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ORIGINAL ARTICLE

Inhibition of Na^+, K^+-ATPase in the presence of crown ethers: modulation of ionic composition or pharmacological effects

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Abstract It is believed that the biological effects of chelating agents such as crown ethers are largely related to their ability to form complexes with ions and/or to facilitate ion transport across membranes. Specific influences are rarely related. Here we present the evidence that even one of the simplest representatives of the crown ether super-family, 1,4,7,10,13,16-hexaoxacyclooctane (18-crown-6), is able to affect the activity of Na^+, K^+-ATPase directly. Using nonlinear regression fitting to kinetic data we have found that the crown ether diminishes the apparent Michaelis constant, K_m, and the maximal rate of ATP hydrolysis, V_m, acting as noncompetitive inhibitors. The apparent dissociation constants, K_i, for the crown interaction with the free ATPase and with the enzyme-substrate complex were established to be of 77 ± 3 mM and 21 ± 2 mM, respectively. So 18-crown-6 possesses weak but "direct" pharmacological activity on Na^+, K^+-ATPase hinders the formation of enzyme–substrate complex and detains the enzyme in this state.

Keywords 18-crown-6 · Crown ether · Inhibition · Na^+, K^+-ATPase

Introduction

The crown ethers exhibit a range of pharmacological effects [1–13]. However, the detailed mechanisms of their action are still poorly understood. Here we investigate interactions between the Na^+, K^+-ATPase and a representative of a large family of crown ethers, 1,4,7,10,13,16-hexaoxacyclooctane (18-crown-6) whose structure is shown in Fig. 1 where the ATP structure was shown too to eye guide comparison. The crown consists of six ethylene oxide (–O–CH_2–CH_2–) units, joined covalently into a relatively rigid macrocyclic ring. It does not possess ionizable groups and, therefore, belongs to nonelectrolytes. However, it has a ring of oxygen atoms surrounded by an external hydrophobic ring. These structural features enable the crown to form stable complexes with alkaline metal ions [16–18] in which the metal ion binds near the center of the oxygen ring. It is believed that crown ether effects are related to their ability to form complexes with ions and/or to facilitate ion transport across membranes. The change in the ionic composition of the aqueous environment caused by crown ether may indirectly affect the performance of numerous enzymes and ion channels. Any of these structures could be used as sensor elements to determine whether the change in ionic composition due to complex formation with ions is the only mechanism of crown ether action, or whether they have direct pharmacological effects. Na^+, K^+-ATPase is ion-dependent. It is a key enzyme in cell volume regulation, maintenance of membrane resting potential, Na^+ and K^+ electrochemical gradients, etc. A single attempt to clarify the mechanism of crown ether action against this enzyme [19] did not permit definitive conclusions, because an indirect method was used to measure Na^+, K^+-ATPase activity. In the present study we have employed a direct measurement of Na^+, K^+-ATPase activity to elucidate the problem.

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Materials and methods

Adenosine 5′-triphosphatase of the porcine cerebral cortex was purchased from Sigma Chemical Co. (St. Louis, MO)

