. Rate constants from the single-exponential fits of the fluorescence decrease are given as a function of the NaCl concentration (*filled circles*) and the $NaNO_3$ concentration (*filled squares*). The *unbroken line* represents a fit of the $NaNO_3$ data to a model with an assumed constant value for $k_{-2} = 44\ s^{-1}$, and the *dotted line* is for a model with $k_{-2} = 1.9 \cdot k_{-1}$. Temperature, 4 °C

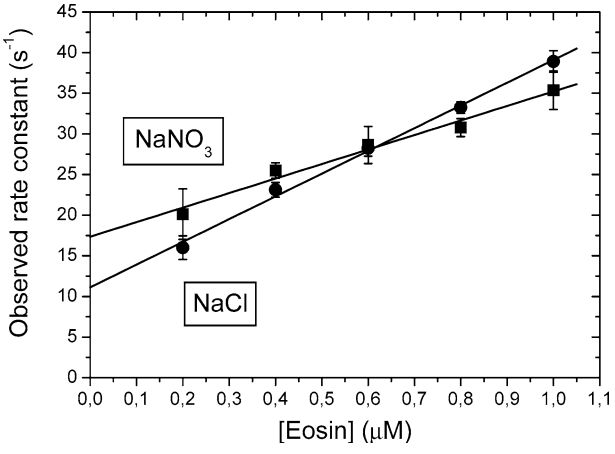


Fig. 5 Observed rate constants of the increase in eosin fluorescence after addition to Na,K-ATPase. Na,K-ATPase (0.1 mg/ml) in 30 mM Na-salt, 10 mM histidine (pH 7.4), 1 mM CDTA was mixed with eosin in the same buffer to final eosin concentrations of 0.2–1.0 μM, and the rate constants from single-exponential fits of the fluorescence increase are given as a function of the eosin concentration in 30 mM NaCl (*filled circles*) and 30 mM $NaNO_3$ (*filled squares*). The *unbroken lines* are the best linear fit of the data. Temperature, 6 °C

both syringes (A and B) contained 10 mM histidine (pH 7.4), 1 mM CDTA and 30 mM NaCl or $NaNO_3$. Syringe A, in addition, contained 0.1 mg/ml Na,K-ATPase membranes and syringe B contained 0.4–2.0 μM eosin. Mixing equal volumes from each syringe gave an instrumental dead time of about 2 ms in both instruments. Data were analysed using Origin 6.0 software (Microcal, Amherst, MA). Observed rate constants from single-exponential fits are given as the average ± SD of three to six determinations.

All reagents were of analytical grade.

Results

Eosin binding with high affinity to the nucleotide site on Na,K-ATPase in the presence of Na^+ leads to a large increase in eosin fluorescence when NaCl is added to Na,K-ATPase in the presence of eosin (Fig. 1). $NaNO_3$ has qualitatively the same effect as NaCl, but the fluorescence increase is smaller. This effect of nitrate is in agreement with results on direct ADP-binding measurements (Nørby and Esmann 1997), where it was shown that substitution of chloride with nitrate leads to a reduced affinity for nucleotide in a competitive manner. The rate of the fluorescence increase is about three-fold larger in the presence of nitrate than with chloride. Nucleotides such as ADP displace eosin, and a large fluorescence decrease is observed upon addition of a large excess of ADP (Fig. 1) due to dissociation of eosin from its binding site. The rate of the decrease is equal to the dissociation rate constant for eosin, and the amplitude ΔF is a measure of the fraction of eosin bound:

$$\Delta F = \Delta F_{max} \cdot [Eo]/\{[Eo] + K_{Eo}\} \quad (3)$$

Figure 2 shows an example of a transient kinetic experiment with displacement of bound eosin by an excess of ADP. Na,K-ATPase in 50 mM NaCl and 0.5 μM eosin was mixed with 200 μM ADP, and the fluorescence decreased with an observed rate constant of 13 s^{-1} (k_{-1} in terms of Scheme 2). When the same experiment was done with 50 mM $NaNO_3$ instead of NaCl, the observed rate constant for fluorescence decrease was about 22 s^{-1}, almost a doubling, and the amplitude was about half (Fig. 2). The decrease in amplitude simply reflects the lower affinity for eosin in the presence of nitrate, whereas the change in the observed rate constant directly shows that Na,K-ATPase binds eosin and nitrate simultaneously. Kinetically, eosin release from the enzyme will be described with an observed rate constant:

$$k_{obs} = k_{-1} \cdot [E \cdot Eo]/\{[E \cdot Eo] + [E \cdot N \cdot Eo]\} + k_{-2} \cdot [E \cdot N \cdot Eo]/\{[E \cdot Eo] + [E \cdot N \cdot Eo]\} \quad (4)$$

