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Topical Review

Functional Properties of Na,K-ATPase, and Their Structural Implications, as Detected with Biophysical Techniques

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Abstract. A full understanding of the molecular mechanism of ion transport and energetics of the Na,K-ATPase will require both structural and functional data. During recent years biophysical methods have provided a number of important pieces of information on ion binding and release and the charge transfer process. This allows the formulation of kinetic models of the transport process. Although a breakthrough has not been obtained due to the lack of detailed knowledge on the three-dimensional structure with a resolution high enough to identify the ion-binding sites and the transport pathway, the functional information has structural implications that create constraints on possible mechanisms of active transport. Here we describe briefly contributions of some biophysical methods to our conceptual understanding of the ion transport process.

Key words: Sodium pump — Mechanistic properties — Binding sites — Ion transport — Structure-function relationship

Introduction

Knowledge of the working of ion translocating membrane proteins such as bacteriorhodopsin, the cytochrome-c oxidase, and the F_0F_1-ATPase was gained only after functional observations and kinetic analyses could be correlated with structural information at atomic resolution. For example, studies on bacteriorhodopsin revealed how structural transitions of the chromophore, following absorption of a photon (energy uptake), induce a movement of the Schiff's base that is used, in a precisely tailored surrounding, to transfer a proton across the central energy barrier in the transport pathway [31, 52, 53, 88]. Recently, convincing proof was given that a mechanical movement of protein subunits, which form a rotor interacting with other parts of the F_0F_1-ATPase, are able to synthesize ATP by a "downhill" movement of protons through the F_0 unit or to pump protons "uphill" on the expense of ATP hydrolysis [22, 57, 63, 69, 96]. In this case concepts of well known macroscopic machines, like a mechanic pump or a water-driven mill, can be used, when scaled down, to describe these highly specific machines with dimensions of a few nanometers.

In the case of the P-type ATPases such a comprehensive understanding is still lacking. Structural details are now just becoming available, and in addition a complete mechanistic concept of the energy transduction has not been formulated so far. Ion pumps need not necessarily work as a scaled-down version of known macroscopic machines.

The Na,K-ATPase is a pump, in that it moves ions across the cell membrane from a lower to a higher electrochemical potential. However, the coupling of the scalar enzymatic process that transfers the Gibbs free energy from the energy-rich ATP to the protein, and the vectorial ion transport from one side of the membrane to the other is poorly understood. The energetics of the P-type ion pumps, in so far as they have been determined, reveal that none of the known reaction steps constitute a "power

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stroke", analogous to that of a mechanical pump, which transfers the energy into the ion-moving process [4, 87, 94].

As discussed below, there are numerous functional observations that emerge from studies of charge movements and other biophysical techniques. The conclusions provide constraints on possible structural features and must be compatible with insights obtained by other approaches such as mutagenesis. Eventually all of the functional data must be explicable in terms of molecular structure when this becomes available.

Structural Features

The Na,K-ATPase, like the gastric H,K-ATPase, consists of two subunits, α and β. The genes for the Na,K-ATPase have been cloned, including isoforms expressed in a variety of different tissues [51, 59, 71, 83]. For the α-subunit, consisting of about 1,000 residues, a variety of experimental techniques [9, 26, 44, 46, 58, 82] have demonstrated the existence of 10 transmembrane segments [45], with both N- and C-termini cytoplasmic. Amino acids that are part of ATP- and cardiac-glycoside binding sites or that are related to the Na^+ and K^+ occlusion sites have been identified mainly by mutation experiments. The β-subunit, consisting of about 300 residues, has a single transmembrane segment, with a cytoplasmic N-terminus, and three conserved S-S bridges and three glycosylation sites in the extracellular domain. The β-subunit does not seem to contain functional sites but is required for stabilization of the α-subunit and passage from the endoplasmic reticulum to the cell membrane. A recent survey of the field is given in the proceedings of the "9th International Conference on Na/K Pump and Related Pumps" [90].

Some information on structural features of Na,K-ATPase has been gained from electron microscopic images [44, 84, 86] and from electron density maps obtained from two-dimensional crystals [32, 33, 85]. These allowed also a three-dimensional reconstruction of the overall shape of the protein with resolution in the order of 25 Å [34, 35, 65, 66]. Much better progress has been made in studies of sarcoplasmic reticulum Ca-ATPase by electron microscopy. This led to a model at 8 Å resolution, showing head, neck, and membrane sectors with 10 transmembrane segments [97].