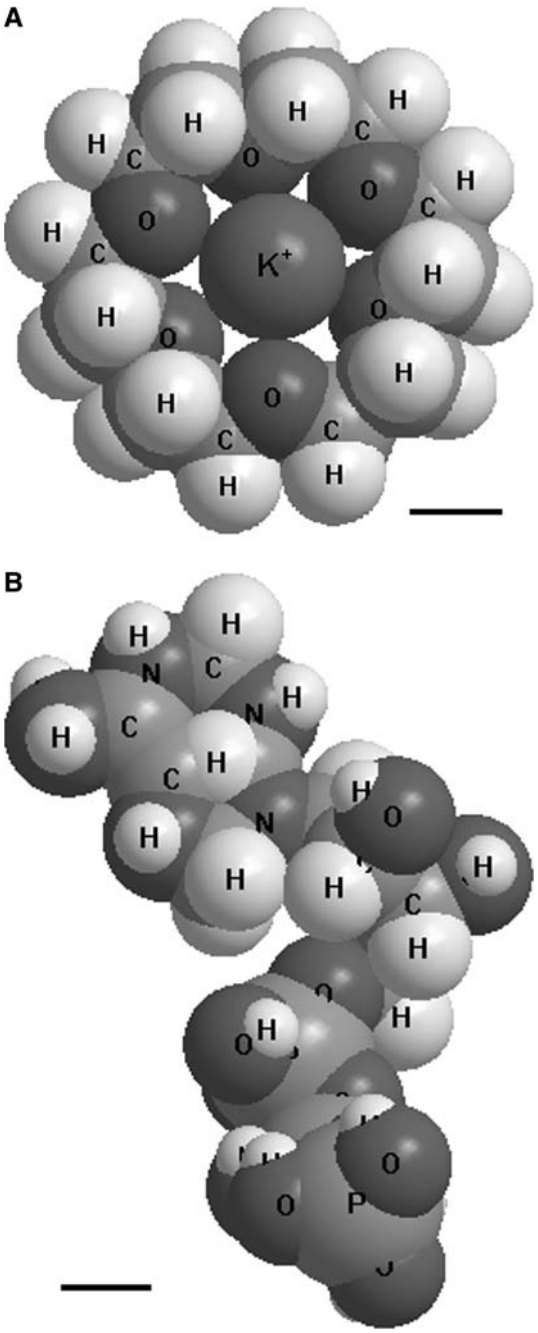

Fig. 1 Space filling model of the crown ether and ATP. CS Chem3D Pro (CambridgeSoft, Cambridge, MA) was used to build the model and to draw the figure. The structures are in agreement with crystallographic data [14, 15]. Atoms of the crown and ATP are marked by respective chemical symbols. Scale bar 0.2 nm

(Cat # A-7510). ATP-Na_2 (Adenosine 5′-triphosphate disodium salt) Sigma ultra, bovine serum albumin, magnesium chloride, sodium chloride and potassium chloride were obtained from Sigma Chemical Co. (USA). 1,4,7,10,13,16-hexaoxacyclooctane (18-crown-6), SDS (Sodium dodecyl sulfate) and ouabain were purchased from Fluka Chemicals GmbH (Germany). TRIS (tris-[hydroxymethyl]aminomethane) ultra pure was supplied by MP Biomedicals (USA). All other reagents were of analytical grade.

Enzyme assays

Na^+, K^+-ATPase activity were measured using a modification of the assay described by Spokas and Spur [20]. The standard reaction medium used to assay contained (mM): 100 NaCl, 10 KCl, 5 $MgCl_2$, 50 TRIS and 0.01% of bovine serum albumin, adjusted to pH 7.8 with HCl. Adenosine 5′-triphosphatase porcine cerebral cortex and 18-crown-6 were added to the reaction mixture in proper concentrations and preincubated for 30–60 min at room temperature (25 ± 1 °C). The enzymatic reaction was initiated by the addition of ATP in concentration of 0.5 ÷ 4 mM adjusted to pH 7.8 by TRIS. Reaction was carried out at 37 °C for appropriated time interval and then stopped by the addition of 10% SDS dissolved in 100 mM sodium acetate, pH 4.0. Concentration of liberated inorganic phosphorus (*Pi*) was determined as described by [20]. Controls with addition of the enzyme preparation after addition of 10% SDS were used to correct for nonenzymatic hydrolysis of the substrate. The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the presence of 1 mM ouabain (ouabain-resistant activity). The difference in *Pi*-liberation seen represents the Na^+, K^+-ATPase activity. The activity was expressed in μmol of *Pi* released per hour per milligram of protein (μmol $Pi \times mg^{-1}$ protein $\times$ h^{-1}). Protein was measured by the Lowry method [21] using bovine serum albumin as standard. In accord with supplier the activity of Na^+, K^+-ATPase was measured of 16.7 ± 1.4 μmol $Pi \times mg^{-1}$ protein $\times h^{-1}$ in standard solution in the presence of 4 mM ATP as substrate.

Software Mathematica 2 (Wolfram Research, Inc.) was utilized to calculate the change in ion concentration in the presence of the crown ether. To account for the effect of changes in ionic composition (due to the crown ether/cation complex formation) on enzyme activity, we employed a control in which the concentrations of K^+ and Na^+ ions were identical to those of the standard solution in the presence of the crown ether. The Na^+, K^+-ATPase activity measured at this adjusted solution was taken as the true control for experiments with the crown. All samples were run at least in triplicate.

