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Conformational Coupling: The Moving Parts of an Ion Pump

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The Na,K-ATPase carries out the coupled functions of ATP hydrolysis and cation transport. These functions are performed by two distinct regions of the protein. ATP binding and hydrolysis is mediated by the large central cytoplasmic loop of about 430 amino-acids. Transmembrane cation transport is accomplished via coordination of the Na and K ions by side-chains of the amino-acids of several of the transmembrane segments. The way in which these two protein domains interact lies at the heart of the molecular mechanism of active transport, or ion pumping. We summarize evidence obtained from protein chemistry studies of the purified renal Na,K-ATPase and from bacterially expressed polypeptides which characterize these separate functions and point to various movements which may occur as the protein transits through its reaction cycle. We then describe recent work using heterologous expression of renal Na,K-ATPase in baculovirus-infected insect cells which provides a suitable system to characterize such protein motions and which can be employed to test specific models arising from recently acquired high resolution structural information on related ion pumps.

KEY WORDS: Active transport; sodium pump; heterologous expression; conformational changes.

INTRODUCTION

It has become clear during the last two decades that the two linked functions of a P-type ATPase or ion pump, the catalysis of ATP hydrolysis, and the transport of cations across the membrane are carried out by two distinct regions of the protein. The P-type ATPases couple these activities in a precise stoichiometric fashion and the way in which the activities of the ATP binding and hydrolysis domain and the cation coordination and transport domain are connected lies at the heart of the mechanism of active transport. In other words, as ATP binds and phosphorylates the protein and ions are occluded and eventually are released at the other membrane surface, which parts or segments of the protein move relative to one another and which parts do not. It has been proposed that a core structure exists for P-type ATPases, based upon these dual functions and that modifications in structure among subgroups reflect changes in the type of transported cation (Lutsenko and Kaplan, 1995). This classification was based upon structural similarities in transmembrane and extramembrane parts of P-type ATPases.

This minireview summarizes data, which has been gathered in recent years, focusing on studies from the author's laboratory, which point to beginnings of answers to these questions. We also describe the development of a strategy, using a heterologous expression system, which will enable us, in the future, to obtain a more complete and precise understanding of the protein movements associated with pumping ions. Most of our work focuses on the Na,K-ATPase or sodium pump, however, one of the most exciting developments in recent years has been made with the SR Ca-ATPase, a closely related member of the P2-ATPase family. This was the attainment of a high-resolution structure of the Ca-ATPase (by Toyoshima and colleagues) from X-ray diffraction studies of a crystallized form of the enzyme, which was thought to be in the E_1Ca_2 conformation (Toyoshima *et al.*, 2000). This model, as well as confirming to a surprising extent what had been concluded from a wide array of biochemical data, provides a new and better basis for further structure–function studies of all the P-type ATPase family.

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ATP BINDING AND HYDROLYSIS

During the period from the mid-1960s until about 1990, most of the techniques to identify which part of the Na,K-ATPase might be associated with ATP binding and hydrolysis had relied upon studies with purified enzyme and employed protein chemistry strategies. These included modification with a variety of chemical reagents, characterization of the modified enzyme, and identification of the site of modification by amino acid sequencing of the modified protein segments (Pedemonte and Kaplan, 1990). All of these studies pointed to the large cytoplasmic loop of the α subunit, between M4 and M5, as containing most of the residues where modification affected ATP binding in a specific way. The site of phosphorylation and the essential aspartate residue (D369) had previously been identified so that it appeared that nucleotide binding and catalysis was performed by the large cytoplasmic loop of about 450 amino acids. In recent years, the great advances in cDNA-based protein expression approaches and purification of appropriately tagged proteins has filled out this picture. It has been possible to express this loop from the Na,K-ATPase in *E. coli*, to purify the polypeptide in milligram quantities and demonstrate that the structured polypeptide in solution had the same nucleotide specificity that had been previously characterized for the native Na,K-ATPase (Gatto *et al*., 1998). That is, ATP and ADP bound, while AMP, UTP, CTP, etc., did not. Thus the nucleotide specificity of the Na,K-ATPase is recapitulated by this isolated loop. It seems likely then that all of the amino acid residues, which define nucleotide specificity, are contained within this structure. Interestingly the specific reactivity of a particular residue (K501) toward fluoroscein isothiocyanate, reported in renal Na,K-ATPase by Karlish several years earlier (Karlish, 1980), is retained in the loop polypeptide and lost on denaturation (Gatto *et al*., 1998). It might be asked to what extent does the detailed structural information obtained from the Ca-ATPase pertain to the Na,K-ATPase structure. Of course, as yet, there is little hard data on which to base a sound comparison. It was only recently that, for the Na,K-ATPase, unambiguous biochemical evidence was provided for the ten transmembrane segment model of the α subunit (Hu and Kaplan, 2000a,b). In studies using DIDS, a bifunctional lysine-directed reagent, with the canine renal Na,K-ATPase, it was shown that inactivation of the enzyme was accompanied by cross-link formation between K480 and K501. The distance between these residues, in the nucleotide binding domain was estimated to be 14 Å (Gatto *et al*., 1997). Examination of the high-resolution structure of the Ca-ATPase reveals that the distance between the corresponding lysine residues in that P-type ATPase is 13.5 Å, an encouraging level of agreement.