This is analogous to a two-compartment analysis of dephosphorylation of Na,K-ATPase (Klodos et al. 1981), with the assumption that there is rapid equilibrium between [E·Eo] and [E·N·Eo] on the millisecond timescale of the stopped-flow experiment (N denotes nitrate). The first term describes eosin dissociation (rate constant k_{-1}) from the fraction of enzyme molecules without nitrate bound, and the latter from those having nitrate bound in addition to eosin (rate constant k_{-2}). The value for k_{-2} cannot be extracted without separate

knowledge of the equilibrium binding constants for nitrate [cf. Eq. (4) and Scheme 2].

The aim was now to substantiate this finding over a broad salt concentration range. First, the dependence of the amplitude of the fluorescence change in NaCl and in $NaNO_3$ was analysed to estimate the equilibrium binding constants for nitrate and subsequently the salt-effects on the observed rate constants for eosin dissociation can be interpreted in terms of Scheme 2.

Dependence of fluorescence amplitudes on NaCl and $NaNO_3$

Figure 3 (filled circles) shows the decrease in the amplitude of fluorescence change at NaCl concentrations between 50 and 600 mM when ADP is added to Na,K-ATPase in 0.5 μM eosin. The amplitude shows the degree of protein saturation with eosin. The decrease is the expected effect of ionic strength, since it reflects the increased equilibrium dissociation constant for eosin, in analogy with increased equilibrium dissociation constant for ADP at increasing ionic strength (Nørby and Esmann 1997). From Eq. (3), it is clear that at a given eosin concentration ΔF will decrease when K_{Eo} increases.

Note that a small increase in ΔF is observed between 20 and 50 mM NaCl (Fig. 3, filled circles). This is due to the fact that 20 mM NaCl does not fully saturate the Na,K-ATPase in terms of inducing the high-affinity nucleotide binding E_1-form (Fedosova and Esmann 2004).

The decrease in amplitude upon increasing salt concentrations is related to a decrease in the affinity for eosin resulting from electrostatic screening of the negatively charged eosin molecule from the positively charged binding site. The analysis of the data was based on Debye-Hückel theory:

$$K_{Eo} = K_{Eo,0} \cdot 10^{-\alpha 1 \cdot \sqrt{I}} \quad (5)$$

where $K_{Eo,0}$ is the equilibrium dissociation constant at zero ionic strength and α1 is the product of the charges of eosin and the binding site ($\alpha 1 = z_E \cdot z_{Eo}$).

The small increase at 20–50 mM NaCl can be accounted for by Na^+-binding to the enzyme with an affinity $K_{Na} = [E] \cdot [Na^+]/[E \cdot Na]$. For simplicity, the multiple and cooperative character of Na^+-binding is ignored in the present analysis.

In the absence of NO_3^-, the amplitude of the fluorescence change will depend on the Na^+ and eosin concentration and the ionic strength as follows from this extension of Eq. (3):

$$\Delta F = \Delta F_{max} \cdot \{[Na^+]/([Na^+] + K_{Na})\} \cdot [Eo]/\left\{[Eo] + K_{Eo,0} \cdot 10^{-\alpha 1 \cdot \sqrt{I}}\right\} \quad (6)$$

where the first bracketed term accounts for the initial increase in amplitude due to binding of Na^+. From a direct measurement of eosin binding to Na,K-ATPase ($K_{Eo} = 0.34$ μM at $I = 120$ mM; see Esmann 1992), we calculate $K_{Eo,0} = 0.1$ μM from Eq. (5).The remaining constants in Eq. (6) were estimated from least-squares fitting to be $\alpha 1 = -1.58 \pm 0.13$, $\Delta F_{max} = 1033 \pm 106$ mV and $K_{Na} = 6.3 \pm 3.6$ mM. The value of about −1.6 for the charge product $\alpha 1 = z_E \cdot z_{Eo}$ [cf. Eq. (1)] is in good agreement with earlier data ($\alpha 1 = -1.5$; Nørby and Esmann 1997). The dissociation constant for Na^+ is clearly not well determined from the present data set and is larger than values of about 3 mM reported earlier (see e.g. Schwappach et al. 1994; Klodos et al. 1995; Fedosova and Esmann 2004). The absolute value for K_{Na} is, however, not critical for determination of α1, which contributes to the decreasing part of the curve mainly at $[Na^+] > 50$ mM. To illustrate this, we have included in Fig. 3 a least-squares analysis of the data where $K_{Na} = 3$ mM is used, thus obtaining only α1 and ΔF_{max} from the fitting procedure. This gives $\alpha 1 = -1.5$ and $\Delta F_{max} = 950$ mV (dotted line in Fig. 3). In the further analysis of the nitrate effects on eosin binding, we have chosen to use the parameters from the best least-squares fit with $\alpha 1 = -1.58$, $\Delta F_{max} = 1{,}033$ mV and $K_{Na} = 6.3$ mM. This choice does not affect any of the conclusions.