Kinetic determinations

To analyze the kinetics of ATPase reaction we have use the nonlinear approaches which offer substantial reduction in

experimental time and is being pursued to determine the minimal experimental design that is compatible with estimation of accurate parameter values [22].

The rate constants

The observed liberation of inorganic phosphate (under influence of Na^+, K^+-ATPase) versus time data were fit according to the nonlinear expression

$$[Pi] = \frac{k_1[ATP_o] - k_{-1}[Pi_o]}{k_1 + k_{-1}}\left(1 - e^{-(k_1+k_{-1})t}\right) \quad (1)$$

where $[Pi]$ is the concentration of liberated inorganic phosphate; $[Pi_o]$ is the measured inorganic phosphate concentration at zero time. Obtained values (0.6μM/l and 4.8μM/l for 0.5 mM/l and 4 mM/l ATP, respectively) are in accord with the specification supplied by the manufacturer; $[ATP_o]$ is the initial concentrations of ATP; k_1 and k_{-1} are the effective rate constants of the direct and the reverse enzymatic reactions and t is time in seconds. Nonlinear regression fitting was accomplished with use of the Microcal Origin 6.0 program (OriginLab Corporation, Northampton, MA, USA).

The type of inhibition was determined using the Lineweaver-Burk double reciprocal plot, by plotting $\frac{1}{V}$ against $\frac{1}{[ATP]}$ analyzed over a range of ATP (0.5–4.0 mM) in the absence and in the presence of crown ether.

The apparent enzymatic constants, K_m^* and V_m^*, were estimated from the average observed rate versus substrate concentration data (in the presence and absence of 18-crown-6) by the nonlinear expression according to Michaelis–Menten equation:

$$V = \frac{V^*_{max}[ATP]}{K^*_m + [ATP]} \quad (2)$$

The inhibitor constants

The average observed reciprocal rate, $\frac{1}{V}$, versus reciprocal substrate concentration, $\frac{1}{[ATP]}$, data (in the presence and absence of 18-crown-6) were fit according to the full Lineweaver–Burk equation for noncompetitive (mixed) enzyme inhibition

$$\frac{1}{V} = \frac{aK_m}{V_{\max}}\frac{1}{[ATP]} + \frac{b}{V_{\max}} \quad (3)$$

where $a = 1 + \frac{[Crown]}{K'_i}$; $b = 1 + \frac{[Crown]}{K''_i}$; K'_i and K''_i is the dissociation constant for inhibitor (crown ether)-binding steps, with the free enzyme ($K'_i = \frac{[E][Crown]}{[ECrown]}$) and with the enzyme–substrate complex ($K''_i = \frac{[ES][Crown]}{[ESCrown]}$), respectively.

In the other instance, the average observed rate versus substrate concentration data (in the presence and absence of 18-crown-6) were fit according to the full nonlinear expression for noncompetitive enzyme inhibition

$$V = \frac{V_{\max}[ATP]}{aK_m + b[ATP]} \quad (4)$$

Regression fitting was accomplished with use of the Microcal Origin 6.0 program (OriginLab Corporation, Northampton, MA, USA). In both approaches, the control data (i.e., no inhibitor, $a = b = 1$) were first analyzed to obtain $V_{\max}$ and K_m. Those estimated values were then fixed in fitting the average rate data obtained from the inhibition experiments for estimation of K_i with Eq. 3 and Eq. 4.

Statistical analysis

The results are presented as means ± S.D. of the number of experiments indicated in the figures.

Results and discussion

To study the influence of chelating agents, like 18-crown-6, on the activity of ion-sensitive enzymes like Na^+, K^+-ATPase one needs to take into account their indirect influence through the change of activity of ions. In other words to control experiments the ion composition of the standard solution should be changed to achieve the concentrations being in the presence of the chelators. 18-crown-6 is a one of those agents. So we start our study of 18-crown-6 influence on the Na^+, K^+-ATPase from the estimation of the changes evoked by it in concentration of the most important (for this enzyme) ions Na^+, K^+ and Mg^{2+} which concentrations in the basic standard solution were 100, 10 and 5 mM, respectively. In accord with [18] and our own recent study [23] the formation constants, K_F, of the crown interaction with Na^+ and K^+ were taken equal 109.65 and 3.72, respectively. We did not find the published value for the crown/Mg^{2+} formation constant in aqueous solution with chloride as anion. So we estimate it by extrapolation of the log K_F versus ionic radii dependence using known [24, 25] values for Ca^{2+}, Sr^{2+} and Ba^{2+}. So, value of log K_F for Mg^{2+} was estimated to be of -1.4. Mathematica 2 software (Wolfram Research, Inc.) was utilized to calculate the actual concentration of Na^+, K^+ and Mg^{2+} in the presence of increasing concentrations of 18-crown-6. As expected, the decrease in Mg^{2+} concentration was negligible (<0.5%). On the other hand, the results demonstrated a considerable decrease in the 'free'