CATION BINDING AND TRANSPORT

Again, the initial ideas about which parts of the protein may be involved in cation binding and transport were obtained from protein chemistry approaches. The essential role of occlusion, or trapping of the transported cations within a coordination center in the protein had arisen from the early and influential studies of Post and co-workers (Post *et al*., 1972). More recent work by Karlish and his colleagues showed that extensive proteolysis of the Na,K-ATPase, which removed major segments of the extra membrane parts of the protein, but left intra-membrane or membrane-associated segments relatively intact, provided a preparation that was still able to occlude Rb or K ions (Karlish *et al*., 1990). This provided critical support for the idea that occlusion occurred within the transmembrane segments, and that at least part of the hydration sphere of Na or K ions in solution might be replaced by side chains of intramembrane parts of the protein as the cation traversed the membrane. Another suggestion was that the important coordinating residues may contain carboxylate groups. The idea here being that charge neutralization would be an effective way of stabilizing the positive charge of the cations in a cation:enzyme complex within the membrane. The first evidence that this may be true was provided by the observation that a specific, positively charged carboxylate-modifying reagent (DEAC), inactivated the renal enzyme by eliminating the capacity to occlude cations (Argüello and Kaplan, 1991). The presence of cations also specifically prevented this inactivation. The single modified residue was subsequently identified as E779 (Argüello and Kaplan, 1994). This pointed to the importance of carboxyl-containing residues in cation coordination by these pumps and drew attention to the M5 segment, which contained this residue.

The identification of M5 as containing residues intimately associated with cation coordination and the presence of the probably closely apposed M6, which contained several acidic functions, immediately provided a clue about the nature of the conformational coupling. Since the cation coordination domain (in M5 and M6) was directly connected (via M5) to the nucleotide binding and hydrolysis domain, the coupling could be direct and essentially mechanical. In other words, movements in the cytoplasmic M4–M5 loop, as ATP bound, would be directly transmitted to M5 and *vice versa*; as cations bound to residues in M5 (and M6), their presence

would be directly transmitted to the M4–M5 loop. The consequences of such changes are seen in the opposing effects of Na and K on ATP affinity (E_1Na vs. E_2K) and the effect of nucleotide binding in reducing the apparent affinity of enzyme for K ions. Subsequent studies utilizing heterologous expression in mammalian cells by a number of groups, but notably Lingrel and co-workers (Jewell-Motz and Lingrel, 1993; Kuntzweiler *et al*., 1996), and Vilsen *et al*. (1997) and in other systems more recently by DePont *et al*. (Swarts *et al*., 1996) and Jorgensen *et al*. (Pedersen *et al*., 1997) have established the importance of a set of residues largely in M5 and M6, with contributions from M4 in cation coordination in the Na,K-ATPase. Similar, somewhat earlier studies in the SR Ca-ATPase by MacLennan and colleagues (Clarke *et al*., 1989) and which were extended by Andersen and co-workers (Vilsen *et al*., 1997) were recently confirmed in the high-resolution structure of the Ca-ATPase and provided a similar picture for the Ca-ATPase.

