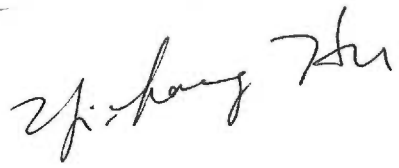
STRUCTURE-FUNCTION STUDIES OF Na,K-ATPase USING SITE-DIRECTED MUTAGENESIS AND CHEMICAL MODIFICATION

by

Yi-Kang Hu



A DISSERTATION

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CERTIFICATE OF APPROVAL

This is certify that the Ph.D. thesis of

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ABSTRACT

This dissertation describes structure-function studies of the sheep renal Na,K-ATPase heterologously expressed in baculovirus-infected insect cells. In one study, we mapped the membrane topology of the Na,K-ATPase α-subunit by using a combination of introduced cysteine mutants and accessibility tests with a membrane-impermeable Cys-directed reagent, MTSEA-biotin. To begin the investigation, two cysteine residues (Cys^{911} and Cys^{964}) of the wild type protein were replaced with serine or alanine to create a background mutant devoid of exposed cysteines (Lutsenko, *et al.*, 1997). Unique cysteines were then introduced into the background in each of the five putative extracellular loops (P118C, T309C, L793C, L876C, and M973C) and the resulting mutants were expressed in baculovirus-infected insect cells. All the mutants were functionally active, as indicated by their ATPase and ligand binding activities, suggesting that they were folded properly in the infected cells. Incubation of the insect cells with the Cys-selective reagent revealed essentially no labeling of the background protein and labeling of all five mutants. Two additional mutants, V969C and L976C, were constructed to further define the M9M10 loop. The lack of labeling of these two mutants showed that while Met^{973} is exposed, Val^{969} and Leu^{976} are not, demonstrating that this method may also be utilized to define membrane boundaries of membrane proteins. Results of our labeling studies are consistent with a specific ten-transmembrane segment model of the Na,K-ATPase α-subunit.

In another study, we replaced all 23 native cysteines of the Na,K-ATPase α-subunit with serine or alanine to investigate the structural and/or functional role(s) of

these amino acid residues. The Cys-less mutant was expressed in baculovirus-infected insect cells, and the resulting cell membrane fractions were isolated for functional characterization. Remarkably, the molecular activity of the cysteine-less mutant in the plasma membranes was close to the wild-type protein (8223 min^{-1} vs. 6655 min^{-1}). Cation and ATP activation of Na,K-ATPase activities revealed that replacing all 23 cysteines resulted in only a 50% reduction of the K_m for Na^+, a two-fold increase in K_m for K^+, and no changes in K_m for ATP. The analysis of distribution of α-subunit among the membranes showed a high percentage of cysteine-less protein in the endoplasmic reticulum and Golgi apparatus compared to the wild-type protein. Furthermore, the cellular stability of the expressed protein appeared reduced in the cysteine-less mutant. Cells harvested after more than 3 days of infection showed extensive degradation of the cysteine-less α-subunit, which is not observed with the wild-type enzyme. Thus the Na,K-ATPase contains no cysteine residues that are critical for function, but the folding and/or assembly pathway of this enzyme is affected by total cysteine substitution. The availability of a Cys-less α-subunit provides the groundwork for future structure-function studies where cysteine residues can be incorporated into different regions of the protein so that spectroscopic methods can be employed to obtain dynamic structure data. Examples of such studies are discussed.

CHAPTER ONE

INTRODUCTION

Na,K-ATPase (sodium pump) is an integral membrane protein found in the cells of all higher eukaryotes. The function of this enzyme is to transport across the plasma membrane sodium ions out the cell and potassium ions into the cell (in a 3:2 ratio) against their potential electrochemical gradient utilizing ATP hydrolysis as the driving force. The sodium pump is a member of the P_2-type ATPase family (Lutsenko & Kaplan, 1995), which includes Ca-ATPases and H,K-ATPases. These enzymes share a similar catalytic cycle that consists of the formation of a phosphorylated protein intermediate. The most commonly accepted model for the reaction mechanism of the sodium pump involves the sequential formation of two acyl-phosphate intermediates (Fig. 1.1). The first intermediate, E_1P, forms on the transfer of the terminal phosphate of ATP to the enzyme in a sodium-dependent fashion. When the sodium ions leave the protein, E_1P is converted to E_2P, which has the highest potassium-binding affinity in the cycle. This is the basis of the Albers-Post model (Post, *et al.*, 1975) for the reaction mechanism of the Na,K-ATPase.

The chemical and electrical gradients generated by the Na,K-ATPase are important for regulating a variety of physiological functions. The electrical gradient is essential for maintaining the resting potential and excitability of nerve and muscle cells. The ion gradient (mostly sodium) is used to regulate cell volume as well as to drive a variety of secondary transport processes including nutrient uptake and waste secretion.

Most clinical studies of the Na,K-ATPase have been focused on its role as the pharmacological target of cardiac glycosides (*e.g.* ouabain and digoxin). Cardiac glycosides extracted from the leaves of digitalis (Fig. 1.2) have been used for the

treatment of cardiac diseases for over 200 years because they increase the myocardial contraction strength and slow the heart rate. The therapeutic mechanisms of the cardiac glycosides are thought to be related to an increase in the intracellular calcium concentration. Partial inhibition of the Na,K-ATPase by the cardiotonic steroids first leads to an increase of intracellular sodium concentration. The increased sodium concentration decreases the driving force of Na/Ca-exchanger and, as a result, calcium ions accumulate in the intracellular space. Calcium release from intracellular space is a key step in muscle contraction and the elevated calcium concentration amplifies the contraction processes.

A numbers of reviews dealing with the physiological roles of Na,K-ATPase have been published (Greig & Sandle, 2000; Greger, 2000; Ziegelhoffer, *et al.*, 2000) and this work will concentrate mainly on the structure-function aspects of the Na,K-ATPase. Recent studies revealing structural information of the Na,K-ATPases, as well as regions and amino acids associated with physiological ligand binding, are discussed.

SUBUNIT COMPOSITION OVERVIEW

Na,K-ATPase is composed of an α-subunit (110 kDa) and a glycosylated β-subunit (~55 kDa). Although the α-subunit contains all the functional domains, interactions between the two subunits are required for function. Four isoforms of the Na,K-ATPase α-subunit (α1, α2, α3 and α4) have been identified and are known to be expressed in several tissues (Shull, *et al.*, 1985; Shull, *et al.*, 1986a; Shamraj & Lingrel, 1994). A higher degree of homology (95%) can be observed among the same isoform of different species than among the different isoforms in one species (Blanco & Mercer,

1998). The α1 isoform is found in most tissues, whereas the α2 is predominant in skeletal muscle and also is detected in the brain and heart tissues. The α3 isoform is essentially present in neuronal cell types and the α4 isoform is restricted to testes. The physiological reasons for the presence of several isoforms has not yet been clearly defined. Biochemical studies on the isoforms revealed modest differences in enzymatic activity and ouabain sensitivity (Sweadner, 1989; Lingrel & Kuntzweiler, 1994; Blanco *et al.*, 1995; James, *et al.*, 1999; Woo, *et al.*, 1999; Blanco, *et al.*, 1999). The presence of these isoforms could allow an organism to regulate Na,K-ATPase activities in specific tissue and cell types. Additionally, the isoforms could have different functional characteristics in the cell types. For example, the α2 isoform is reported to have a unique functional role in calcium handling in cardiac myocytes (James, *et al.*, 1999), and the α4 isoform has been shown to play a role in sperm motility (Woo, *et al.*, 2000).

Three isoforms of the β-subunit (β1, β2 and β3) have been described and they share a relatively low degree of sequence similarity. The β1 isoform is expressed in most cells (Kawakami, *et al.*, 1986; Mercer, *et al.*, 1986; Shull, *et al.*, 1986b) while the β2 is found predominantly in neuronal tissues (Martin-Vasallo, *et al.*, 1989). The β3 isoform was initially identified in *Xenopus* and *Bufo marinus* (Jaisser, *et al*, 1992; Good, *et al.*, 1990). Recent studies have also detected this isoform in mammals, particularly in the lungs and testes (Malik, *et al.*, 1996). The β-subunit appears to be involved in the maturation and the localization of the Na,K-ATPase (Chow & Forte, 1995; McDonough, *et al.*, 1990). Data from our studies have demonstrated that heterologously expressed α-subunit alone does not reach the plasma membrane (Gatto, *et al.*, 2001). Once the αβ heterodimer is localized to the plasma membrane, the β-subunit seems to play a role in

the modulation of the potassium activation of the sodium pump. This laboratory has previously reported that reducing the disulfide bridges of the β-subunit resulted in a 50% loss in rubidium (a potassium congener) occlusion by the purified enzyme (Lutsenko & Kaplan, 1993).

An additional protein, γ-subunit, has been described to be specifically associated with the Na,K-ATPases in some tissue types (Forbush, *et al.*, 1978; Reeves, *et al.*, 1980). This 10-kDa protein, though not required for function, appears to regulate the enzyme's affinity for cations (Therien, *et al.*, 1999; Arystarkhova, *et al.*, 1999).

MEMBRANE TOPOLOGY

Assigning transmembrane segments of large membrane proteins based on hydropathy plot can be a complex process. Assumptions about the shape, length and charge compositions of each transmembrane segment must be made, and discrepancies often occur. There is general agreement, however, based on antibody epitope localization studies (Canfield & Levenson, 1993; Yoon & Guidotti, 1994), that both the amino and carboxyl-termini of the α-subunit are at the cytoplasmic side. Therefore, the α-subunit should contain an even number of transmembrane domains.

The hydropathy plot of the Na,K-ATPase α-subunit divides the protein into three large domains of similar size (Fig. 1.3A). The amino-terminal third of the protein appears to contain four membrane-spanning regions, and this prediction is supported by data obtained from protease accessibility and immunochemical studies (Karlish, *et al.*, 1993; Lutsenko & Kaplan, 1994; Yoon & Guidotti, 1994; Canfield, *et al.*, 1996). These regions are followed by a large cytoplasmic domain, where ATP binds and hydrolysis

takes place. The hydropathy profile of the carboxyl-terminal third of the α-subunit is more ambiguous, and the existence of from two to six transmembrane helices has been proposed. Structural comparison with other P-type ATPases is difficult due to the lack of sequence homology in this region.