The effect of nitrate on the affinity for eosin can be analysed using Scheme 2, as outlined for ADP-binding (Nørby and Esmann 1997). It is assumed that eosin binds with high affinity in the absence of nitrate, and substitution of chloride for nitrate leads to a reduction in apparent eosin affinity due to binding of nitrate with a dissociation constant K_N.

Figure 3 (filled squares) also shows the amplitude of eosin fluorescence response to ADP in the presence of 20–600 mM $NaNO_3$. The amplitude decreases much more upon an increase in $[NaNO_3]$ than upon a similar increase in [NaCl]. Note that the initial increase in fluorescence at 20–50 mM salt is absent here—the nitrate effect manifests itself before $[Na^+]$ becomes saturated.

The nitrate data are least-squares fitted by an expansion of Eq. (6):

$$\Delta F = \Delta F_{max} \cdot \{[Na^+]/([Na^+] + K_{Na})\} \cdot \{[Eo]/K_{Eo} + [Eo] \cdot [N]/(K_{N \cdot Eo} \cdot K_N)\}/ \{1 + [Eo]/K_{Eo} + [Eo] \cdot [N]/(K_{N \cdot Eo} \cdot K_N) + [N]/K_N\} \quad (7)$$

where the last two bracketed terms give the fraction of enzyme with eosin bound as a function of the eosin and nitrate concentration (cf. Scheme 2) and the first term is the Na^+ dependence as before. The dissociation constant for nitrate is denoted K_N. The effect of ionic strength on K_{Eo} is taken from the NaCl data ($K_{Eo,0} = 0.1$ μM, $\alpha 1 = -1.6$, $K_{Na} = 6.3$ mM and $\Delta F_{max} = 1033$ mV are here fixed values), and, in addition, it is assumed that the dissociation constant for eosin binding to the E·N-form ($K_{N \cdot Eo}$) depends on the ionic strength in the same manner as eosin-binding to E:

$$K_{N\cdot Eo} = K_{N\cdot Eo,0} \cdot 10^{-\alpha 1\sqrt{I}} \quad (8)$$

The least-squares fit of the nitrate data in Fig. 3 gives $K_N = 12 \pm 1.5$ mM and $K_{N\cdot Eo,0} = 1.23 \pm 0.2$ μM. Nitrate binding thus leads to a 12-fold reduction in affinity for eosin. From the principle of microscopic reversibility, the dissociation constant for nitrate binding to enzyme with eosin bound ($K_{Eo\cdot N}$) can be derived from $K_{Eo\cdot N} = K_N \cdot K_{N\cdot Eo}/K_{Eo} = 153$ mM. The magnitude of the dissociation constant for nitrate, 12 mM, can be considered in agreement with the value of about 30 mM deduced earlier (Nørby and Esmann 1997), taking into account the differences in experimental conditions.

Dependence of the rate constant for fluorescence decrease on NaCl and $NaNO_3$

Figure 4 shows the dependence of the dissociation rate constant for eosin on the NaCl and $NaNO_3$ concentration. There is a marked increase in the observed rate constant from 20 to 200 mM salt, with no further increase up to 400 mM salt. At all concentrations, the observed rate constant is higher in $NaNO_3$ than in NaCl.

The equilibrium dissociation constant for eosin depends on the ionic strength in a manner described well by the Debye-Hückel theory [Eq. (5)], whereas the relationship between the ionic strength and the rate constants for the eosin binding/dissociation reactions is not clear at present. The data in Fig. 4 suggest a complex behaviour of the dissociation rate constants (k_{-1} and k_{-2}) in relation to the ionic strength.