An event of outstanding importance is the recent publication of the 2.6 Å crystal structure of sarcoplasmic reticulum Ca-ATPase [91]. Since the Ca-ATPase and the Na,K-ATPase are close members of the same family of proteins [92], presumably the structure of Na,K-ATPase will resemble that of Ca-ATPase, particularly within the cytoplasmic domains with greatest sequence similarity, but show detailed differences related to the cation specificities, and the presence of a β-subunit. The structure confirms the topological organization of ten transmembrane helices deduced for Ca, Na,K-, H,K- and H-pumps by biochemical techniques [61], and reveals several unexpected features. It was found [91] that (i) both ions are located side by side with a distance of 5.7 Å approximately in the middle of the transmembrane section of the protein, (ii) the ion binding sites are surrounded by the transmembrane helices M4–M6 and M8, (iii) the α helices M4 and M6 are partly unwound to provide efficient coordination geometry for the two Ca^{2+} ions, and (iv) a cavity with a rather wide opening, surrounded by M2, M4 and M6 is discussed as an access structure on the cytoplasmic side. The outlet of Ca^{2+} is likely to be located in the area surrounded by M3–M5. The details of Ca^{2+} occlusion sites fit well with that deduced in extensive mutagenesis studies [14, 62]. The cytoplasmic sector is divided into three domains, two domains N (nucleotide) and P (phosphorylation) within the loop between M4 and M5, well separated from a third A (actuator or anchor) domain containing the loop between M2 and M3 and the strand leading into M1. The fold of the P-domain is like that of L-2-haloacid dehalogenase and related proteins with homologies to P-type pumps in conserved cytoplasmic sequences [6, 77]. Comparison of the crystal structure (an $E_1$2Ca conformation) with cryoelectron microscope images of Ca-ATPase in both E_1 or E_2 conformations [20], suggested that in E_2 a large movement of domain A brings it into contact with the P/N domain (*see below*). The latter suggestion fits well with our recent evidence for conformation-dependent domain movements based on Fe-catalyzed oxidative cleavage [27, 28, 72]. The large separation between cation sites within transmembrane segments and ATP sites within the cytoplasmic domains, seen in the X-ray structure and inferred previously from mutagenesis and proteolysis, raises the issue as to the nature of their interaction. It is proposed that the cytoplasmic loop between M6 and M7, which also makes contacts with the P-domain, plays a crucial role in the energy transduction process.

Crystal structures of soluble monovalent cation binding proteins show that the bound cations are largely dehydrated and ligated by several oxygen-containing ligands, including carboxylate, serine and threonine hydroxyls, amide oxygens and backbone carbonyl oxygens. Based on these precedents intensive mutagenesis studies of Ca-ATPase and Na,K-ATPase of such residues within transmembrane segments have been carried out [1, 14]. For Na,K-ATPase, which is the main topic of this essay, there is evidence that E327 in M4, S775, T774, N776 and E779 in M5, D804 and D808 in M6 are involved in monovalent cation binding and occlusion [43, 68, 70, 73].