concentration of both monovalent cations in the presence of 18-crown-6 (Fig. 2): the higher the concentration of the crown, the more pronounced the decrease in cation concentration. By reason of the specificity of 18-crown-6, the decrease in K^+ concentration is much more pronounced. At the highest concentration of 18-crown-6 used (40 mM), the effective concentrations of Na^+ and K^+ decreased from 100 mM and 10 mM (standard solution) to ~92 mM and ~3 mM, respectively. To account for the effect of changes in ionic composition (due to the crown ether/cation complex formation) on enzyme activity, we employed a control in which the concentrations of K^+ and Na^+ ions were identical to those of the standard solution in the presence of the crown ether.

Examples of time courses of Na^+, K^+-ATPase activity are shown in Fig. 3. As expected, decreasing the effective concentration of Na^+ and K^+ from 100 mM and 10 mM to ~92 mM and ~3 mM (to imitate the chelator's effect of 40 mM 18-crown-6) leads to a moderate reduction in enzyme activity. The effective rate constant of *Pi* liberation, *K*, measured in the presence of 4 mM ATP under these true control condition (see Methods) was established to be of $1.7 \pm 0.1 \times 10^{-6}$ s^{-1}. In the presence of 40 mM 18-crown-6, the effective rate constant is decreased almost two fold ($p < 0.001$) to $0.9 \pm 0.1 \times 10^{-6}$ s^{-1} (Fig. 3). The inhibition is not overcome by increasing the substrate (ATP) concentration from 0.5 mM to 4.0 mM (Fig. 3**B**).

To learn about the type of the inhibition, the addition experiments were done with different concentrations of ATP. One hour incubation time was chosen to make the crown effects more significant. Results of the typical experiments obtained in the presence of 40 mM crown are presented in Fig. 4. From Lineweaver-Burk plot (Fig. 4**A**) one could suppose the crown inhibits the ATPase by "noncompetitive" (mixed) mechanism. Nonlinear regression fitting of the data like those presented in Fig. 4**B** with

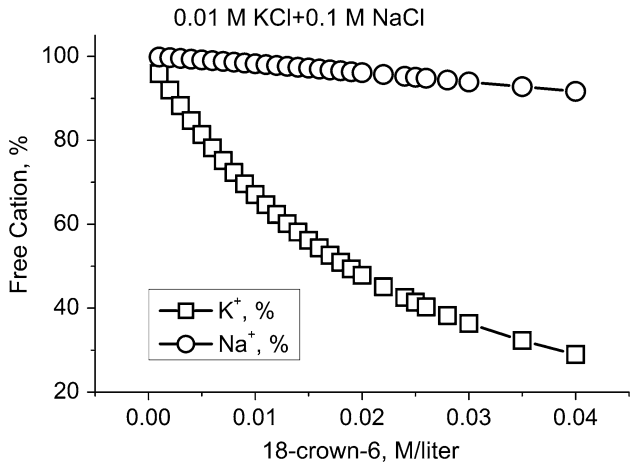

Fig. 2 The influence of 18-crown-6 on the concentration, %, of Na^+ and K^+ in the standard solution