In summary, the P2-type ATPases have a cation coordination and transport domain provided by some of the intramembrane segments (through a series of highly conserved residues) and a cytoplasmic extramembrane domain for the catalytic hydrolysis of ATP within the central M4M5 cytoplasmic loop. In order to couple the activities of these regions, changes in protein conformation occur and, with this, movements of the various protein segments.

MOVEMENT OF PROTEIN SEGMENTS

It has long been appreciated that during the reaction cycle of the Na,K-ATPase, the protein undergoes a series of conformational changes. This is implicit in the idea that internally accessible Na binding sites were exposed to the extracellular medium to complete Na expulsion and that K binding sites traverse the membrane in the opposite direction. The first important demonstration of these conformational changes at the protein level involved the use of controlled proteolysis with trypsin. In these studies, Jorgensen demonstrated that the proteolytic cleavage sites were altered, depending on the presence of either Na or K ions (Jorgensen, 1975). This demonstrated a clear structural change imposed on the protein by the binding of the transported and activating cations. Some of these cleavage sites are in the M4–M5 cytoplasmic loop. Kinetic evidence for related changes in this region on the sequential binding of K ions has also been reported (Kaplan *et al*., 1998).

An extension of these kind of proteolysis studies led to a more detailed picture of the likely structural changes occurring (Lutsenko and Kaplan, 1994). In this latter work, extensive tryptic digestion was performed and the parts of the protein remaining associated with the membrane were analyzed. Although extensive proteolysis was performed, the nature of the product was governed by the ligands present during proteolytic digestion. Three main structural forms were revealed in these studies. The most compact form (i.e., most resistant to digestion) was found in the presence of ADP (or ATP), where the ATP binding domain appears to interact most closely with the intramembrane regions. When cations bind, an intermediate form is produced and the least compact form is produced when the enzyme is phosphorylated. A series of specific changes in orientation of some segments of the protein, e.g., a rearrangement of the cytoplasmic M2–M3 loop (the activator domain in the recent Ca-ATPase structure) upon phosphorylation, and the protection of the cleavage of the carboxy-terminus on cation binding or ouabain binding, were described.

In summary, these studies provided evidence for relaxed or open states of the α subunit and closed states, which are produced by the different protein conformations populated as the Na,K-ATPase progresses through the reaction cycle. These movements were interpreted in terms of the compactness of the extramembrane protein structure and in terms of the proximity of parts of the central cytoplasmic loop to the membrane. These movements resemble the changes that have been suggested to occur in the SR Ca-ATPase in the light of the high-resolution structure recently reported (MacLennan and Green, 2000; Toyoshima *et al*., 2000). It will be very interesting to reexamine these in the context of the high-resolution structure of the Ca-ATPase.

The proteolysis studies referred to above by Karlish and co-workers (Karlish *et al*., 1990) defined a post-tryptic complex of polypeptides, which remain associated with the membranes when digestion is carried out in the presence of K ions. This complex of membrane peptides is still able to occlude K ions and presumably still contains major parts of the cation occlusion structure of the native protein. When this post-tryptic preparation is incubated in the absence of K ions, a dramatic change takes place (Lutsenko *et al*., 1995). A single peptide is initially released from the membrane phase to the aqueous solution. This peptide contains the M5M6 loop. The loop contains the majority of the residues which have been identified as cation-coordinating sites for the occluded cations (Kaplan *et al*., 1997). The segment contains three carboxylic acid residues, which are intramembranous, and at least one residue, which is exposed to the extracellular milieu (Hu *et al*., 2000). The negatively charged residues have been found to play an important role in cation binding, specificity, and transport. The observation of the specific loss