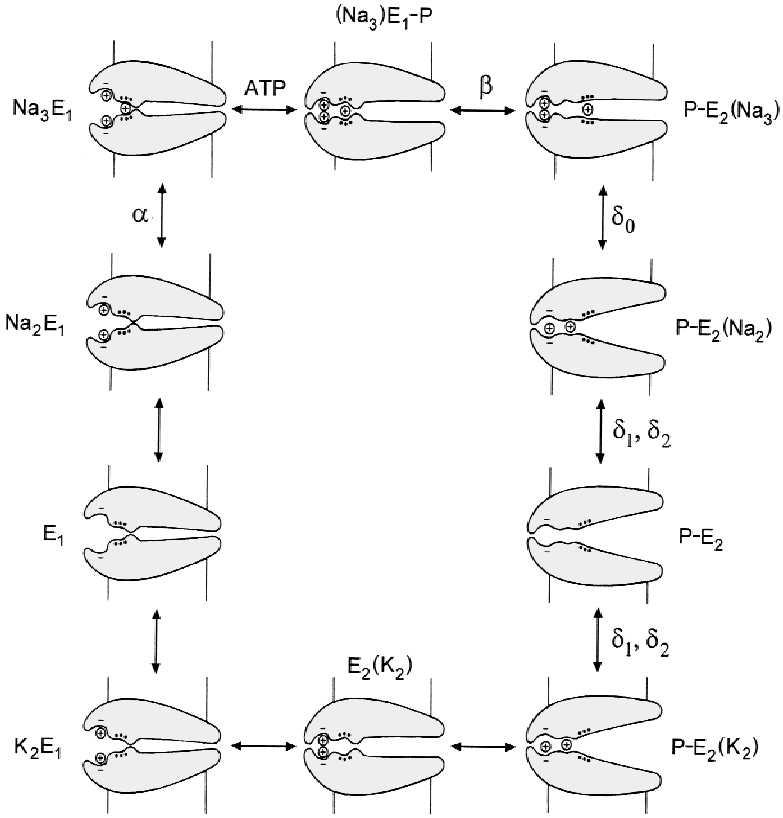

Fig. 1. Mechanistic model of the ion transport through the Na,K-ATPase on the basis of the so-called Post-Albers cycle under physiological buffer conditions (adapted from [95]). The representation of the ion pump is schematic and does not imply real structural elements. Those reaction steps that are marked with Greek letters indicate the electrogenic processes that were found and corresponding dielectric coefficients are $\alpha = 0.25$, $\beta \leq 0.1$, $\delta_0 = 0.7$, $\delta_1 = \delta_2 = 0.1$–$0.2$ [19, 37, 39, 79]. For details *see* text.

Functional Properties with Structural Implications

During recent years biophysical experiments using a variety of techniques have been focusing on the ion movements through the Na,K-ATPase [4, 18, 75]. The conceptual framework for such studies is the so-called Post-Albers cycle (Fig. 1) which is the generally accepted scheme to describe the sequence of reaction steps of coupled ion transport and enzymatic reactions. It has been established that Na^+ and K^+ transport occur by a "Ping-Pong" mechanism, meaning that the two ion species are transported sequentially. Na^+ movement out of the cells is associated with phosphorylation by ATP and then the conformational change E_1-P $\rightarrow$ E_2-P, while the counter transport of K^+ ions is associated with rapid dephosphorylation of E_2-P and the conformational change $E_2(2K) \rightarrow E_1$ which is accelerated by binding of ATP with a low affinity [55]. A characteristic property of the reaction sequence is the existence of occluded states, E_1-P(3Na) and E_2(2K), in which the ions are unable to exchange with either aqueous phase [25]. The stoichiometry of 3 Na^+/2 K^+ per ATP hydrolyzed is maintained in a wide range of electrolyte compositions, deviations being observed only at extremely low Na^+ concentrations or at extremes of pH [5, 10, 15, 16, 29]. Due to the unequal quantities of charge transported in opposite directions the pump produces an electric current in which a net positive charge is extruded from the cell. This property of the ion pump is called electrogenicity [55]. An effective way to investigate ion transport is the detection of charge movements in the course of the transport process [2, 18, 55, 75]. Because of the Ping-Pong mechanism it is possible to study separately the Na^+- and K^+-transport pathways and the associated charge movements. The ion-translocating partial reactions have been scrutinized extensively with respect to their kinetics, energetics and electrogenicity, and the experimental findings place a number of constraints on any mechanistic model of the Na,K-ATPase. As can be seen from Fig. 1 the charge-translocating steps of the transport process are mainly correlated with ion binding and release (for a recent review *see* [18]).