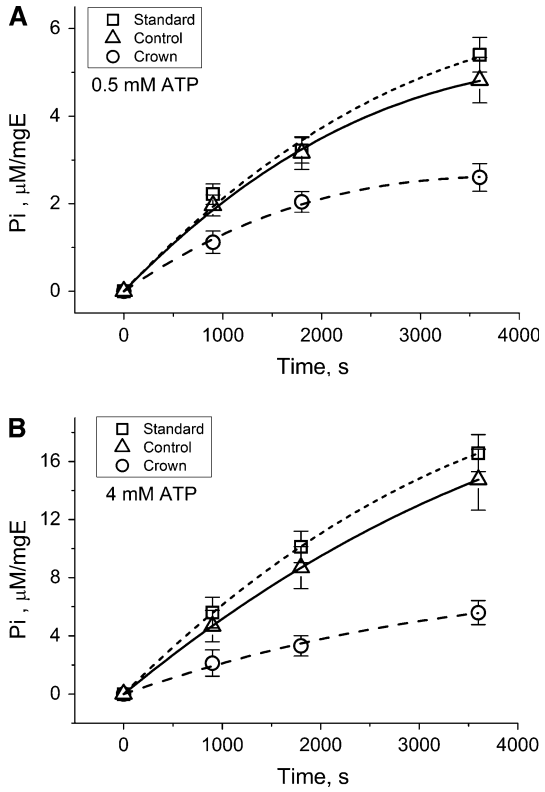

Fig. 3 Typical kinetic courses of *Pi*-liberation under Na^+, K^+-ATPase (0.05 mg) action in the presence of 0.5 mM/l (**A**) and 4 mM/l (**B**) ATP. The final crown concentration was 40 mM. The control reaction medium used to assay contained (mM): 91.64 NaCl, 2.9 KCl, 5 $MgCl_2$, 50 TRIS and 0.01% of bovine serum albumin, adjusted to pH 7.8 with HCl. Other experiments were carried out with the standard medium as described in Methods. Each point represents averages (± SD) of three independent experiments. Data sets were fit according to the nonlinear Eq. 1. Note the change in the *Pi*-scale from (A) to (B)

Eq. 2 confirms the assumption, because the crown decreased both parameters of the enzymatic reaction, K_m^* and V_m^*, from 1.25 ± 0.2 mM and 19 ± 2 μmol *Pi* × mg protein^{-1} × h^{-1} to 0.65 ± 0.06 *mM* and 6.5 ± 0.2μmol *Pi* × mg protein^{-1} × h^{-1}, respectively. This type of the inhibition supposes that both the enzyme and the enzyme-substrate complex bind inhibitor. Presumably 18-crown-6 binds to sites of Na^+, K^+ ATPase that participate in both ATP binding and hydrolysis. Another words, 18-crown-6 can bind to ATPase at it free from the substrate state and by this manner prevent or difficult the formation of enzyme–substrate complex. Moreover, it can bind to the enzyme when the complex is already formed and stopped the enzyme at this step. This kind of inhibition cannot be overcome by increasing the substrate concentration (Fig. 3). So, 18-crown-6 acts as a noncompetitive "mixed" inhibitor.

In the preceding publication [19] suffer from two incorrectnesses. First, the authors did not take into