of the M5M6 loop, following the removal of K ions, has several interesting consequences: (1) it appears that the M5M6 loop is more stable within the membrane when cations are bound than when it is cation free; (2) this, in turn, suggests that just as the M5M6 residues stabilize the cations, which are within the membrane phase, the reverse may also be true, i.e., the coordinated cations stabilize the negatively charged loop within the membrane; (3) such modulation of the stability of the loop within the membrane may facilitate the motion of these transmembrane segments during protein conformational changes. The notion here is that the loss of M5M6 from the membrane of proteolyzed Na,K-ATPase preparations is an amplified form of small excursions of the loop as cations bind and leave the intact protein during ion pumping. Of course, in the intact native portion, such movements are damped as the M5M6 is anchored by its connection to other membrane segments and the large cytoplasmic domain. The possibility that such movements may play a general role in P2-type ATPase function received support from the observation that this selective and directional loss (to the extracellular compartment) of M5M6 on the removal of K ions also occurred in the closely related gastric H, K-ATPase (Gatto *et al*., 1999). The movements, which that might be expected to occur in the native α subunit, would be motions of M5M6 in a direction that is perpendicular to the plane of the membrane. It is interesting that in the high-resolution structure of the Ca-ATPase, these helixes (especially TM5) are among the longest helical structures in the protein and extend from the external surface of the membrane to the phosphorylation domain (Toyoshima *et al*., 2000). Whether such postulated movements may occur via pistonlike motions or via rotations of the transmembrane helixes awaits further characterization. A prediction that was made following the studies describing this specific loss of M5M6 for the membrane was that these two helixes were probably anchored by protein–protein interaction in the intact protein. It was suggested that M5 and M6 would be internal helixes in the two-dimensional arrangement of the transembrane helixes. Examination of the structure of Toyoshima *et al.* (2000) reveals this arrangement—M5M6 are held at the center of the Ca-ATPase intramembrane array of helixes, surrounded by other transmembrane segments.

There now exists evidence for movements in the large cytoplasmic loop of the Na,K-ATPase and the transmembrane segments M5 and M6. Do other segments also alter their relative positions during the reaction cycle? Experiments using nonpenetrating cysteine-directed reagents has supplied suggestive evidence that segments of the protein in M8 and M9 may also move during the reaction cycle (Lutsenko *et al*., 1997). In labeling studies, it was shown that the residues C911 and C964 were the only cysteine residues exposed to the extracellular medium and that C964 was the most readily accessible (Lutsenko *et al*., 1997). When the enzyme bound K ions, C964, was no longer accessible to a hydrophilic nonpenetrating reagent, suggesting that C964, which is probably close to the extracellular boundary of M9 (Hu and Kaplan, 2000a) may be mobile and become more buried in the E_2K enzyme form. This is in contrast to the E_2P form, where C964 is readily accessible. Thus, the M9M10 loop may also be involved in protein movements during the pumping cycle. These data can be interpreted either as showing that the reactive residues become more exposed by the region around them "opening up," making them more accessible to extracellular reagents, or, alternatively, they may be moving with respect to the bilayer:aqueous interface and becoming more or less buried in the membrane.

Although most attention has been paid to the α subunit and movements associated with its conformational changes, it is by no means clear that the β subunit can be ignored. There is considerable evidence from a variety of mutagenesis studies that changes in the β subunit can influence the affinity of the $\alpha\beta$ complex for cations (Eakle *et al*., 1992, 1994, 1995; Hasler *et al*., 1998; Koenderink *et al*., 1999). It is also clear that disruption of the β subunit by reducing agents, which cleave one or more of the three S–S bridges in the β subunit extracellular domain, also disrupts cation occlusion and enzyme function (Lutsenko and Kaplan, 1992, 1993). The susceptibility of these S-S bonds to small reducing agents is prevented by the enzyme adopting the E_2K or cation-bound conformation. Such changes may also point to structural motion in the β subunit. There is little direct evidence for changes in β subunit conformation as a consequence of the conformational changes undergone by the β subunit. Proteolysis studies have again provided some suggestive evidence of such changes. Clear differences were seen in the digestion pattern of the β subunit when sealed vesicles containing the Na,K-ATPase were digested in the presence of cations or with MgP_i (Lutsenko and Kaplan, 1994). In the presence of Rb ions, producing the E_2Rb form, digestion produced a membrane-bound 14-kD fragment, via cleavage between R135 and G136 of the canine renal enzyme. A second cleavage point is exposed in the phosphorylated form of the enzyme produced in the presence of Mg and P_i. It was proposed that Rb binding caused movements in the loop within the first S–S bridge of the extracellular segment of the β subunit that resulted in burying the RG cleavage site, which had been exposed on phosphorylation.