K^+ Transport

There is convincing experimental evidence that K^+ transport is electroneutral, i.e., no net charge is moved within the protein between states E_2-P(2K) and E_1 [8, 19, 30, 76]. Only the extracellular binding (or release) of the ions is electrogenic [12, 76]. This partial reaction can be investigated in the so-called K^+/K^+ exchange mode, in

the presence of K^+, with or without Mg^{2+} and inorganic phosphate, P_i, and ATP [50]. The findings can be understood by assuming that (i) the positive charges of the two ions are counterbalanced by two negative charges of the protein, (ii) the binding sites in state E_2-P are located inside the protein and are accessible through a narrow ion well [12] and that (iii) in the E_1 conformation binding of K^+ (or its congeners Li^+, Rb^+, Cs^+, Tl^+, NH_4^+) is electroneutral, i.e., the binding sites are not buried inside the protein [17, 19, 30, 95]. In the absence of K^+ and Na^+ it is possible to phosphorylate the enzyme by P_i and the apparent rates of this pathway are pH dependent, the lower the pH the faster is the rate [3]. From the analysis of the kinetics it was concluded that the transition E_2-P $\rightarrow E_1$ occurs with two protons bound and the transition with empty binding sites is either extremely slow or absent. As a consequence, in the so-called Na-only mode, when no extracellular K^+ is present, the transition from E_2-P back to E_1 occurs when the binding sites are occupied by 2 protons (as congeners of K^+), and thus the Na^+-only transport is actually a 3 Na^+/2 H^+ transport mode [3]. This can be understood in the framework of the energetics of the ion pump since movement against the negative membrane potential of the two negative charges in the binding sites is not a favorable event [3]. There is also evidence that the Na^+/H^+ stoichiometry can vary with pH, and at pH 8.5 a slow rate of electrogenic Na transport without exchange with protons appears to be possible [29].

Na^+ Transport

Forward Na^+ transport ($E_1 \rightarrow E_2$-P, i.e., out of the cell) requires ATP and, except in conditions of very low Na concentrations [11, 29, 74, 89], ATP hydrolysis occurs only with 3 Na^+ bound to the protein. Even if only Na^+ ions are present on the cytoplasmic side of the pump, virtually no transition into state E_2(2Na) has been found, by contrast to other congener cations (K^+, Rb^+, Cs^+, NH_4^+, Tl^+), which antagonize Na^+ binding and cause a conformational change into the occluded E_2 state after two ions have bound. The virtual absence of the E_2(2Na) state is in agreement with the observation that binding of the third Na^+ ion occurs with a higher affinity than binding of the second and stabilizes the protein in the Na_3E_1 state. This can be understood only by assuming that the third Na binding site becomes available after two Na^+ ions have already bound [78]. Binding of the first two Na^+ is electroneutral (like binding of 2 K^+ ions or their congeners); binding of the third Na^+ is electrogenic with a dielectric coefficient of 0.25 [19, 95]. Occupation of the third, highly selective Na^+ site, which is formed by transmembrane parts of the Na,K-ATPase [13, 43, 81], is strictly correlated with an effect on the fluorescent FITC label that is bound to a lysine, K501, inside the nucleotide binding site [21, 47, 60]. The mechanism of this label (its chromophore is a fluorescein) is that of a pH indicator which detects (small) changes of the local proton concentration. Such changes may be produced by rearrangements of the amino acids which form the ATP binding pocket [24, 91]. In the case of the Na,K-ATPase, FITC responds preferentially to the conformational transition $E_1 \rightleftharpoons E_2$, which is known to alter the ATP-binding affinity between low (E_2) and high (E_1) [47]. A smaller but significant fluorescence decrease upon binding of the third Na^+ can be interpreted as a minor conformational relaxation in the nucleotide binding site, probably including a movement of aspartate D371 into a position where it coordinates with the γ_A phosphate of the ATP bound in the nucleotide binding site. Thus, binding of the third Na^+ makes the enzyme competent to become phosphorylated, and ensures that no ATP is wasted without three Na^+ bound to the pump [79].