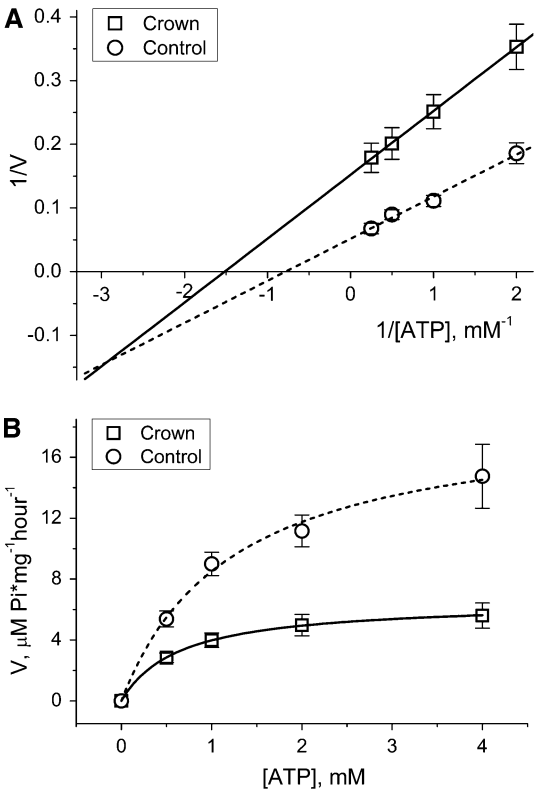

Fig. 4 Lineweaver–Burk (**A**) and Michaelis–Menten (**B**) plot of the inhibition of Na^+, K^+ activated ATPase (0.05 mg/ml) by 40 mM 18-crown-6. Data points represent averages (± SD) over at least three separate experiments. The dashed line illustrates the fitting of the control data (in the absence of 18-crown-6) to the Lineweaver–Burk equation, $\frac{1}{V} = \frac{K_m^*}{V_{max}^*}\frac{1}{[ATP]} + \frac{1}{V_{max}^*}$ (A) or to the Michaelis–Menten equation (B; Eq. 2). V_{max}^* and K_m^* values obtained from these control data fits (i.e., no inhibitor) were then fixed in fitting the average rate data obtained from the inhibition experiments for estimation of K_i. The solid line illustrates such fitting of the data (in the presence of 18-crown-6) to the full Lineweaver–Burk (A, Eq. 3) or to the Michaelis–Menten (B, Eq. 4) equations for mixed inhibition

consideration the changes in ionic composition of the reaction mixture (Fig. 2) due complex formation by crown ethers that considerably alter the Na^+,K^+ ATPase activity (Fig. 3). Moreover, they have used the indirect method (NADH coupled assay) to evaluate the effect of crown ethers on the kinetics of Na^+, K^+ ATPase. In those assay, the hydrolysis of ATP is coupled to the oxidation of NADH, using pyruvate kinase and lactic dehydrogenase [26]. For each ADP produced, one phosphoenol is converted to pyruvate, restoring the ATP. The pyruvate, in turn, reacts with NADH to produce lactate and oxidizes the NADH to NAD^+, which was monitored at 340 nm and assumes to reflect the hydrolysis of ATP. A priori, crown ethers can affect all enzymes used in the assay preventing the precise conclusion concerning the Na^+, K^+ ATPase. Because both incorrectnesses, the authors so were able to indicate that the crown inhibits the Na^+, K^+ ATPase possibly through uncompetitive or noncompetitive mechanisms.

We have used the direct method of measurement of Na^+, K^+ ATPase activity via record of the *Pi*-liberation. So, our study removes all doubts being left by the preceding publication [19], permitting conclusion that the inhibition effect of the 18-crown-6 is the result of it direct interaction with the enzyme and that 18-crown-6 acts as a noncompetitive "mixed" inhibitor.

To establish the inhibition constants for the crown binding to these two states of Na^+, K^+ ATPase, the average observed rates versus substrate concentration data (in the presence of the crown) were fit according to the full nonlinear expressions for noncompetitive enzyme inhibition (Eqs. 3 and 4). As the result, the apparent dissociation constants, K_i, for the crown interaction with the free enzyme and with the enzyme–substrate complex were established to be of 77 ± 3 mM and 21 ± 2 mM, respectively. The result points out that 18-crown-6 do not have a strong affinity to enzyme. However, there is a difference in the inhibition constants which could be related to postulated conformational changes during the enzymatic cycle of ATPase. The crown interacts stronger with the enzyme–substrate complex freezing the enzyme at this state. Such ability of the crown together with it close relation with linear poly-(ethylene glycol) (PEGs), polymers widely utilized in crystallization of proteins [27, 28] permits to set up a hypothesis that the crown ethers (and 18-crown-6, in particular) could be useful in crystallization of Na^+, K^+ ATPase and, as a consequence of this, in the establishing (decoding) its three-dimensional structure.

Conclusion remarks

We used a 18-crown-6 and Na^+, K^+-ATPase of the porcine cerebral cortex as a molecular probes to detect possible pharmacological effects of the crown ethers on ion dependent enzymes. The choice was motivated by the importance of the enzyme and 18-crown-6 in cell physiology and in developing new medicines, respectively. The results and tentative conclusions are:

- The crown ether possesses pharmacological effects on the Na^+, K^+-ATPase activity in addition to the expected indirect influence mediated through the changes in the ionic composition of the aqueous solutions.
- The crown acts as "noncompetitive" or "mixed" inhibitor decreasing both main kinetic parameters: the Michaelis constant, K_m, and the maximal rate of ATP hydrolysis, V_m.
- Crown ether binds to "free" enzyme and to enzyme–substrate complex. Binding to the enzyme–substrate

complex is about four times stronger than to the "free" enzyme. Hence, in the presence of the crown Na^+, K^+-ATPase spend more time in the complex with substrate.

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