Over the last decade, results from several biochemical and molecular biological studies favor a 10-transmembane model for the α-subunit (Karlish, *et al.*, 1993; Lutsenko & Kaplan, 1994; Yoon & Guidotti, 1994; Canfield, *et al.*, 1996). However, other studies, including a recent investigation using epitope accessibility tests (Lee & Guidotti, 1998), claim to establish four transmembrane segments in the carboxyl-terminal region and a total of eight transmembrane segment for the α-subunit. Besides having produced conflicting secondary structural information, the experimental methods employed in these studies suffer from inherent limitations and need further confirmation by other methods. Chapter three discusses these limitations in detail and describes an alternative approach we used to map the membrane topology of the Na,K-ATPase α-subunit. This new approach overcomes the limitations and deals exclusively with functional Na,K-ATPase molecules in their native states. The results from our study are consistent with a specific 10-transmembrane segment topological model of the Na,K-ATPase α-subunit.

The hydropathy plot of the β-subunit predicts one transmembrane segment region for the protein (Fig. 1.3B), and a recent molecular biological study of the protein further defined this model (Hasler, *et al.*, 2000). The β-subunit is a type II membrane protein; in other words, the amino-terminal is cytoplasmic and the carboxyl-terminal is extracellular. The amino-terminal portion of the protein forms an intracellular tail of about 40 amino acid residues. This is followed by an α-helical transmembrane segment of 26 residues.

The remaining ~260 residues form a large extracellular domain, which contains three disulfide bridges and three *N*-glycosylation sites.

Cation Binding and Occlusion Sites

The translocation of cations by Na,K-ATPase involves several coordinated steps, which are represented by a simplified Albers-Post scheme in Fig. 1.1. The binding of three cytoplasmic sodium ions stimulates the transfer of the terminal-phosphate of the bound ATP to the enzyme and the formation of E_1P. A conformational change takes place, and the sodium-binding site is exposed to the extracellular domain. At this E_2P conformation, the sodium ions leave the protein and the binding of two extracellular potassium ions follows. Protein dephosphorylation and ATP binding occur, and the protein again changes its conformation, which allows the release of potassium ions in the cytoplasm. During parts of the reaction cycle, the cations are occluded by the Na,K-ATPase and are not accessible to either surface.

The cations have to physically pass through the membrane during the transport process, thus it was reasonable to hypothesize that amino acids involved in cation binding and/or occlusion reside in the transmembrane regions. These residues are likely to be negatively charged to accommodate the cations in the hydrophobic environment. Chemical modification and site-directed mutagenesis studies have identified several of these transmembrane residues, and proteolytic digestion of the protein in the presence and absence of the cations has revealed specific transmembrane loops involved in ion coordination during the catalytic cycle.

One of the earliest chemical modification studies reported the labeling of Glu^{953} in the transmembrane segment 9 (M9) by *N,N'*-dicyclohexycarbodiimide in a rubidium-protectable manner (Goldshleger, *et al.*, 1992). However, the interpretation that Glu^{953} is essential for cation binding is challenged by a subsequent mutagenesis study that showed replacing Glu^{953} produced functional enzyme (Vasilets, *et al.*, 1998). Another carboxyl modifying reagent, 4-(diazomethyl)-7-(diethylamino)coumarine inactivated cation occlusion of Na,K-ATPase and the inactivation was prevented by potassium ions. Glu^{779} in M5 was identified as the modified residue (Arguello & Kaplan, 1994). Comparably, site-directed mutagenesis study showed that when the E779L mutation is introduced into an ouabain-resistant form of α-subunit, mammalian cells expressing this α-subunit were not able to survive under ouabain-selective pressure (Jewell-Motz & Lingrel, 1993). In other words, the E779L mutation seemed to inactivate the protein.

Additional mutagenesis studies on Glu^{779}, as well as several other transmembrane residues, have been carried out. The results are summarized in Table 1.1, which shows that substituting several amino acid residues in M4, M5, M6 or M8 could change the sodium and/or potassium binding affinities of the enzyme. Interestingly, results of the mutagenesis studies on Pro^{326}, Ser^{775} and the Thr residues demonstrate that cation binding of the Na,K-ATPase is not limited to residues with carboxyl side-chains (Table 1.1). Ion coordination may also involve oxygen-containing side chains and/or the carbonyl groups of the polypeptide backbone. Furthermore, the involvement of the proline residue suggests that a "kink" in the proposed helical structure of M4 is important for cation coordination.

Perhaps the most striking evidence supporting the involvement of transmembrane residues in cation coordination came from proteolytic digestion studies on the Na,K-ATPase. Extensive tryptic treatment of purified renal Na,K-ATPase digests away most extramembrane portions of the α-subunit, and yet leaves the transmembrane regions and the β-subunit essentially intact. This post-tryptic preparation alone, shown in Fig. 1.4, is capable of occluding rubidium (Karlish, *et al.*, 1990; Lutsenko & Kaplan, 1994), suggesting it contains all the amino acid residues necessary for ion coordination. Furthermore, this laboratory reported that if rubidium ions are removed from the post-tryptic preparation, the transmembrane hairpin M5M6 is released (Lutsenko, *et al.*, 1995) to the extracellular domain (Gatto, *et al.*, 1999a). Because of the charged amino-acid composition in M5M6, it is likely that membrane localization of this hairpin is partially stabilized by association with cations.

ATP Binding Domain

The energy source for the active transport mediated by the sodium pump is the hydrolysis of ATP molecules. To date, all the amino acid residues associated with ATP binding and hydrolysis have been located, via chemical modification and mutagenesis studies, to the large cytoplasmic loop between M4 and M5. Asp^{369} is the site of phosphorylation during the catalytic cycle of Na,K-ATPase and substitution of this residue, not surprisingly, leads to an inactive protein (Kuntzweiler, *et al.*, 1995). Several other residues in this loop, including Cys^{367}, Cys^{456}, Lys^{480}, Lys^{501}, Gly^{502}, Asp^{710}, Asp^{714}, Lys^{719}, Lys^{767} (reviewed in Pedemonte & Kaplan, 1990), and more recently Cys^{577} (Gatto, *et al.*, 1999b), have been implicated as sites of contact for ATP. Chemical modifications

of these residues take place in the absence of ATP, but not in its presence. Limited mutagenesis studies have been performed on these residues. Chapter four describes the construction of a functional Na,K-ATPase α-subunit with all its native cysteines replaced. We found that the cysteine-less form of the sodium pump displays similar ATP affinity as the wild-type enzyme, suggesting that none of the cysteines in the ATP-binding loop participate in direct ATP binding. Mutations of Asp^{710} and Asp^{714}, on the other hand, produced nonfunctional enzymes (Lane, *et al.*, 1993), implying that the side chains of these charged residues may play an important role in ATP binding and/or hydrolysis.

Our laboratory has overexpressed the large cytoplasmic loop (Lys^{354}-Lys^{774}) of the rat Na,K-ATPase α1-subunit in *Escherichia coli.* The purified loop displayed ordered secondary folding structures and had nucleotide affinities similar to those observed for the E_2 conformation of the native enzyme (Gatto, *et al.*, 1998). This is a further demonstration that the amino acid residues in this loop are the determinants of ATP binding.

OUABAIN BINDING SITES

The Na,K-ATPase is the receptor for ouabain. Recent studies have detected endogenous ouabain in mammals (Hamlyn, *et al.*, 1991; Kawamura, *et al.*, 1999), suggesting that the cardiac glycoside may have a regulatory role in animal physiology.

Ouabain inhibits the Na,K-ATPase by binding to the extracellular domain of the protein at the E_2P conformation. The ouabain-binding site is complex based on the following observations: (i) Affinity-labeling experiments have identified the α-subunit as the primary binding site and yet some labeling of the β- and even the γ-subunits also

occur (Forbush, *et al.*, 1978). (ii) Mutagenesis studies have found that most of the amino acid residues responsible for ouabain sensitivity reside in the M1M2 region of the α-subunit (Price, *et al.*, 1989; Price, *et al.*, 1990). For example, substitutions of Leu^{111} and Gln^{122} in this region with charged residues (Arg and Asp, respectively) alter the ouabain sensitivity of sheep α1-subunit approximately 1000-fold (Price & Lingrel, 1988). However, a number of other residues involved in ouabain sensitivity have been identified throughout M3-M10 (Croyle, *et al.*, 1997), as depicted in Fig. 1.5. (iii) Substitutions of any of these resides do not abolish ouabain sensitivity. (iv) H,K-ATPase is insensitive to ouabain, but a chimeric H,K-ATPase containing the M3M4 and M5M6 regions of the Na,K-ATPase has high affinity for ouabain (Koenderink, *et al.*, 2000). (v) NMR studies have shown that although the steroid head group of the ouabain appears to associate tightly with the Na,K-ATPase, the sugar moiety of the molecule displays a relatively high degree of rotational freedom when bound to the protein (Middleton, *et al.*, 2000). Ouabain is approximately 20 Å in length, and it is likely that the two head groups (Fig. 1.2) interact with separate regions of the Na,K-ATPase.

Experimental results from studies to date suggest that multiple extracellular and transmembrane regions are involved in determining ouabain sensitivity. However, the actual ouabain-binding sites remain to be elucidated.

AIMS AND OUTLINES OF THIS THESIS

How does the Na,K-ATPase couple the energy of ATP hydrolysis with active cation transport? Since the discovery of the sodium pump more than four decades ago, scientists have been actively pursuing the answers to this fundamental and yet complex

question. Extensive research thus far has provided a better understanding of the functional domains of the enzyme. The transmembrane segments are involved in cation coordination and transport, while ATP binding and hydrolysis are restricted to the large cytoplasmic loop between M4M5. Conformational changes in one area of the protein must be accompanied by the changes of the other because cation selectivity in the transmembrane region is determined by the nucleotide binding status in the cytoplasmic loop (and *vise versa*). Additionally, studies have shown that alterations of amino acid residues in one region of the protein also affect the catalytic properties of the other (Ellis-Davies & Kaplan, 1993; Arguello & Lingrel, 1995; Koster, *et al.*, 1996). Despite the extensive research, however, we have yet to elucidate the molecular mechanisms in which the separate domains communicate and work together. The pursuit for understanding the structure-function relationships of the Na,K-ATPase continues .