Some of the most specific, testable predictions about interdomain movements in P2-type ATPases have been made recently as a consequence of the appearance of the

high-resolution situation of SR Ca-ATPase (MacLennan and Green, 2000; Toyoshima *et al*., 2000). The proposed changes involve closing and rotations of the extramembrane nucleotide binding and activator domains during the reaction cycle. Specific suggestions have also been made about the role of the M6M7 loop on phosphorylation, but the possibility of alterations in the transmembrane helixes during the transport cycle have not yet been addressed.

If we believe that a satisfactory explanation of how ion pumps work necessarily involves a description of the relative motions of different parts of the protein during its pumping cycle, how is this data to be obtained? The remaining section of this article will outline a long-term approach aimed at developing experimental strategies that will provide such data.

CHARACTERIZATION OF PROTEIN MOTIONS

The ability to measure and characterize movements in proteins depends on having an appropriate experimental parameter, which is sensitive to changes in environment or location. In recent years, optical methods using fluorescence or phosphorescence techniques to report such changes have been used with some success in the ion channel field. A requirement of such approaches is the ability to place the extrinsic probe in a known location in a protein. In order to characterize the protein movements in the Na,K-ATPase molecule, it will be necessary to be able to locate probes at many different specified locations in the protein. The most plausible strategy at this time would utilize a robust expression system, with the capacity to produce mutant Na,K-ATPase molecules modified in such a way that this goal can be attained.

The only expression systems available for the Na,K-ATPase, which lack endogenous activity use either yeast or insect cells. The former using transfection, the latter baculovirus infection. The yeast system has been employed by Jorgenson and co-workers (see this issue) and by Farley and colleagues (Eakle *et al*., 1994) for a variety of structure–function studies. The insect cell system has been developed by Mercer *et al*. (Koster *et al*., 1996) and also by De Pont and co-workers for their studies of Na,K-ATPase and H,K-ATPase mechanism and regulation (Koenderink *et al*., 1999). We have also recently begun to exploit the baculovirus-infected sf9 and High-Five insect cell system for the heterologous expression of the renal Na,K-ATPase. We achieved specific activities at least one order of magnitude higher than previously obtained with this system (Hu and Kaplan, 2000a). We have been able to confirm our previous observation, obtained with purified renal enzyme, that C911 and C964 were the only externally exposed cys residues of the α subunit and have exploited this to provide unambiguous chemical confirmation of a specific ten-transmembrane segment topology of the α subunit (Hu and Kaplan, 2000a). These studies also demonstrated the ability to label, with nonpenetrating fluorescent probes, the heterologously expressed α subunit of the renal Na,K-ATPase (Hu and Kaplan, 2000b). In a further development, following the lead of Kaback and colleagues in the *lac* permease, we have been able to express a fully functional sheep renal Na,K-ATPase in which all twenty-three of the endogenous cysteine residues of the α subunit have been replaced by serine or alanine residues (Hu *et al*., 2000). This cys-less α subunit has the same phosphoenzyme turnover rate as wild-type enzyme and studies of the cation activation of the ouabain-sensitive ATPase activity revealed that the half-maximal activation by Na ions was around 9 mM and the half-maximal activation by K ions was 7 mM. This compares well with values for the wild-type protein, expressed in the same cells of 16 mM Na and 3 mM K. We suggested that this change may be the result of a shift in the equilibrium between the E_1 (Na-favoring) conformation and the E_2 (K-favoring) conformation, so that in the cys-less mutant the E_1/E_2 ratio was higher than in the wild-type protein. This prediction is borne out by studies on the apparent affinity with which vanadate inhibits the wild-type and cys-less activities. Vandate acts on E_2 forms and, if our suggestion is correct, we would expect to see a right shift (to higher concentrations) in the concentration-dependence of vanadate inhibition. Such an effect is seen and shown in Fig. 1. As well as