When phosphorylated by ATP, the enzyme performs a conformational transition into its E_2-P states, in which the bound Na^+ ions are successively deoccluded and released [37, 38, 40, 95]. While the conformational relaxation is of minor electrogenicity, the release of the first Na^+ to the extracellular aqueous phase is the dominant charge-carrying step. It was found that this ion moves through ~70% of the protein dielectric [40, 95]. This may be explained by a narrow and deep access channel or "ion well" between the binding site in the protein and the aqueous outside of the protein [55, 56]. The release of the first Na^+ is followed by another conformational relaxation that brings the remaining two Na^+ ions closer to the extracellular aqueous phase, because they have to cross only 10–20% of the membrane dielectric when they are released in the next reaction steps [40, 95]. (In the E_1 conformation these two ions are bound to a position close to the "dielectric" surface, i.e., their binding is not electrogenic, in contrast to the third Na^+, *see above.* Therefore, they are placed "behind" the Na^+ which is first released to the extracellular side.) The kinetics of the deocclusion/release reactions have been analyzed recently and were found to occur with increasing rate constants from the first ion ($\lesssim$1,000 sec^{-1}) to the third ($\geq 10^6$ sec^{-1}) [40]. The reduced electrogenicity of the second and third Na^+ release step (*see* Fig. 1) is matched by a corresponding value of K^+ binding, which follows under physiological conditions [37, 76]. Thus overall the release process may be explained by assuming that the first Na ion is released from a deep access channel while the next two Na ions are released in a shallow channel. At least two different mechanisms could explain the change from a "deep" to "shallow" ion-well: (i) In a major structural rearrangement of the protein the narrow ion well widens to become a large vestibule which is then filled by electrolytes so that the electric potential surface is

deformed and bends into the vestibule, close to the binding sites, or (ii) water molecules penetrate the protein matrix and increase the dielectric constant of the environment of the binding site significantly. This process would also deform the course of the electric potential within the protein [95]. The latter mechanism would allow the immediate rehydration of the ions when they are released from the sites without having to migrate as unscreened charges through the protein matter whose polarization would be rather energy consuming.

Competition of Ions at the Binding Sites

In both principal conformations, in which the ion-binding sites are accessible from the aqueous phases, competition between cations is observed. On the extracellular side, where under physiological conditions the Na^+ concentration is 20–30-fold higher than that of K^+, it was found that K^+ has a 1,000-fold higher affinity than Na^+ [37]. This property of the binding sites in the E_2-P states shifts the ion binding and release sequence, E_2-P(3Na) $\rightleftharpoons$ E_2-P(2Na) $\rightleftharpoons$ E_2-P $\rightleftharpoons$ E_2-P(2K) $\rightleftharpoons$ E_2(2K), strongly to the right side, and avoids K^+-binding becoming a rate-limiting step under turnover conditions. Larger monovalent organic cations, such as amines, which are neither occluded nor transported, affect K^+ (or Rb^+) binding from the extracellular side by blocking one of the ion sites [23].

On the cytoplasmic side the binding affinity for Na^+ ions is significantly higher than at the extracellular side. Whereas in equilibrium titration experiments the apparent half-saturating Na^+ concentration in the E_2-P state was in the order of 500 mM [37], in the E_1 state a value of 4 mM was found when 5 mM Mg^{2+} were present [78, 79]. The apparent affinity for K^+ ions was not markedly different in both conformations, E_2-P and E_1, with an apparent half-saturating concentration of 0.1 mM [12, 36, 47, 79]. The effective cytoplasmic affinity of 0.1 mM is the consequence of a low intrinsic K_M in E_1 which is amplified due to the trapping in E_2(2K) [47]. Bearing in mind that in the cytoplasm the concentration ratio of K^+/Na^+ is of the order of 30, under equilibrium conditions the ion-binding and release sequence, $E_1$2K $\rightleftharpoons$ E_1 $\rightleftharpoons$ $E_1$2Na $\rightleftharpoons$ $E_1$3Na, should be shifted quantitatively to the left, with only less than 1% of the pumps in the E_1 state with all sites occupied by Na^+, ($E_1$3Na). In normal physiological conditions the pumping rate is about 25% of maximal and it is limited by the cytoplasmic Na concentration. There are two factors that raise the degree of occupation above the 1% just predicted. In each case the ion-binding and release reaction are fast (perhaps, diffusion controlled) in comparison to other steps that raise the effective affinity for Na^+ ions. First, ATP at millimolar concentration in the cell accelerates the rate of E_2(2K) $\rightarrow$ E_1 transition by about 1,000-fold and reduces the apparent affinity for K^+ at cytoplasmic sites to approximately 10 mM, thus making K^+ ions a much less effective competitor with cytoplasmic Na ions. Second, in the presence of ATP the reaction step which follows Na^+ binding, $E_1$3Na · ATP $\rightarrow$ E_1-P(3Na), continuously drains the preceding states, disturbing the virtual binding equilibrium in E_1 and thus producing an apparent higher affinity of Na^+ [49, 80].