The majority of our knowledge about the structure-function relationships of the sodium pump has been obtained from biochemical studies on purified native enzymes. Accessibility tests using proteolytic and chemical reagents in the presence of the various physiological ligands have revealed the dynamic nature of the Na,K-ATPases. These tests take advantage of the unique chemical properties of several amino acid residues to identify the regions involved in catalysis. Synthesis of functional Na,K-ATPase molecules with these reactive residues engineered at pre-determined locations would greatly expand the capability of these biochemical methods. For example, with the recent advances in spectroscopic technology, it is conceivable that an engineered site can be introduced into a catalytic domain so that it is selectively labeled with a fluorescent probe and its movements can be monitored during the reaction cycle. This type of study has

been performed to obtain dynamic structure data on other membrane proteins (Cha, *et al.*, 1999; Dunham & Farrens, 1999).

One major aim of this thesis is to develop and optimize an expression system that produces engineered Na,K-ATPase molecules suitable for labeling and other biochemical studies. Most mutagenesis studies of the sodium pump until now have relied on established mammalian expression systems to produce the desired proteins. Distinction between the endogenous and the exogenous Na,K-ATPase activity is made based on ouabain sensitivity. The endogenous protein is inhibited in the presence of nanomolar ouabain, whereas the expressed proteins (*i.e.* the rat α1-subunit or mutated α-subunits) are much less sensitive (K_I about 200 μM) and are fully functional in the presence of 1 μM ouabain. Although applications of this type of expression system have been described, the presence of endogenous Na,K-ATPase would greatly complicate the interpretation of the results of the proposed labeling experiments.

One way to avoid the problem of the endogenous protein is to use an expression system with little or no detectable Na,K-ATPase activity. Yeast cells are completely devoid of Na,K-ATPase, however, the abundance of endogenous H-ATPases may contribute to a high background signal in measurements. Insects are reported to contain detectable Na,K-ATPase activities only in the nervous system. Mercer and co-workers have expressed the sodium pump in the sf9 cell line, derived from the ovary of the fall armyworm *Spodoptera frugiperda*, using a baculovirus expression system. In their initial studies, the highest specific activity of the expressed proteins was 5 μmol P*i* · mg^{-1} protein · h^{-1} (DeTomaso, *et al.*, 1993). We have explored an alternative baculovirus expression system for our studies. This system offers a quicker and easier way for

selecting the desired recombinant baculovirus and it saves more than two weeks of laboratory time per mutant production than the system employed by Mercer. We do not detect endogenous Na,K-ATPase in the insect cell lines and the highest specific activity of our expressed proteins is 37 μmol $Pi \cdot mg^{-1}$ protein $\cdot h^{-1}$ (Hu & Kaplan, 2000), which is perhaps the best among any system reported (Pedersen, *et al.*, 1996). The methodology and background information of this expression system are described in chapter two.

Another aim of this thesis is to demonstrate that the combination of protein engineering and chemical modification provides structure-function information that was hitherto unobtainable using other methods. As mentioned earlier and also discussed in depth in chapter three, the membrane topology of the Na,K-ATPase α-subunit remains poorly characterized in spite of extensive research. Using our baculovirus expression system, I have constructed a panel of Na,K-ATPase mutants each with a unique cysteine residue in the putative extracellular loops. The chemical accessibility of these cysteines has allowed us to characterize the topogenic properties of these fully functional Na,K-ATPase molecules and dissect this protein's transmembrane secondary structure.
In chapter four, we continue with the theme of sulfhydryl chemistry and describe the construction of a Na,K-ATPase α-subunit with all its 23 native cysteine residues replaced. Remarkably, we found that this cysteine-less protein is active and displays similar ligand binding affinities as the wild-type enzyme. We are now in a position where cysteine residues can be introduced back into the protein to carry out further biochemical studies. The potential applications of this cysteine-less protein, as well as some of our current projects, are discussed in chapter five.

Table 1.1. Consequences of Substituting Transmembrane Residues of the Na,K-ATPase α-subunit in the Protein's Cation Affinities

Substitution	Location	Cation Binding Affinity, relative to wild type	References
P326A	M4	K, -3×; Na, -2×	Vilsen, 1992
E327Q		K, -47×*	Nielsen, *et al.*, 1998
E327A		K, -91×*	
E327L		K, -1.9×; Na, -2.8×	Jewell-Motz & Lingrel, 1993
E327Q		K, -3.1×; Na, -1.7×	
E327D		produced inactive protein+	
L330A		K, -2.5×; Na, +2×	Vilsen, 1992
Y771F	M5	K, -9×*; Na, -17×#	Pedersen, *et al.*, 1998
T772A		K, -3.3×*; Na, -5×#	Pedersen, *et al.*, 1998
T772S		K, -1.7×*; Na, -4×#	
T774A		K, +2×*; Na, -20×#	Pedersen, *et al.*, 1998
T774S		K, -1.7×*; Na, -5.5×#	
S775A≠		K, -29×*; Na, -11×#	Pedersen, *et al.*, 1998
S775T		K, -19×*; Na, -6×#	
S775A		K, -31×; Na, no change	Arguello & Lingrel, 1995
S775C		K, -13×, Na, no change	
S775Y		produced inactive protein+	
N776A		K, -52×*; Na, -19×#	Pedersen, *et al.*, 1998
N776Q		K, -4×*; Na, -32×#	
E779D		K, -91×*	Nielsen, *et al.*, 1998
E779Q		K, -3×*	
E779P		K, -1.5×; Na, -1.5×	Koster, *et al.*, 1996
E779A**		K, -3.3×; Na, -2×	
E779K		K, -2.5×; Na, +1.7×	
E779L		produced inactive protein+	Jewell-Motz & Lingrel, 1993
T781A		K, +1.6×*; Na, +1.3×#	Pedersen, *et al.*, 1998
T781S		K, -1.2×*; Na, -1.9×#	
D804E,N	M6	K, -91×*	Nielsen, *et al.*, 1998
D804L,E,N		produced inactive protein+	Jewell-Motz & Lingrel, 1993
D808E		K, -21×*	Nielsen, *et al.*, 1998
D808N		K, -91×*	
D926L	M8	K, -1.9×; Na, -1.8×	Jewell-Motz & Lingrel, 1993
D926N		K, +1.8×; Na, -3.7×	

*The K affinity was determined by ATP replacement studies.
+This substitution was introduced into an ouabain-resistant form of the Na,K-ATPase α-subunit. Mammalian cells expressing this mutant was not able to survive in media containing 1 μM ouabain, suggesting that this substitution inactivated the protein.
#The Na affinity was determined by ATP phosphorylation studies.
≠This mutant could only occlude one Tl (K) ion.
**This mutant lost ion selectivity and displayed Na-ATPase activities.

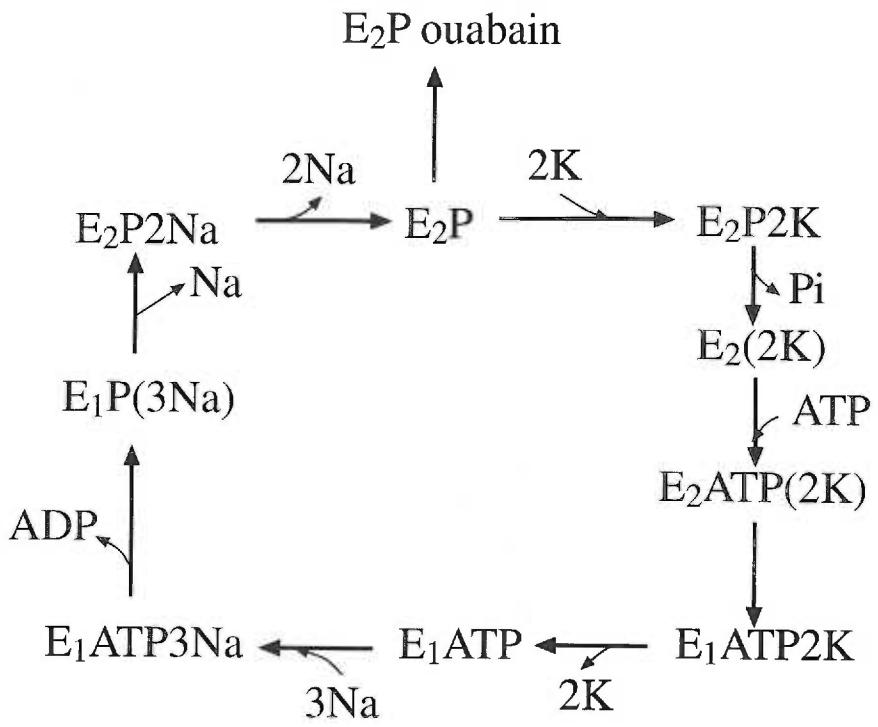
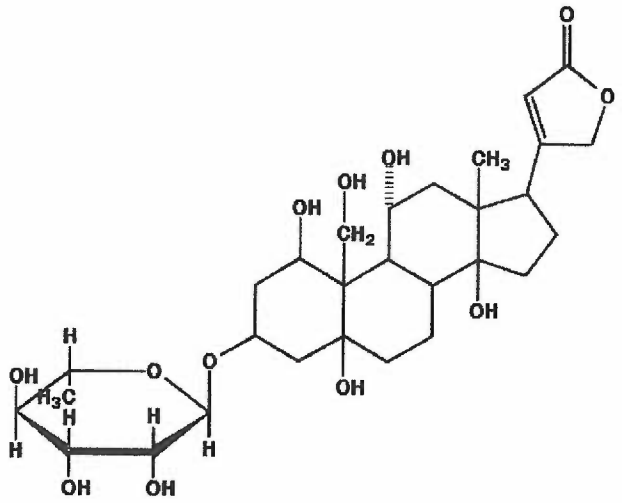


Fig. 1.1. A basic Albers-Post Model of Na,K-ATPase describing the catalytic cycle of the enzyme. Occluded ions are depicted in *parentheses*.

A



B

Fig. 1.2. Figures of **A**, ouabain; and **B**, foxglove plant, a digitalis.