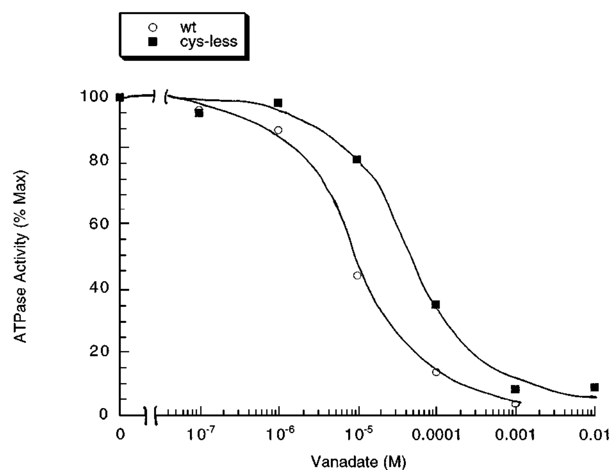

Fig. 1. Vanadate inhibition of Na,K-ATPase in insect cells. Na,K-ATPase activity of purified plasma membranes from sf9 insect cells containing heterologously expressed sheep renal Na pump molecules. The effects of increasing concentrations of vanadate on the activity of wild-type and cys-less Na,K-ATPase α subunits are compared.

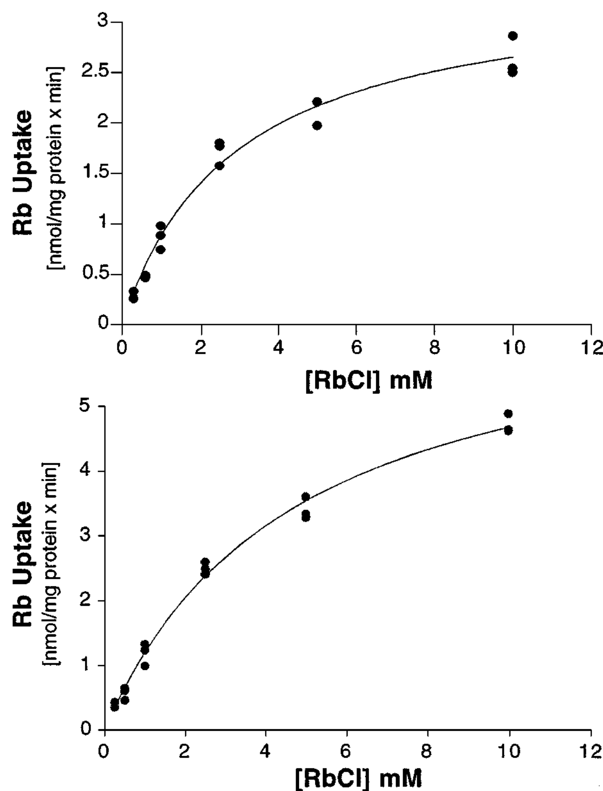

Fig. 2. Cation transport of heterologously expressed Na,K-ATPase. ^{86}Rb uptake was measured in sf9 insect cells, which had been infected with baculovirus particles containing the cDNA for Na,K-ATPase heterodimers containing either wild-type (A) or cys-less (B) α subunits. The dependence of the uptake rates on extracellular Rb concentrations are compared. The apparent k_m values observed from these data are 2.9 mM Rb for the wild-type enzyme and 4.8 mM Rb for the cys-less mutant.

being able to measure, in the insect cell system, many of the biochemical activities of the Na,K-ATPase, it is also possible to obtain transport data and estimate the apparent cation affinities in the transport process. Results of such experiments are shown in Fig. 2. In these experiments, we have compared the Rb activation of ouabain-sensitive ^{86}Rb uptake by wild-type (2A) or cys-less (2B) Na pump molecules in the sf9 system. The data yields half-maximal values, which differ by about two-fold, in agreement with similar studies on the activation of ATPase activity of the isolated plasma membranes (Hu *et al.*, 2000). Studies are underway in which Na,K-ATPase molecules are now being produced with single or double cys residues being reintroduced in a cys-less background. Modification of such mutant Na pumps with reporter molecules will provide data on changes of location or alterations in distance between predetermined sites. Since the pump can be held in different conformations in the presence of different ligands, such data can initially be obtained in a static situation. The longer term goals are to follow dynamic optical changes following the activation of pump molecules by the rapid introduction of substrates through photorelease techniques using caged ATP (Kaplan *et al.*, 1978) or caged Mg reagents (Kaplan and Ellis-Davies, 1988) as has been developed and exploited by Bamberg and co-workers (see this issue).

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