We therefore chose to analyse the data in a manner eliminating some of the complexity. At a given [NaCl], the observed rate constant for eosin dissociation (k_{-1}) is determined assuming a single enzyme species E·Eo being present [cf. Eq. (2)]. In $NaNO_3$ at the same salt concentration, an additional species with eosin bound is formed (E·N·Eo). The rate of dissociation of eosin from Na,K-ATPase in the presence of nitrate follows the kinetics of a two-pool model where eosin can dissociate from the two different enzyme species, E·Eo and E·N·Eo, with rate constants k_{-1} and k_{-2} (cf. Scheme 2):

$$\begin{array}{ccc} \leftarrow & [E\cdot Eo \leftrightarrow E\cdot N\cdot Eo] & \rightarrow \\ k_{-1} & K_{Eo\cdot N} & k_{-2} \end{array} \quad (9)$$

The time dependence of the fluorescence decrease (i.e. eosin dissociation) follows a single exponential function, suggesting that the nitrate binding is at equilibrium on the timescale of these experiments. The observed rate constant for eosin dissociation depends on the nitrate concentration [cf. Eq. (4)]:

$$k_{obs} = k_{-1}/\{1 + [N]/K_{Eo\cdot N}\} + k_{-2}/\{1 + K_{Eo\cdot N}/[N]\} \quad (10)$$

where $K_{Eo\cdot N} = [N]\cdot[E\cdot Eo]/[E\cdot N\cdot Eo]$ is substituted into Eq. (4). The dissociation rate constant in chloride k_{-1} is determined separately at each NaCl concentration (Fig. 4, filled circles). The unbroken line fitted to the nitrate data in Fig. 4 (filled squares) shows k_{obs} calculated using Eq. (10) with $K_{Eo\cdot N,\ 0} = 153$ mM and $k_{-2} = 44\ s^{-1}$. Here, k_{-2} is taken to be constant over the range of nitrate concentrations tested, i.e. independent of the ionic strength. The dotted line gives k_{obs} for a model where k_{-2} is taken to be 1.9-fold larger than k_{-1} at each salt concentration, i.e. imposing the same ionic strength dependence on k_{-2} as found for k_{-1}.

The increased dissociation rate observed when chloride is substituted with nitrate is also found with other anions. At 20 °C in the presence of 50 mM Na-salt, the observed dissociation rate constants (cf. Fig. 2) were $20.2 \pm 0.4\ s^{-1}$ (Cl^-), $< 25.5 \pm 0.3\ s^{-1}$ (Br^-), $< 28.6 \pm 0.4\ s^{-1}$ (NO_3^-), $< 35.3 \pm 1.4\ s^{-1}$ (I^-), $\approx 35.7 \pm 1.0\ s^{-1}$ (ClO_4^-), $< 42.7 \pm 0.9\ s^{-1}$ (SCN^-). The equilibrium dissociation constant for ADP was also found to increase in the same order (Nørby and Esmann 1997).

Estimation of the binding-rate constant for eosin in chloride and nitrate

We have experimentally determined the binding-rate constants k_1 and k_2 (Scheme 2) in stopped-flow experiments in 30 mM NaCl or $NaNO_3$ as shown in Fig. 5. The fluorescence increase upon addition of eosin to final concentrations between 0.2 and 1.0 μM is fitted to a single exponential function. The relation between k_{obs} and the individual rate constants of Scheme 2 describing eosin binding to enzyme with and without nitrate bound follows from an expansion of Eqs. (2) and (10):

$$k_{obs} = (k_1/\{1 + [N]/K_N\} + k_2/\{1 + K_N/[N]\}) \cdot [Eo] + k_{-1}/\{1 + [N]/K_{Eo\cdot N}\} + k_{-2}/\{1 + K_{Eo\cdot N}/[N]\} \quad (11)$$

For NaCl experiments, Eq. (11) is reduced to Eq. (2) (since $[N] = 0$) and the observed rate constant for the fluorescence increase upon eosin binding is linearly related to the eosin concentration (cf. Fig. 5), yielding a binding-rate constant (equal to the slope) of about $28 \pm 1.1\ \mu M^{-1}\cdot s^{-1}$ ($= k_1$) and a dissociation rate constant (the intercept) of $k_{-1} = 11.1 \pm 0.7\ s^{-1}$ (Fig. 5), in good agreement with the dissociation rate constant deduced from ADP displacement of eosin (cf. Fig. 4, circles).