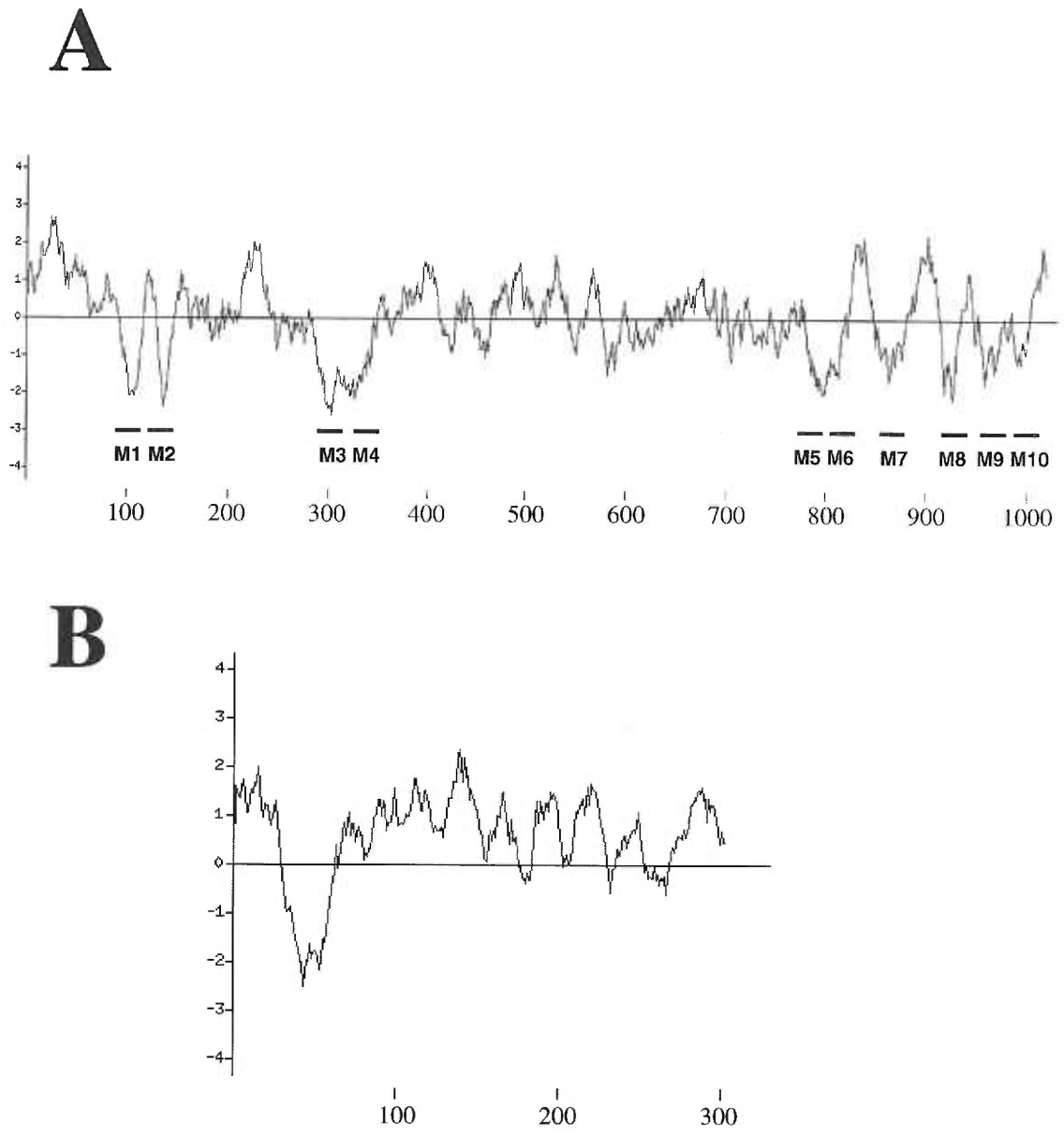


Fig. 1.3. Hydropathy profiles of **A**, the α-subunit; and **B**, the β-subunit of the Na,K-ATPase. The profiles were generated, based on the Kyte-Doolittle algorithm, using 17 amino acid residues as an averaging window.

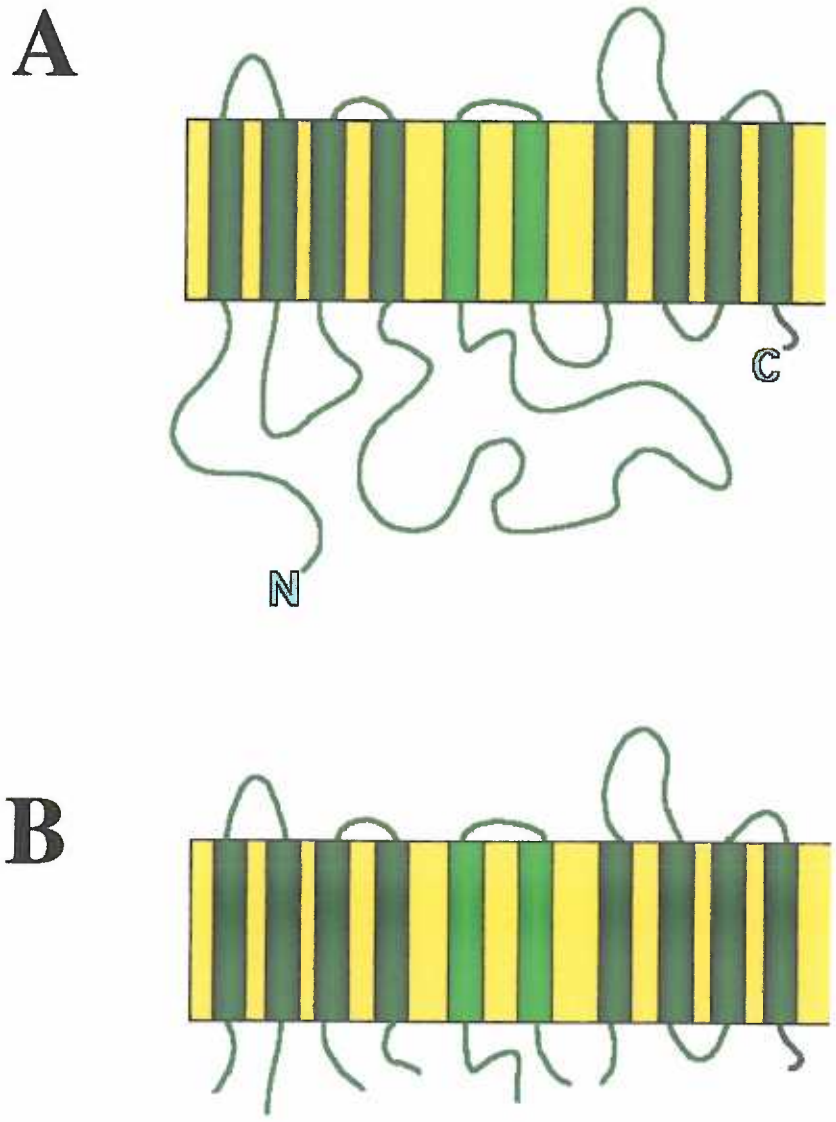


Fig. 1.4. The Na,K-ATPase α-subunit before (**A**) and after (**B**) extensive trypsin digestion in the presence of K ions.

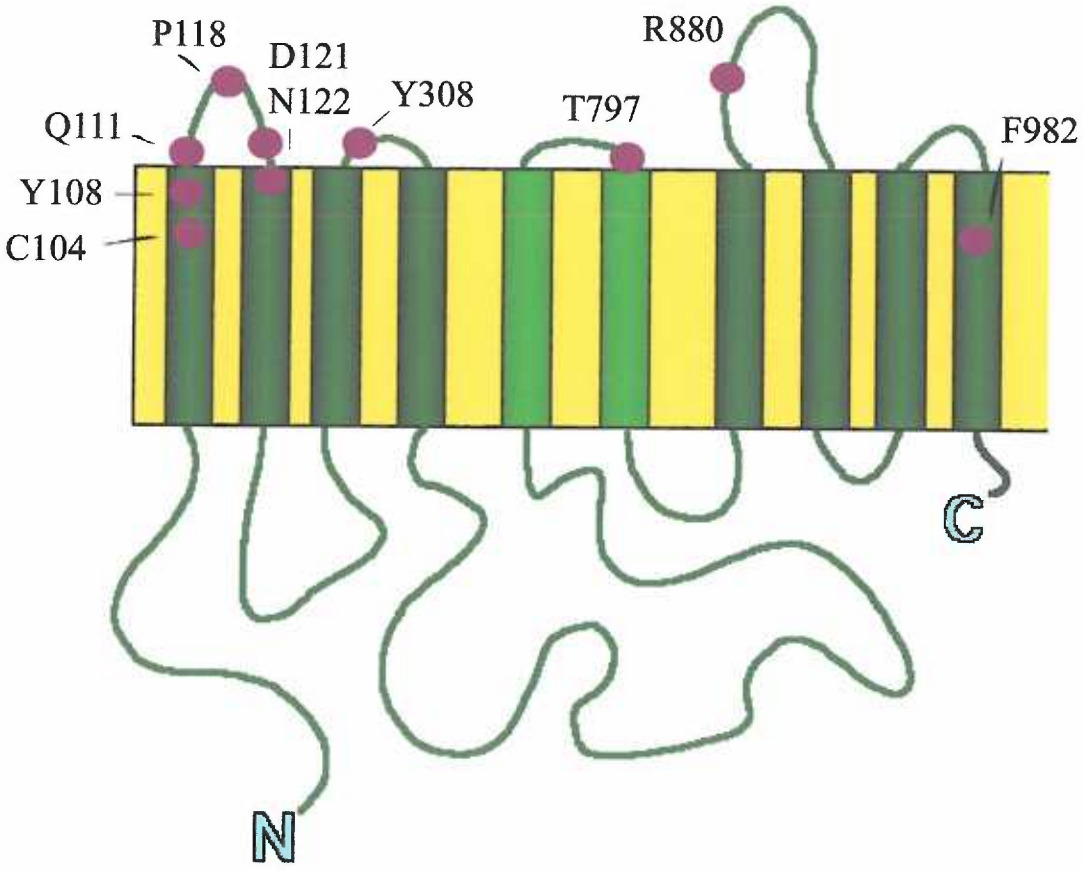


Fig. 1.5. Determinants of Ouabain Sensitivity of the Na,K-ATPase α-subunit.