Kinetics studies of Na^+ binding in the presence of K^+, and vice versa, revealed reciprocal effects on the apparent binding affinities, which may be explained by mutual competition for the same two sites to which ion binding is not electrogenic [78, 79], and it turned out that the affinity of the monovalent cations depends strongly on the radius of the dehydrated ion, being at a maximum at an ion radius of 1.4 Å, i.e., for K^+ and Rb^+ [78, 98].

The effect of the cytoplasmic Mg^{2+} on the apparent affinity of Na^+ has been known for a long time [37, 64]. Kinetic studies have shown that Mg^{2+} ions are able to bind at two different sites, in a competitive and noncompetitive manner [78]. The noncompetitive binding disappears in the experiments with 19kDa membranes produced by extensive tryptic digestion of renal Na,K-ATPase. This preparation consists of membrane-embedded fragments corresponding to transmembrane segments and intact short hairpin loops on the extracellular side whereas most of the cytoplasmic parts of the protein, including the ATP-binding site, have been digested away [13, 48, 78]. The competitive binding can be explained by a reaction scheme in which Mg^{2+} (or any other cation) is allowed to bind to the first site whereas, to the second site near the membrane surface, only monovalent cations are able to bind (Fig. 2). Other divalent cations, such as Ca^{2+}, Ba^{2+}, Sr^{2+}, behave similarly to Mg^{2+}, and Ca^{2+} is the strongest competitor of Na^+ [78, 98]. This observation parallels findings of Shainskaya et al. [82] who found that Ca^{2+} binds to a motif at the cytoplasmic loop between the transmembrane segments M6 and M7, which is thought to be involved in the ion binding in the Na,K-ATPase [82] and SR Ca-ATPase [67].

As demonstrated some years ago, the large trivalent organic cation Br_2TITU, a trivalent aromatic isothiouronium derivative, or guanidinium derivatives m-XPG and p-XPG, block Na^+ and K^+ binding and occlusion in a competitive fashion [41, 99]. A detailed analysis of the kinetics proved that these compounds behave very similarly to the divalent cations (Fig. 2), although the affinity is higher by 3–4 orders of magnitude (K_D values of 5–10 μM for m-XPG and p-XPG and 0.2–0.5 μM for Br_2-TITU). The properties of Br_2-TITU and m- or p-XPG show that they are not themselves occluded. Thus competitive blocking of Na^+ or K^+ occlusion indicates that occlusion occurs in at least two stages. At the first stage

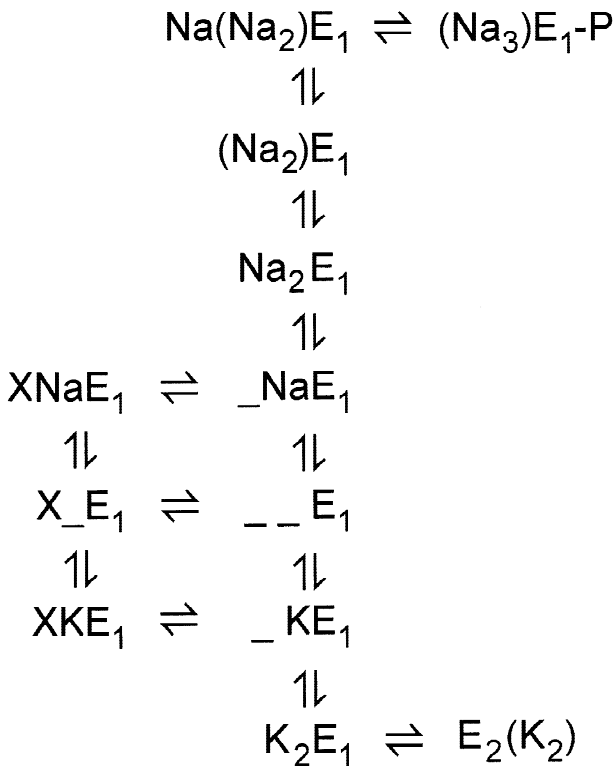

Fig. 2. Reaction scheme of the cytoplasmic ion binding mechanism that accounts for the observed competing effects of Na^+, K^+ (and its congeners), Mg^{2+} (or other divalent cations) and Br_2TITU. "X" represents any ion. For the sake of simplicity of the model it was assumed that the blocker X prevents binding of a second transported cation.

