First publ. in: Annals of the New York Academy of Science 986 (2003), pp. 159-162

Kinetic Investigations of the Mechanism of the Rate-Determining Step of the Na^+,K^+-ATPase Pump Cycle

RONALD J. CLARKE,[a] PAUL A. HUMPHREY,[a] CHRISTIAN LÜPFERT,[b] HANS-JÜRGEN APELL,[c] AND FLEMMING CORNELIUS[d]

[a] *School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia*

[b] *Department of Biophysical Chemistry, Max-Planck-Institut für Biophysik, D-60596 Frankfurt am Main, Germany*

[c] *Department of Biology, University of Konstanz, D-78435 Konstanz, Germany*

[d] *Department of Biophysics, University of Aarhus, Ole Worms Allé 185, DK-8000 Aarhus C, Denmark*

ABSTRACT: The kinetics of the $E_2 \rightarrow E_1$ conformational change of unphosphorylated Na^+,K^+-ATPase from rabbit kidney were investigated via the stopped-flow technique using the fluorescent label RH421 (pH 7.4, 24°C). The enzyme was preequilibrated in a solution containing 25 mM histidine and 0.1 mM EDTA to initially stabilize the E_2 conformation. On mixing enzyme with NaCl alone, tris-ATP alone, or NaCl and tris-ATP simultaneously, a fluorescence decrease was observed. The reciprocal relaxation time, $1/\tau$, of the fluorescent transient was found to increase with increasing NaCl concentration and reached a saturating value in the presence of 1 mM tris-ATP of 54 (±3) s^{-1}. The experimental behavior could be described by a binding of Na^+ to the enzyme in the E_2 state with a dissociation constant of 31 (±7) mM, which induces a subsequent rate-limiting conformational change to the E_1 state. Similar behavior, but with a decreased saturating value of $1/\tau$, was found when NaCl was replaced by choline chloride. Experiments performed with enzyme from shark rectal gland showed similar effects, but with a lower amplitude of the fluorescence change and a higher saturating value of $1/\tau$ for both the NaCl and choline chloride titrations. The results suggest that Na^+ ions or salt in general play a regulatory role, similar to ATP, in enhancing the rate of the rate-limiting $E_2 \rightarrow E_1$ conformational transition by interaction with the E_2 state.

KEYWORDS: stopped-flow; fluorescence; voltage-sensitive dye; rate constant; regulation

The kinetics of the $E_2 \rightarrow E_1$ conformational change of unphosphorylated Na^+,K^+-ATPase from rabbit kidney and shark rectal gland were investigated[1] via the stopped-flow technique using the fluorescent label RH421 (pH 7.4, 24°C). This

Address for correspondence: Ronald J. Clarke, School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia. Voice: +61 2 9351 4406; fax: +61 2 9351 3329.
r.clarke@chem.usyd.edu.au

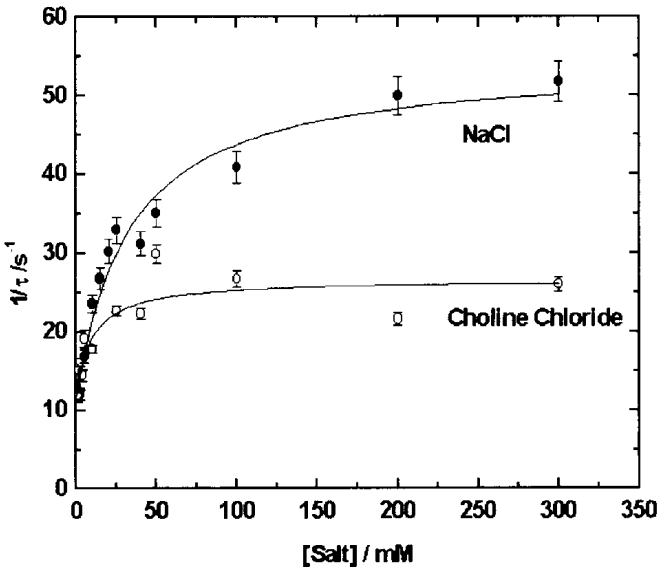

FIGURE 1. Effect of varying salt concentration, that is, NaCl (*filled circles*) and choline chloride (*open circles*), on the reciprocal relaxation time ($1/\tau$) of RH421 fluorescence transients of rabbit kidney Na^+,K^+-ATPase induced by mixing simultaneously with salt plus 2 mM of tris-ATP; excitation wavelength = 577 nm, emission wavelength ≥ 665 nm. The *solid lines* represent nonlinear least-square fits of equation 1 to the data.