CHAPTER TWO

THE BACULOVIRUS EXPRESSION SYSTEM: A SURVEY ON PRINCIPLES AND METHODS

The baculovirus expression system is becoming one of the most popular eukaryotic expression systems available. Features of this system that are advantageous include the following:

i. High expression levels - The baculovirus system has been used to produce both soluble and complex membrane proteins. The most distinguishing feature of this expression system is the potential for very high level of expression of a heterologous gene. The highest expression level reported is 30-45% of the total cellular protein, which is approximately 1 gram of the desired protein product per 1 liter of cell culture (Janne, *et al.*, 1993; Lou, *et al.*, 1995).

ii. Capacity for large inserts – The baculovirus genome (134 kbp) is capable of accommodating large DNA inserts, which means that multiple foreign proteins can be expressed with one viral construct. The largest insert reported in our study thus far is ~8 kbp, but the potential for larger inserts is yet to be determined. The inserts can be a genomic DNA containing unspliced introns. However, the use of cDNA inserts is recommended for a higher expression level.

iii. Post-translational modifications – The insect cells provide a eukaryotic environment that can facilitate the folding, modification and assembly of eukaryotic proteins. The post-translational modifications that have been reported include signal and proteolytic cleavage, *N*- and *O*-glycosylation, acylation, amidation, phosphorylation, prenylation, and carboxymethylation (reviewed in O'Reilly, *et al.*, 1994).

iv. Ease in cell culture maintenance - The most commonly used cell lines in the baculovirus system are sf9 and High Five cells. The sf9 cells originated from the fall armyworm *Spodoptera frugiperda* ovarian tissues, and the High Five cells were derived

from the eggs of the cabbage looper *Trichoplusia ni.* Both cell lines are maintained at 27 °C (or room temperature), do not require CO_2 supplementation, and can be easily adapted as attached or suspended cultures to adjust the needs of the user. They double every 16-24 hours and routine cell maintenance can be performed every three to four days. Fetal bovine serum is no longer needed as culture media containing nutritional substitutes became commercially available. These media eliminate the variability of serum supply and simplify the purification process of the expressed proteins (especially secreted proteins).

v. Safety considerations – Baculoviruses have a relatively narrow host range and they do not replicate in mammalian cells (Carbonell, *et al.*, 1985; Carbonell & Miller., 1987). They are safer to work with than most mammalian viruses. Proper laboratory precautions (*i.e.* wear gloves, no food or drink, and wash hands) and sterile techniques suffice when working with baculoviruses.

Expression Systems Overview

Baculoviruses are a diverse group of viruses found mostly in insects. The DNA of the baculovirus commonly used for expression vector work is *Autographa californica* nuclear polyhedrosis virus (AcMNPV). Expression of foreign proteins in sf9 or High Five insect cells can be established by replacing a viral gene, that is not essential for propagation of the viruses, with the coding sequences of the proteins. Two frequently used AcMNPV genes are the polyhedrin and p10 genes. Both genes contain very strong promoters and are activated during the very late phase of the infection (>24 hours).

Several other less strong promoters, activated in earlier stages of infection, have also been successfully used for expression studies (O'Reilly, *et al.*, 1994).

The large size of the AcMNPV genome (134 kbp), which contains recognition sites for nearly all known restriction endonucleases, poses a great challenge for DNA manipulations with the standard cloning techniques. Therefore, allelic replacement was adopted in the classical methods for inserting the genes of interest. The allelic replacement strategies are based on a homologous recombination event, in the insect cells, between the wild-type AcMNPV DNA and an appropriate transfer vector containing the gene of interest. This procedure is schematically illustrated in Fig. 2.1. One starts by cloning the gene of interest downstream of a viral promoter in the transfer vector so that both the gene and the promoter are flanked on both sides by viral sequences. The resulting transfer vector and the wild-type baculovirus DNA are cotransfected into insect cells, and enzymes in the cells recognize the homologous sequences and recombine the DNAs. The recombinant viruses are then purified from the non-recombinant wild-type viruses after they have been amplified. Although allelic replacement simplifies the cloning process, it is a rather inconvenient system to work with. Typically, only 0.1% to 1% of the viruses produced are recombinant. Selections for the recombinant viruses are made by standard plaque assay methods (described below), which are based on the morphology of the infected insect cells. The desired morphology is not always obvious and the assays need to be repeated several times. It is crucial that the recombinant baculoviruses are isolated in the amplification process because the non-recombinant wild-type baculoviruses replicate ~10^3 times more

efficiently. For the reasons above, the entire procedure can take as long as 6 weeks to properly rid the viral isolate of the non-recombinant viruses.

More recently, the development of a baculovirus shuttle vector, bacmid, allows researchers to produce recombinant baculoviruses more rapidly and efficiently (Luckow, *et al.*, 1993). This system, shown in Fig. 2.2, is based on site-directed transposition of the shuttle vector propagated in *E. coli*. To start constructing the recombinant virus, a gene of interest is cloned into a donor plasmid so that the gene is downstream of a viral promoter and that both the gene and promoter are flanked on both sides by Tn7 transposon inverted repeats. *E. coli* cells containing the bacmids are transformed with the donor plasmid, and transposition takes place to produce recombinant bacmids. The bacmid contains an attTn7 site (the target site for the bacterial transposon Tn7) engineered inside a *lac*Z gene, which is disrupted upon insertion of the transposable element containing the gene of interest. Therefore, the white *E. coli* colonies containing the recombinant bacmids are easily distinguishable from the blue colonies harboring the unaltered *lac*Z gene. The resulting recombinant bacmids are used to transfect insect cells to produce the desired baculoviruses. Several rounds of plaque purification are no longer needed because all of the baculoviruses produced by this method are recombinant. In average, one can save 2-4 weeks of laboratory time using the bacmids to produce recombinant baculoviruses.

Using the principles of the bacmid method, Life Technologies Incorporated has developed the Bac-to-Bac Expression System for producing foreign proteins in insect cells. The company offers several donor plasmids for cloning the gene of interest, and we have used pFASTBACDUAL for our expression studies. As shown in Fig. 2.3, this

plasmid offers two multiple cloning sites, I and II, into which were inserted the sheep β1- and α1-subunit cDNAs, respectively. The α-subunit is cloned under the p10 promoter and the β-subunit is under the polyhedrin promoter. The resulting donor plasmid, pFASTBACDUALαβ, is transformed into the DH10BAC cells, which contains the target bacmid, capable of producing the recombinant bacmids via transposition. Detailed protocols for using this system to generate recombinant baculoviruses are described in the following two chapters.

CELL MAINTENANCE

As discussed earlier, maintaining the sf9 and High Five insect cells is less complicated than many other cell lines. We use Ex-Cell 420 medium for the Sf9 and Ex-Cell 405 for the High Five cells. Both media are purchased from JRH Biosciences. Our cell stocks are kept in suspension at 27 °C with a density between 0.5 x 10^6 and 4 x 10^6 cells per ml, which is measured using a hemacytometer. The same batch of cells is not used for more than 2 months, and the new batch, brought back from liquid nitrogen stocks, is not used for expression studies until it has been subcultured daily as attached cultures for two weeks. We found that the expression level is the highest when the cells are infected during these two months of "mid-life" period.

THE PLAQUE ASSAY

The plaque assay is the most frequently used method for purifying recombinant baculoviruses and for determining viral titer. Although the Bac-to-Bac Expression

System eliminates the needs for multiple rounds of plaque purification, it is still advantageous to determine the viral titers of newly generated baculoviruses for the following reasons. (i) Defective virus particles have been demonstrated to accumulate when too many viral particles are used for viral amplication. These defective particles contain extensive mutations in viral genes and require co-infection with the healthy viruses for replication. The replication of the defective particles interferes with the replication of the healthy viruses, thus it is important to know the viral titer and to keep it low during amplication. (ii) Expression studies are best performed using a good-quality high-titer viral stock so that cell infection and harvest are synchronous. Additionally, infecting cells with a consistent number of viral particles is vital for reproducible results in expression studies.

To begin the plaque assay, healthy sf9 or High Five cells are seeded at a density of $1x10^6$ cells per well (~50% confluent) in a 6-well culture plate. After one hour of incubation at 27 °C, the cell medium is replaced with 1 ml of the baculovirus at various dilutions (usually 10^{-6}-10^{-9}), and the mixture is allowed to incubate at 27 °C for an additional hour. After infection, the virus is removed and quickly replaced with 2 ml of agarose overlay pre-warmed at 42 °C. The overlay is made by combining 25 ml 2X Grace's Insect medium (Life Technologies Inc.), 5 ml heat-inactivated fetal bovine serum, 0.5 ml 100X antibiotics cocktail (5000 unit/ml penicillin, 5 mg/ml streptomycin, and 10 mg/ml neomycin), and 20 ml 2.5% liquefied low-melting agarose in water. The cells are placed at room temperature for 5 mins to cool off the overlay, and then at 27 °C for 4-10 days for plaque formation. Viral plaques are observed as an area in the cell monolayer that is ringed by infected cells. The infected cells are morphologically distinct

from the uninfected cells in that they are generally larger and show signs of cell lysis (O'Reilly, *et al.*, 1994). However, uninfected cells also display this type of morphology if they are overgrown or suffer from environmental stress (*i.e.* changes in temperature, media compositions, or contamination), which often happen in the plaque assays. Thus, the task of identifying viral plaques is not trivial.

Using Green Fluorescent Protein as a Reporter Group in Baculovirus Infection Studies

Very recently, I have constructed a novel baculovirus donor plasmid to simplify the plaque identification process. This plasmid, pFastBacDUALαβGFPQ, is produced by cloning the cDNA of a green fluorescent protein (GFP) into the transposable region of the pFastBacDUALαβ vector. The strategy is to express simultaneously the Na,K-ATPase and GFP, in the infected cells, using one viral construct so that the viral plaques can be readily and unambiguously identified with fluorescent microscopy. A vector map of the pFastBacDUALαβGFPQ plasmid is shown in Fig. 2.4. The GFP cDNA is cloned upstream of an SV40 polyadenylation signal sequence and downstream of a baculovirus early promoter, IE1, and its enhancer, hr5. The IE1 directs the expression of the GFP at 6-hour post-infection, allowing for a faster plaque identification process. Additionally, the IE1 is a relatively weak promoter; thus the expression of the GFP is less likely to overload the protein production machinery in the infected insect cells.

To construct the pFastBacDUALαβGFPQ donor plasmid, the cDNA of the SuperGlo GFP was first cut out of the pQBI25 plasmid (Qbiogene) using *SacII* and

EcoRV, and was cloned into the multiple cloning region of the pAcP(+)IE1-3 vector (Novagen), which contains the IE1 promoter and hr5 enhancer, using the unique *SacII* and *StuI* sites. An SV40 polyadenylation signal, excised from the pFASTBACDUALαβ vector as a *SpeI/AvrII* fragment, was then inserted into the unique *SpeI* site downstream of the GFP cDNA. The resulting plasmid was sequenced to ensure the proper orientation of the insert and was used as the template for the following step. A DNA fragment containing the hr5, IE1, GFP cDNA and SV40 polyadenylation signal was amplified by PCR using a forward and a reverse primer each containing an engineered *AvrII* site, and the fragment was cloned into the transposable region of the pFASTBACDUALαβ plasmid using *AvrII*. The resulting donor plasmid was transformed into the DH10BAC cells to generate recombinant bacmids, which were then used to transfect insect cells to produce recombinant baculoviruses.