Na^+ or K^+ ions bind to a site that is affected by the blocker, and in a second stage the Na^+ or K^+ ions become occluded. More recent findings of Shainskaya et al. [82] imply that the competitive blockers including Br_2-TITU, m- or p-XPG, Ca^{2+}, Mg^{2+} bind to a motif at the cytoplasmic loop between the transmembrane segments M6 and M7, which may be thought of as the initial recognition site for cation occlusion in the Na,K-ATPase [82] and SR Ca-ATPase [67].

Structural Implications and Questions

In the E_2-P state the binding sites are inside the membrane dielectric. Although the release of all ions is electrogenic, only the Na^+ in the third site is released through a narrow access channel, which produces the major electrogenic contribution to the pump-induced transmembrane current, whereas all other ions (Na^+ or K^+) migrate through a wider access channel in contact with the extracellular aqueous phase. A proposal from the structure of the SR-Ca-ATPase in state E_2 at 8 Å resolution, in which a cavity within the transmembrane domain is claimed to be visible [97], is not confirmed by the 2.6 Å structure in the unphosphorylated (E_1) form of the pump [91]. Thus a major issue concerns the physical nature of an ion well. Whereas the major charge-carrying process, the dissociation of Na^+ ions at the extracellular surface, may occur in a deep "ion-well" within a channel-like structure, as explained above, the concept of the electrical "ion-well" reflects only the dielectric constant of the medium experienced by the moving charge, and does not necessarily imply the existence of a fixed structural feature.

Until more precise structural data are available, the current evidence from charge transfer studies suggests that the Na,K-ATPase possesses two charged sites for two Na^+ or two K^+ ions and a third neutral site specific for a Na^+ ion. In E_1 the third Na site is situated inside the protein at a position where the Na^+ has crossed about 25% of the membrane potential. This site becomes accessible only when the first two sites are occupied by two Na^+ ions. Obviously, these conclusions bear on the mutagenesis studies mentioned previously. Can the two charged sites be identified with individual carboxylate residues (E327, E771, D804, D808)? Are the two charged Na^+ binding sites identical with the two charged sites for K^+ ions? Is there overlap in the ligating groups for the Na^+ and K^+ ions as we have suggested? Is the neutral site for the third Na^+ ion to be identified with uncharged oxygen ligands already implicated (T774, N776?) or backbone carbonyls? Are all the charged residues identified by mutagenesis to be considered direct contact residues or could they participate in related structures such as "ion wells"? Apart from the requirement of molecular structure, there is an obvious need to carry out similar biophysical studies with the mutated pumps as have been done with the wild-type enzyme. In one case a mutation, E779A, has been suggested to affect the extracellular ion-well [7].

The recognition that cation sites are located within transmembrane segments, that deocclusion at the extracellular surface occurs within a deep "ion-well", and that the Na^+ transport-linked conformational change E_1-P $\rightarrow$ E_2-P is itself associated with only a minor charge movement [40, 95], effectively excludes a mobile "carrier" mechanism of transport. The alternative is the so-called moving "barrier" mechanism [54]. A "barrier" is an impediment to free diffusion of occluded states through the protein. In E_1-P(3Na) and E_2(2K) "barriers" are closed at both surfaces. In E_2-P an extracellular "barrier" is open and a cytoplasmic "barrier" is closed while in E_1 the cytoplasmic "barrier" is open and an extracellular "barrier" is closed. Obviously the question arises as to the structural meaning of "barrier". In the Ca-ATPase structure there is no obvious "barrier" to prevent dissociation of the Ca^{2+} ions other than the ligating groups themselves [91]. Presumably the alternate accessibility of the cation sites at the two surfaces could be the result

of alteration in the twist or tilt or perhaps stretch of the relevant transmembrane helices that reduces the ligation of the occluded cations and allows dissociation to one or other side.