method has the advantage over other techniques that the probe RH421 does not interfere with the ATP binding site, so that measurements can be made with high time resolution in very close to physiological conditions. The enzyme was preequilibrated in a solution containing 25 mM histidine and 0.1 mM EDTA to stabilize initially the E_2 conformation. On mixing rabbit kidney enzyme with NaCl alone, tris-ATP alone, or NaCl and tris-ATP simultaneously, a fluorescence decrease was observed. The reciprocal relaxation time, $1/\tau$, of the fluorescent transient was found to increase with increasing NaCl concentration and reached a saturating value in the presence of 1 mM tris-ATP of $54 \pm 3\ s^{-1}$ in the case of rabbit kidney enzyme (see FIG. 1). Similar behavior, but with a decreased saturating value of $1/\tau$ was found when NaCl was replaced by choline chloride. Analogous experiments performed with enzyme from shark rectal gland showed similar effects, but with a significantly lower amplitude of the fluorescence change and a higher saturating value of $1/\tau$ for both the NaCl and choline chloride titrations.

The observed increase in $1/\tau$ with increasing NaCl or choline chloride implies that Na^+, $choline^+$, or salt bind to the enzyme in the E_2 state and stimulate the $E_2 \rightarrow E_1$ conformational change. Similar behavior has previously been observed with ATP,[2] which is thought to bind to a low-affinity regulatory site and stimulate the $E_2 \rightarrow E_1$ transition. The effects of both Na^+ and ATP on the conformational transition can be summarized in the generalized reaction scheme shown in FIGURE 2. Under conditions of saturating ATP concentrations, that is, the experimental condition for

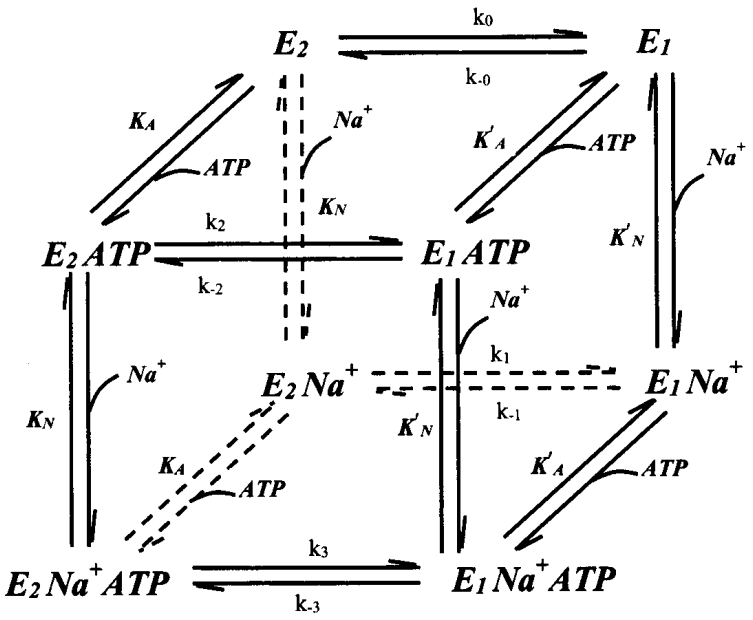

FIGURE 2. Generalized reaction scheme for the conformational change, Na^+ binding and ATP binding of unphosphorylated Na^+,K^+-ATPase.

the results shown in FIGURE 1, this reaction scheme predicts the following dependence of $1/\tau$ on the Na^+ concentration:

$$\frac{1}{\tau} \approx \frac{k_2 + k_{-2} + (k_3 + k_{-3})[Na^+]/K}{1 + ([Na^+]/K)} \tag{1}$$

where K is the dissociation constant of the enzyme for Na^+ ions, k_2 and k_{-2} are the forward and backward rate constants of the transition $E_2 \cdot ATP \leftrightarrow E_1 \cdot ATP$, and k_3 and k_{-3} are the forward and backward rate constants of the transition $E_2 \cdot ATP \cdot Na^+ \leftrightarrow E_1 \cdot ATP \cdot Na^+$. Fitting of the NaCl titration data shown in FIGURE 1 to this equation yields the values $k_2 + k_{-2} \approx 11 \pm 1\ s^{-1}$, $k_3 + k_{-3} \approx 54 \pm 3\ s^{-1}$, and $K \approx 31 \pm 7$ mM. Similarly, replacing $[Na^+]$ by $[choline^+]$ in the preceding equation and fitting the choline chloride titration data yields $k_2 + k_{-2} \approx 13 \pm 2\ s^{-1}$, $k_3 + k_{-3} \approx 27 \pm 2\ s^{-1}$, and $K \approx 9 \pm 5$ mM.