GFP expression in the baculovirus-infected insect cells can be visualized under a fluorescent microscope, as shown in Fig. 2.5. Additionally, the plaque assays performed with this baculovirus yielded viral plaques that are readily identifiable (Fig. 2.6). Using the formula:

$$\text{viral titer (pfu/ml)} = \text{number of plaques} \times \frac{1}{\text{dilution factor}},$$

we determined that a high-titer viral stock (~10^9 pfu/ml) was obtained after two rounds of the standard amplification procedure.

For our initial expression study, High Five cells (200 ml) were infected with the high-titer baculovirus at a multiplicity of infection of 5 for five days. The multiplicity of infection is calculated by the formula:

$$\text{multiplicity of infection} = \frac{\text{titer of virus (pfu/ml)} \times \text{ml of virus used}}{\text{number of cells}}.$$

Expressions of the Na,K-ATPase α- and β-subunits were detected in the infected High Five cell plasma membranes (Fig. 2.7), which had a specific activity of 13 μmol P*i* · mg^{-1} protein · h^{-1} for the expressed proteins. As reported in chapters three and four, using our baculoviruses devoid of the GFP gene, we routinely obtained a specific activity of 11 μmol P*i* · mg^{-1} protein · h^{-1} for the expressed wild-type proteins. It seems likely that the expression of GFP does not impede the expression of the Na,K-ATPases in the baculovirus-infected insect cells.

In summary, a green fluorescent protein (GFP) gene was transplanted into a recombinant baculovirus that directs the expression of the Na,K-ATPase α- and β-subunits in insect cells. The co-expression of GFP enabled us to monitor infection with fluorescent microscopy and facilitated efficient titer determination without the use of costly enzyme substrates or antibodies. Our study should be useful to researchers who utilize the baculovirus system for expression studies as the Na,K-ATPase genes can be easily excised to accommodate other genes of interest.

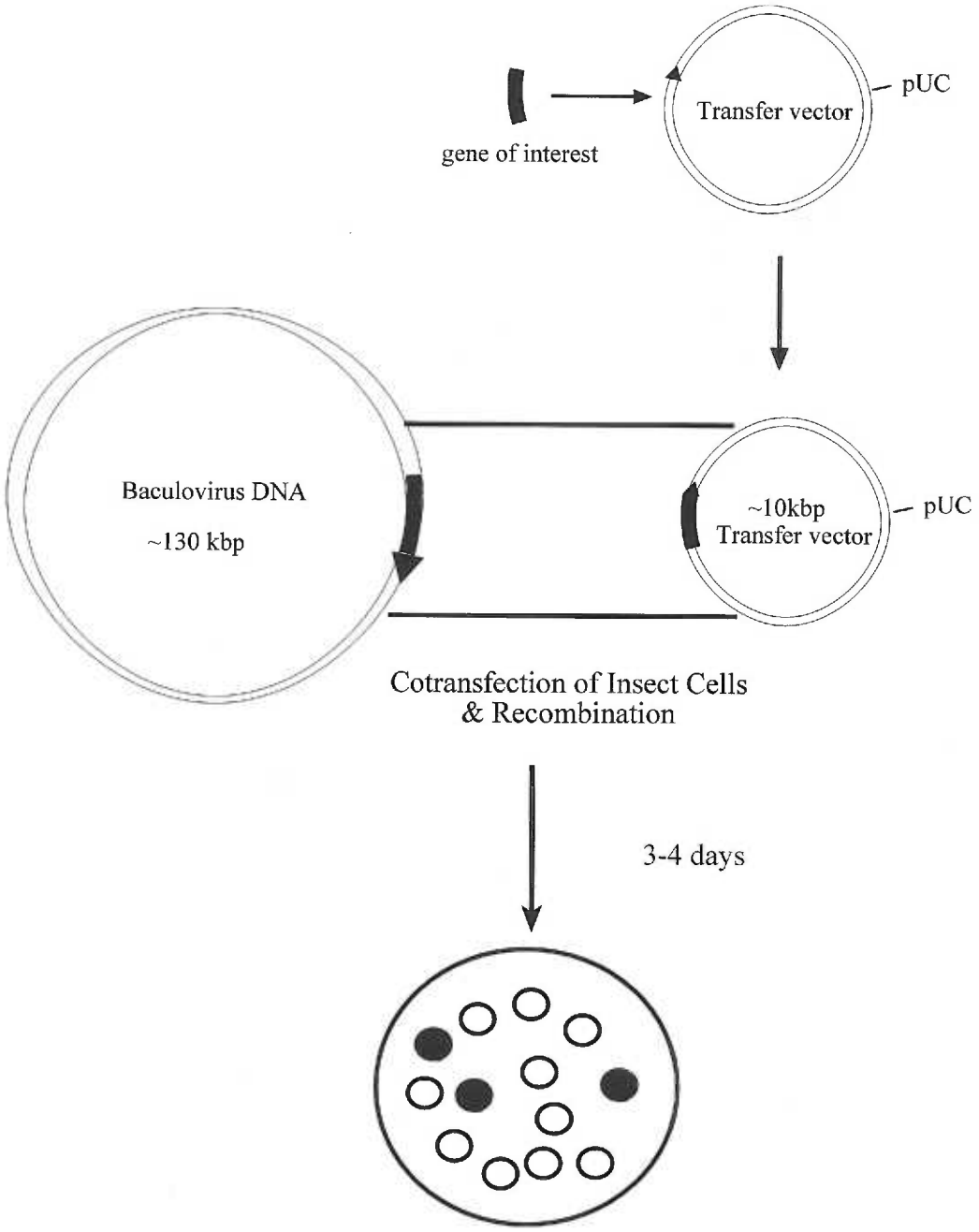


Fig. 2.1. Schematics of the Allelic Replacement Method for Producing Recombinant Baculovirus.

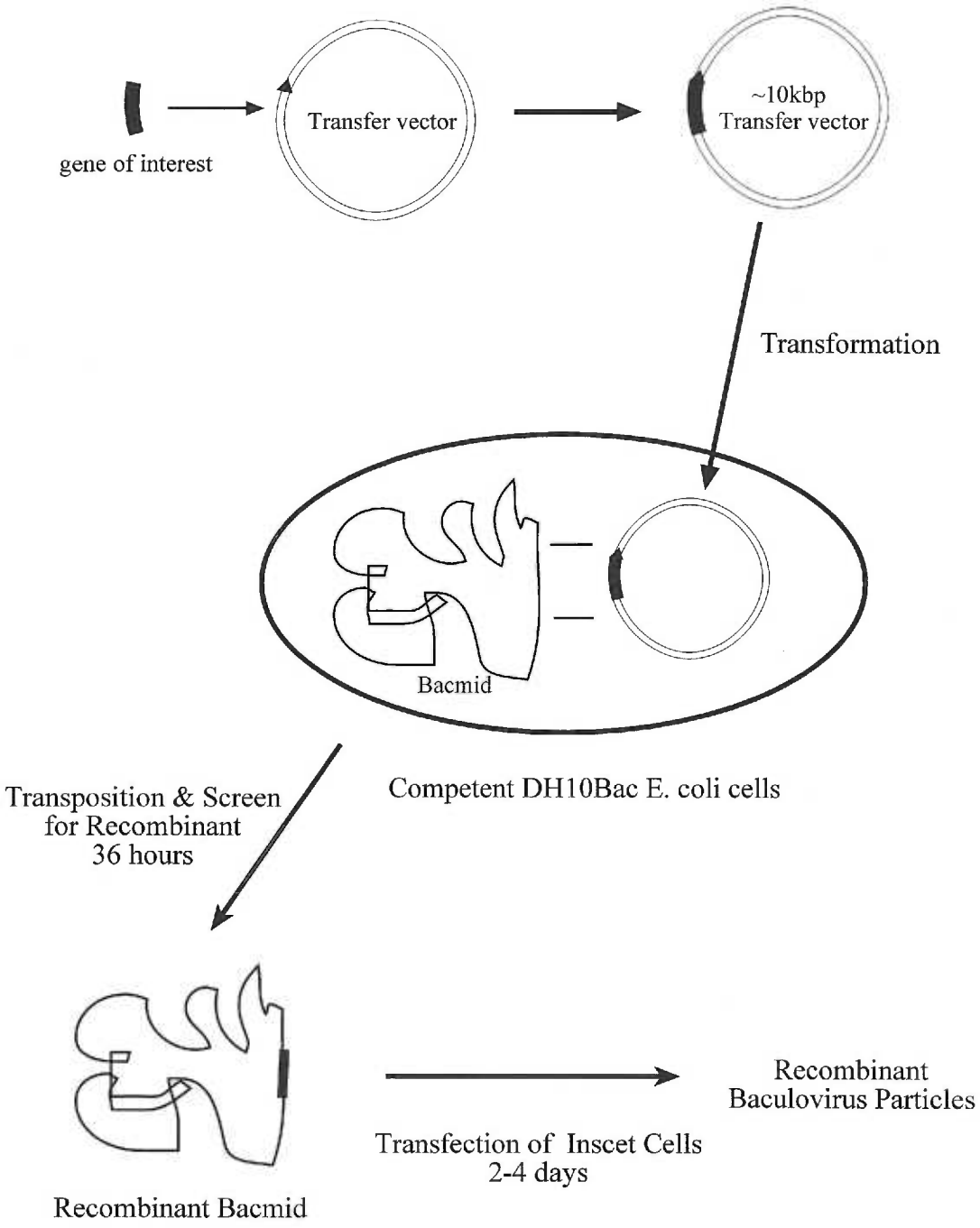


Fig. 2.2. Schematics of the Bacmid Method for Producing Recombinant Baculovirus.

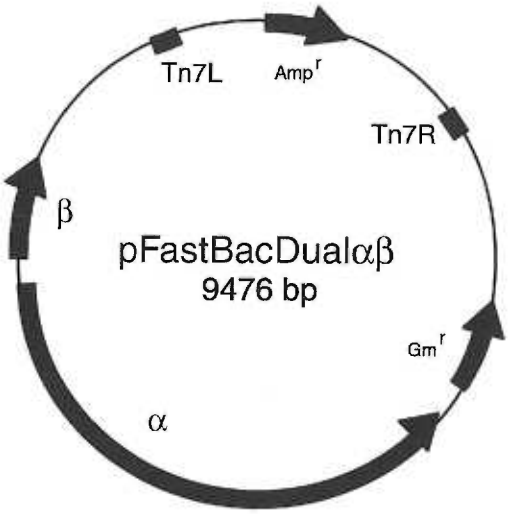


Fig. 2.3. Vector Map of the Donor Plasmid pFASTBACDUALαβ. The α-subunit is cloned under the p10 promoter and the β-subunit is cloned under the polyhedrin promoter. Both promoters are expressed at the very late stage (>24 h) of insect cell infection.