In the E_1 conformation the structural and functional information presented before allow the presentation of a schematic but more detailed structural proposal for the Na,K-ATPase to explain the known functional details (Fig. 3). We propose an adaptation of a model to explain an ordered binding sequence for the SR Ca-ATPase [1, 42, 62] based on an initial proposal of Menguy et al. [71]. The ordered entry and side-by-side alignment of the two Ca^{2+} ions was confirmed by the high resolution structure [91]. An initial recognition site is accessible for cations of any valence, probably located within the cytoplasmic loop between transmembrane segments M6 and M7. To this site ions of any valence may bind, especially bulky organic cations such as Br_2-Titu [82]. Two charged sites within M4, M5, M6 and M8 are located beyond this loop. If a di- or trivalent cation is bound to L6/7, Na^+ or K^+ may be able to enter into the charged sites, although the apparent affinity is reduced and no occlusion will be possible. When a mixed population of Na^+ and K^+ occupies the charged sites, an occluded state is either not formed or is too short-lived to allow any subsequent reaction. The charged sites within the transmembrane segments can be occupied only by monovalent cations, and the binding pocket is structured in a way that dehydrated ions with a radius of 1.4 Å fit most tightly. Nevertheless, these sites are not buried at a significant distance from the cytoplasmic surface inside the protein dielectric, because no detectable dielectric coefficient is found when K^+ (or its congeners) bind to it. When two K^+ ions are bound to the sites (and no ATP is present) the quasi-occluded state, $E_1(2K)$, is able to undergo a (rapid) transition to the genuinely occluded state $E_2(2K)$. In the case that two Na^+ ions are bound, a quasi-occluded state, $E_1(2Na)$, may be formed and the third and Na^+-specific binding site inside the protein dielectric may become accessible; the third Na^+ binds electrogenically. Such an ordered (but non-single file) mechanism could easily explain a higher affinity of the third binding site than that of the second. This sodium-specific step triggers a structural rearrangement at the nucleotide-binding site, which then becomes competent to be phosphorylated by ATP, $E_1(3Na) \cdot ATP \rightarrow E_1\text{-}P(3Na) + ADP$. To meet the kinetic requirements for this partial reaction, i.e., appropriate rate constants, the only condition besides the listed constraints would be that ion movement in and out of the sites is fast (diffusion controlled) compared to the rates in subsequent reaction steps of the pump cycle. This model, of course, raises the question of the structural organization of cation sites within the transmembrane segments—single file or side by side. It also raises the issue of the proposed role of L6/7. It could serve as the entry port for cations [67, 82]: It does not seem impossible that the charged residues in this loop could serve both as a low affinity initial recognition site for cations on their way to becoming occluded and also as a coupling device to link transmembrane segments and the phosphorylation domain as proposed in ref. [91]: The Ca-ATPase structure in an E_1 conformation showed that the loop also makes contact with the phosphorylation domain, which, in the case of the Na,K-ATPase, may hint at the mechanism observed which requires the third Na^+ ion bound in order to enable phosphorylation of the enzyme [79].

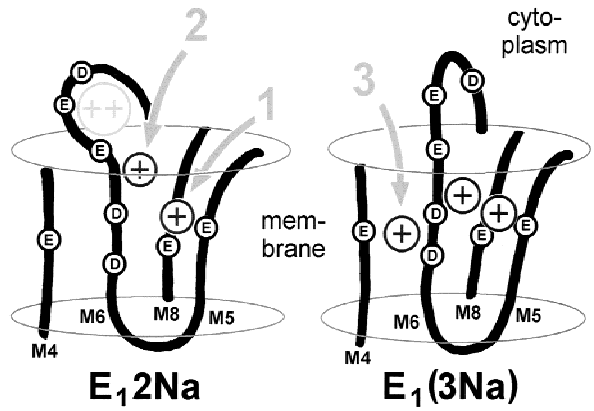

Fig. 3. Hypothetical structure-function relation of the cytoplasmic ion-binding sites of the Na,K-ATPase. Binding of three Na^+ ions is proposed on the basis of a model for the SR-Ca-ATPase [71] as discussed in the text. The negatively charged amino acids D (aspartate) and E (glutamate) are placed according to a proposal of Vilsen et al. [93]. The divalent cation presented in the left cartoon in light gray represents a blocker which affects binding of the occluded ions.

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