The conclusion that Na^+ can bind to the E_2 state would at first sight seem to be contrary to the generally accepted view that Na^+ ions bind to transport sites of the enzyme in the E_1 state of the enzyme. Forbush[3] and Hasenauer *et al.*[4] also found, however, from rapid filtration studies that Na^+ ions increased the rate of radioactive K^+ or Rb^+ release by enhancing the rate of the $E_2 \rightarrow E_1$ transition. From their measurements they concluded that other cation sites can be occupied at the same time as the K^+ transport sites. We propose, therefore, that Na^+ ions or salt in general play a

regulatory role, similar to low-affinity ATP binding, in enhancing the rate of the rate-limiting $E_2 \rightarrow E_1$ conformational transition by interaction with the E_2 state.

Since the experiments reported here were carried out on open membrane fragments with both sides of the enzyme accessible to Na^+ ions, it is not possible from the experiments described here alone to decide whether the Na^+ ions are binding to the enzyme from the cytoplasmic or the extracellular face. Experiments of van der Hijden and de Pont[5] on rabbit kidney enzyme reconstituted into lipid vesicles, however, showed that extracellular Na^+ ions increase the steady-state phosphorylation level of the enzyme, most likely by inducing a transition of the enzyme to the E_1 conformation, from which it can be phosphorylated. Based on these results, it therefore appears very likely that the Na^+-induced stimulation of the $E_2 \rightarrow E_1$ transition found here is in fact due to Na^+ binding to regulatory extracellular sites.

Whether or not such extracellular Na^+ binding plays an important regulatory role under physiological conditions depends on the extracellular Na^+ concentration. According to electron microprobe results of Thurau,[6] the Na^+ concentration in the extracellular fluid of kidney tubule cells is approximately 160 mM. Comparison with the results shown in FIGURE 1 shows that at this concentration the Na^+ effect on the reciprocal relaxation time has virtually reached saturation. Variations in the extracellular Na^+ concentration over a fairly wide range therefore would be expected to have a relatively minor influence on the rate of the $E_2 \rightarrow E_1$ transition. Thus, it seems unlikely that Na^+-induced stimulation of the $E_2 \rightarrow E_1$ transition would play an important regulatory role *in vivo*. The effect could be seen rather as an evolutionary optimization of enzyme operation under the normal physiological conditions. The normally high extracellular Na^+ or salt concentration can be thought of as facilitating the spontaneous relaxation of the enzyme back into the E_1 state following its dephosphorylation, so that it is ready once again to become phosphorylated by ATP and transport Na^+ ions.

REFERENCES

1. HUMPHREY, P.A. *et al.* 2002. Mechanism of the rate-determining step of the Na^+,K^+-ATPase pump cycle. Biochemistry **41:** 9496–9507.
2. CLARKE, R.J. *et al.* 1998. Kinetics of Na^+-dependent conformational changes of rabbit kidney Na^+,K^+-ATPase. Biophys. J. **75:** 1340–1353.
3. FORBUSH, B., III. 1987. Rapid release of ^{42}K and ^{86}Rb from an occluded state of the Na,K-pump in the presence of ATP or ADP. J. Biol. Chem. **262:** 11104–11115.
4. HASENAUER, J. *et al.* 1993. Allosteric regulation of the access channels to the Rb^+ occlusion sites of $(Na^+ + K^+)$-ATPase. J. Biol. Chem. **268:** 3289–3297.
5. VAN DER HIJDEN, H.T.W.M. *et al.* 1989. Cation sidedness in the phosphorylation step of Na^+/K^+-ATPase. Biochim. Biophys. Acta **983:** 142–152.
6. THURAU, K. 1979. Nephrology—A look into the future. Kidney Int. **15:** 1–6.