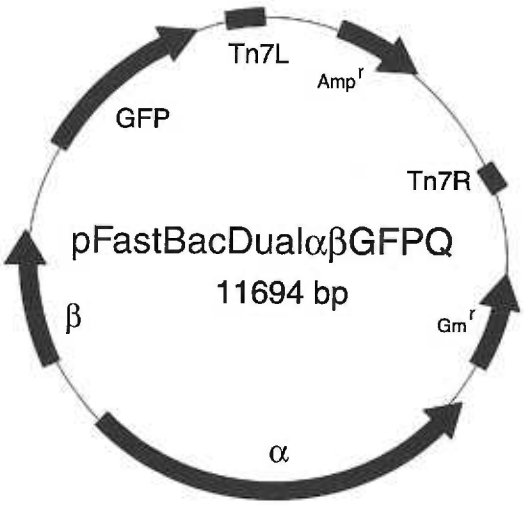


Fig. 2.4. Vector Map of the Novel Donor Plasmid pFASTBACDUALαβGFPQ. The GFP is cloned under the IE1 promoter and its enhancer, hr5. The IE1 promoter directs the expression of GFP at 6 h post-infection.

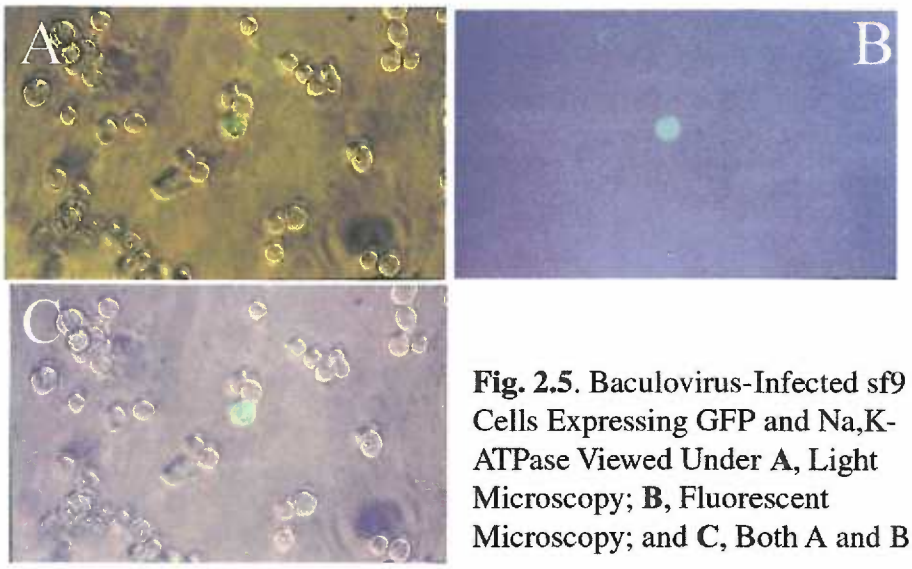


Fig. 2.5. Baculovirus-Infected sf9 Cells Expressing GFP and Na,K-ATPase Viewed Under **A**, Light Microscopy; **B**, Fluorescent Microscopy; and **C**, Both A and B.

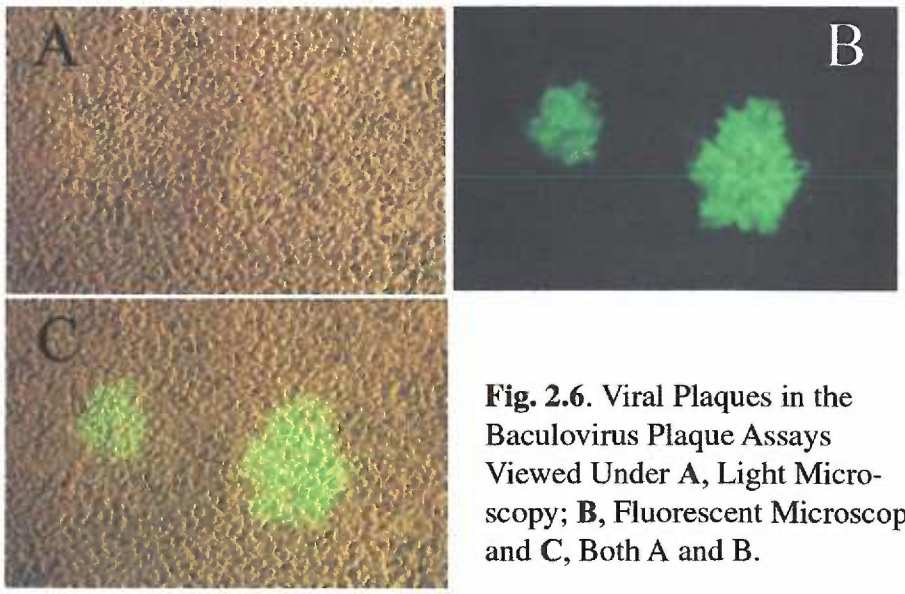


Fig. 2.6. Viral Plaques in the Baculovirus Plaque Assays Viewed Under **A**, Light Microscopy; **B**, Fluorescent Microscopy; and **C**, Both A and B.

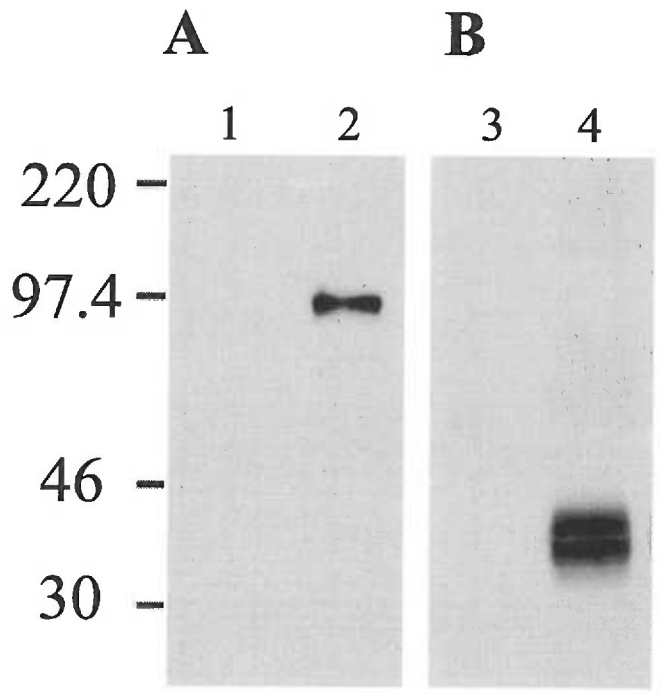


Fig. 2.7. Immunoblots of Uninfected (*lanes* 1 and 3) and Baculovirus-Infected (*lanes* 2 and 4) High Five Cells. The baculovirus contains the cDNA encoding the Na,K-ATPase and GFP. The cells were fractionated under a five-step sucrose gradient, and the plasma membrane fractions were collected and were separated in 7.5% acrylamide gels. The protein samples were transferred onto nitrocellulose membranes and probed with **A**, anti-α1 antibody; and **B**, anti-β1 antibody.

CHAPTER THREE

SITE-DIRECTED CHEMICAL LABELING OF EXTRACELLULAR LOOPS IN A MEMBRANE PROTEIN: THE TOPOLOGY OF THE NA,K-ATPASE α-SUBUNIT

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The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; MTSEA-Biotin, N-biotinylaminoethyl methanethiosulfonate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

SUMMARY

We have mapped the membrane topology of the renal Na,K-ATPase α-subunit by using a combination of introduced cysteine mutants and surface labeling with a membrane-impermeable Cys-directed reagent, *N*-biotinylaminoethyl methanethiosulfonate (MTSEA-Biotin). To begin our investigation, two cysteine residues (Cys^{911} and Cys^{964}) in the wild-type α-subunit were substituted to create a background mutant devoid of exposed cysteines (Lutsenko *et al.*, 1997). Into this background construct were then introduced single cysteines in each of the five putative extracellular loops (P118C, T309C, L793C, L876C and M973C) and the resulting α-subunit mutants were co-expressed with the β-subunit in baculovirus-infected insect cells. All of our expressed Na,K-ATPase mutants were functionally active. Their ATPase, phosphorylation and ouabain-binding activities were measured and the turnover of the phosphoenzyme intermediate was close to wild-type enzyme, suggesting that they are folded properly in the infected cells. Incubation of the insect cells with the cysteine-selective reagent revealed essentially no labeling of the α-subunit of the background construct and labeling of all five mutants with single cysteine residues in putative extracellular loops. Two additional mutants, V969C and L976C, were created to further define the M9M10 loop. The lack of labeling for these two mutants showed that while Met^{973} is apparently exposed, Val^{969} and Leu^{976} are not, demonstrating that this method may also be utilized to define membrane-aqueous boundaries of membrane proteins. Our labeling studies are consistent with a specific ten-transmembrane segment model of the Na,K-ATPase α-subunit. This strategy utilized only functional Na,K-ATPase mutants to

establish the membrane topology of the entire α-subunit, in contrast to most previously applied methods.

INTRODUCTION

Na,K-ATPase (sodium pump) is an integral membrane protein that is present in most animal cells. The enzyme consists of two subunits: a large catalytic α-subunit (about 110 kDa) and a glycosylated β-subunit (~55 kDa); both subunits are required for enzymatic activity (Noguchi, *et al.*, 1987; Horowitz, *et al.*, 1990; DeTomaso, *et al.*, 1993). Na,K-ATPase utilizes the energy derived from ATP hydrolysis to transport Na^+ and K^+ ions across plasma membranes against their electrochemical gradients and is a member of the P_2-ATPase family (Lutsenko & Kaplan, 1995). The ion gradients generated by the sodium pump are important for regulating a variety of physiological functions such as cell excitability, contractility, and secondary active transport.