In 30 mM nitrate, the intercept increases to $17.4 \pm 1.0\ s^{-1}$ and the slope decreases to $18 \pm 1.5\ \mu M^{-1}\cdot s^{-1}$. Using the values for the equilibrium dissociation constants for nitrate binding ($K_N = 12$ mM and $K_{Eo\cdot N} = 153$ mM; see above) and the binding/dissociation rate constants in NaCl as determined above ($k_1 = 28\ \mu M^{-1}\cdot s^{-1}$ and $k_{-1} = 11\ s^{-1}$), the dissociation rate constant k_{-2} for eosin from the E·N·Eo-form can then be calculated using Eq. (11) to be about $25\ s^{-1}$, and a binding rate constant k_2 of about $14\ \mu M^{-1}\cdot s^{-1}$, two-fold lower than for binding in NaCl. The binding-rate experiments thus show a decreased binding-rate

constant for eosin in the presence of nitrate and confirm the results from the ADP experiments shown in Figs. 2 and 4, namely an increased dissociation-rate constant in nitrate. The calculation of $k_{-2} \approx 25\ s^{-1}$, close to two-fold larger than k_{-1} at this $[Na^+]$, suggests that the model with a constant ratio of about two between k_{-2} and k_{-1} (dotted line in Fig. 4) is the better approximation (see above). This implies that the ionic strength-dependent change in the 20–100 mM range of the structure of the nucleotide site—leading to increased dissociation rate constants—occurs in parallel in chloride and nitrate.

Discussion

Anions have in recent years been shown to affect partial as well as overall enzymatic reactions of the Na,K-ATPase. Nucleotide binding, phosphorylation and shifts in phosphoenzyme equilibria have been studied in detail, and the anion effects appeared to follow the Hofmeister series (Post and Suzuki 1991; Klodos 1991; Nørby and Esmann 1997; Ganea et al. 1999; Sokolov et al. 2001). The mechanism of the interactions between the Hofmeister salt and the solute in general is not known; in case of protein stability, the suggested explanations are based on either a ''site-binding model'' or a ''weak interaction model'' (Baldwin 1996). Here, we characterize the details of anion (NO_3^-) interaction with the Na,K-ATPase using changes in eosin binding properties of the enzyme as a detection method. Our description is based on the site binding model, where the Hofmeister anion acts as a chemical reactant, since previous experiments demonstrated nearly competitive interactions between nucleotide and nitrate (Nørby and Esmann 1997).

It was shown that nucleotide binding is equally affected by a variation in ionic strength in media containing NaCl, Na-acetate or Na-sulfate, suggesting a non-specific Debye screening of nucleotide binding. In fact, the dependence of the equilibrium dissociation constant follows the relationships derived by Debye and Hückel (1923) over a large concentration range (0.1–1 M salt; Nørby and Esmann 1997; Fedosova et al. 2002). However, a number of anions, such as nitrate and perchlorate, exert additional effects leading to a much reduced affinity for the nucleotide at a given salt concentration (relative to that in NaCl). It correlates with the position of these anions in the Hofmeister series, although the effective concentrations are significantly lower: thus, the nitrate effect is observed in the range 0–0.1 M salt (cf. Fig. 3), rather than the 0.1–1 M range for which the Hofmeister-type effects are usually described (for discussion see Post and Suzuki 1991; Ramos and Baldwin 2002).

Determination of the effect of the nitrate concentration on the observed equilibrium dissociation constants for nucleotide or eosin binding suggests the presence of a site with an equilibrium dissociation constant for nitrate of about 10–30 mM (this paper and Nørby and Esmann 1997). This anion site could potentially be identical to the part of the nucleotide binding site which accommodates three to four negative charges from bound ADP or ATP. In this case, nitrate and nucleotide binding should be mutually exclusive, following the model in Scheme 1 (left). Alternatively, the anion site could be separate from the nucleotide site, with bound nitrate exerting its effect through a change in the local protein structure of the nucleotide site leading to a decreased affinity for nucleotide.

The rates of eosin binding and dissociation determined by stopped-flow fluorometry are both affected by the presence of nitrate. The dissociation rate for eosin is significantly higher in the presence of nitrate than in chloride, which is indicative of simultaneous binding of eosin and nitrate. Analysis of the data in Fig. 5 suggests that nitrate binding leads to the formation of an enzyme species with a reduced binding-rate constant for eosin as well as an increased dissociation rate constant, i.e. interaction with the nitrate results in a change in the structure of the nucleotide binding site. Both effects lead to a reduction in affinity by an order of magnitude (the equilibrium dissociation constant increases from 0.1 to 1.2 μM).

In conclusion, we show simultaneous binding of nitrate and eosin to the Na,K-ATPase. The equilibrium dissociation constant for nitrate derived from the kinetic analysis is about 10 mM. This relatively high affinity justifies application of the site binding model, although additional effects of anions exerted via the membrane dielectric cannot be excluded.

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