Several isoforms of the α- and β-subunits have been cloned from various species and the primary sequences have been described (Lingrel & Kuntzweiler, 1994). The secondary structural information on the protein, on the other hand, remains controversial in spite of extensive investigation; such information is essential for understanding structure/function relationships of the Na,K-ATPase. Based on hydropathy analysis (Kyte & Doolittle, 1982), protease accessibility (Karlish, *et al.*, 1993; Lutsenko & Kaplan, 1994) and immunochemical studies (Yoon & Guidotti, 1994; Canfield, *et al.*, 1996), the amino-terminal third of the α-subunit appears to contain four membrane-spanning regions. These regions are followed by a large cytoplasmic loop that has been identified as the ATP-binding domain. Chemical reagents which inactivate the Na,K-ATPase (and abolish high affinity ATP binding) in an ATP-protectable manner all modify amino acid residues in this loop (reviewed by Lingrel & Kuntzweiler, 1994). This loop has recently been overexpressed in *Escherichia coli* and exhibits the same

nucleotide-binding specificities as native Na,K-ATPase (Gatto, *et al.*, 1998). Studies on the carboxyl-terminal third of the α-subunit have produced conflicting results (Karlish, *et al.*, 1993; Lutsenko & Kaplan, 1994; Yoon & Guidotti, 1994; Canfield, *et al.*, 1996; Lee & Guidotti, 1998; Xie, *et al.*, 1996; Fiedler & Scheiner-Bobis, 1996; Shimon, *et al.*, 1998). Recent data using epitope accessibility claim to establish four transmembrane segments in the carboxyl-terminal region and have a total of eight transmembrane segments (Lee & Guidotti, 1998), while several other studies claim to establish that there are 10 transmembrane segments (Xie, *et al.*, 1996; Fiedler & Scheiner-Bobis, 1996; Shimon, *et al.*, 1998).

Most methods used until now to establish membrane topology of the Na,K-ATPase suffer from inherent limitations. Several methods employ proteolytic digestion in sealed vesicles or with purified protein (Karlish, *et al.*, 1993; Shimon, *et al.*, 1998). It is not clear in such studies whether after the initial protease cleavages that the protein retains what can be considered as its native structure. In more recent cleavage methods, based on metal-ion catalyzed cleavages (Shimon, *et al.*, 1998), assumptions are made that the cleavage event occurs close to the metal binding sites, and the locations of the bound metals are also unknown. Indeed with this and other cleavage approaches, interpretations based on minor fragmentation products need further confirmation by other methods. In the use of epitope accessibility, assumptions must be made about the effects of permeablilizing detergents; *i.e.* that such detergent treatments do not alter protein structure (Yoon & Guidotti, 1994; Canfield, *et al.*, 19969; Moller, *et al.*, 1997). These methods are particularly prone to provide misleading information with a protein such as the Na,K-ATPase where abundant evidence exists demonstrating the mobility or

flexibility of its carboxyl-terminal regions. In other methods, originally introduced in studies of prokaryotic membrane protein topology, the amino-terminal domain truncations are attached to reporter proteins in order to determine the topogenic properties of the fusion region (Xie, *et al.*, 1996; Fiedler & Scheiner-Bobis, 1996). However, since the complete structure of the protein of interest is not used and functional tests are not then available, it is assumed that the membrane orientation of the truncations faithfully reflects their orientation in the complete proteins.

In this paper, we describe a different approach to determine the correct number of transmembrane spanning regions of the Na,K-ATPase α-subunit. This method uses data obtained from functional Na,K-ATPase molecules in their native states. Two cysteine residues (Cys^{911} and Cys^{964}) in the α-subunit are changed into serine or alanine residues; the resulting α "null" mutant protein no longer has available cysteine residues for extracellular chemical modification (Lutsenko, *et al.*, 1997). Using this null protein as a starting point, our strategy is to introduce one cysteine residue into each of the putative extracellular loops so the membrane topology of the α-subunit can be probed by surface labeling with membrane-impermeable sulfhydryl reagents. The Na,K-ATPase mutants are heterologously expressed in insect cells using a baculovirus expression system. We are able to characterize and perform labeling experiments exclusively on expressed proteins since the insect cells (sf9 and High Five cells) contain no detectable amount of endogenous Na,K-ATPase activity (DeTomaso, *et al.*, 1993; Blanco, *et al.*, 1994). Our results are consistent with a specific ten-transmembrane segment topological model of the Na,K-ATPase α-subunit. A preliminary report of some of our findings has been published previously (Hu & Kaplan, 1999).

EXPERIMENTAL PROCEDURES

A full-length cDNA clone encoding the wild-type sheep Na,K-ATPase α_1- and β_1-subunits was a gift of Dr. Elmer Price (University of Missouri, Columbia). The Bac-To-Bac™ baculovirus expression system was obtained from Life Technologies, Inc.

Plasmids and Construction of Mutant-- Site-directed mutagenesis of the α-subunit was carried out in the pST100 vector via the polymerase chain reaction overlap extension mutagenesis method (Higuchi, *et al.*, 1988; Ho, *et al.*, 1989) using primers listed in Table 3.1. The plasmid pST100 was constructed by subcloning the wild-type sheep α-subunit cDNA into the multiple cloning site of pOCUS-2 vector (Novagen) as a *Not*I and *Sse*8387I fragment; two silent mutations were then introduced into the α-subunit cDNA to facilitate cassette mutagenesis (see Fig. 3.1A). Using the C911S/C964A mutant as the background construct, cysteine residues listed in Table 3.1 were introduced in the α-subunit individually.

Recombinant baculovirus was produced by following the Bac-To-Bac™ system protocols provided by the manufacturer. Briefly, the donor plasmid pFASTBACDUALαβ (Fig. 3.1B) was constructed by subcloning the wild-type β- and α-subunit cDNA into the multiple cloning sites I and II of the pFASTBACDUAL vector respectively. The β-subunit cDNA was introduced into the vector as an *EcoR*I/*Spe*I fragment and the α-subunit cDNA as a *Sma*I/*Stu*I fragment. To construct donor plasmids containing the cysteine mutations, the wild-type α-subunit cDNA in the pFASTBACDUALαβ was replaced with the α mutants using *Afl*II and *Sac*I. DH10BAC™ cells were transformed with pFASTBACDUALαβ vectors to obtain recombinant baculovirus shuttle vectors (bacmids), which were used to transfect insect cells to generate recombinant baculoviruses. The genomic DNA of the

recombinant baculoviruses were isolated by using the Easy-DNA Kit (Invitrogen) and were sequenced to ensure the appropriate cysteine mutations in the α-subunits.

Cells and Viral Infections—High Five cells were maintained at 27 °C in 250 ml suspension cultures and were split every 3 days with fresh Ex-Cell™405 medium (JRH Biosciences) to keep cell density between 0.5 and 4 x 10^6 cells/ml. For viral amplification, log phase high viability High Five cells (>98%, as determined by trypan blue exclusion) were infected with recombinant baculovirus at a multiplicity of infection of 0.1-1. After 5 days, cells were centrifuged (500 x g, 10 min) and the resulting supernatants were collected as viral stocks. For protein expression, log phase high viability High Five cells in medium containing 1% ethanol (v/v) was infected with viral stocks at a multiplicity of infection of 10-15. High Five cells were harvested (100 x g, 10 min) 4 days after infection.

Plasma Membrane Isolation--Harvested High Five cells (~4 x 10^8 cells) were frozen at -20 °C for an hour and then were resuspended in 6 ml ice-cold homogenizing buffer (250 mM sucrose, 10 mM Tris-HCl, pH7.4) containing a protease inhibitor mixture (antipain, 1 μg/ml; pepstatin, 1 μg/ml; leupeptin, 1 μg/ml; phenyl-methanesulfonyl fluoride, 20 μg/ml). The cells were disrupted via Dounce homogenization and the cell mixture was centrifuged for 15 min at 500 x g to remove intact cells and debris. Into the supernatant containing cell membranes was added an equal volume of 2.55 M sucrose (made in 2 mM EDTA and 10 mM Tris-HCl, pH7.4) and the membrane mixture was fractionated on a 5-step sucrose gradient as described (Yang, *et al.*, 1997). Briefly, the sucrose gradients (3 ml of 2 M sucrose, 6 ml of 1.6 M sucrose, 12 ml of cell membrane mixture, 12 ml of 1.2 M sucrose, 6 ml of 0.8 M sucrose) were

centrifuged in a Beckman SW 28 rotor at 25,000 rpm for 2.5 h, and the endoplasmic reticulum, golgi apparatus and plasma membrane fractions were discernible at the 1.5 M, 1.3 M and 1 M density regions, respectively. The collected plasma membrane fraction was diluted with buffer (25 mM imidazole, 1 mM EDTA, pH7.4), concentrated by centrifugation (Beckman 60Ti rotor, 40,000 rpm, 30 min) and resuspended in a small volume of the homogenizing buffer. The plasma membrane proteins were kept at 4 °C for short-term storage and at -20 °C for long-term storage (~4 weeks).

ATPase Assay---A typical Na,K-ATPase assay contained 500 µl of assay medium (mM: EGTA, 0.6; NaCl, 156; KCl, 24; $MgCl_2$, 3.6; ATP, 3.6; imidazole, 60; Na Azide, 10; pH7.2) and 100 µl of cell membrane preparations containing 8 µg of protein. The protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The assay mixture was incubated at 37 °C for 30 min and the phosphate release was determined as reported by Brotherus *et al.* (1981). The Na,K-ATPase activity was the difference between the ATP hydrolysis measured in the presence and absence of 83 µM ouabain.

Phosphorylation with [^{32}P]ATP--This procedure was carried out essentially as described (Pedemonte & Kaplan, 1986) in a 50 µl medium containing: 1 M NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH7.0, and 50 µg of membrane protein. The reaction was initiated by the addition of ATP ([γ-^{32}P]ATP, NEN Life Science Products, and 7.27 µM Tris-ATP) on ice for 60 s, and the phosphorylation was quenched with 750 µl of ice-cold 5% (v/v) trichloroacetic acid containing 0.5 mM Tris-ATP and 1.5 mM Tris-phosphate The samples were filtered through Millipore filters (pore size 0.45 µm), washed 3 times with 2 ml quenching buffer, and counted in a scintillation counter. Phosphorylation of

the Na,K-ATPase enzyme was calculated from the difference between [^{32}P]phosphate incorporation in the medium above and that measured in a medium containing 1 M KCl instead of NaCl.

Equilibrium [^{3}H]Ouabain Binding--Ouabain binding of the expressed proteins was measured as described (Pedersen, *et al.*, 1996) with the following modifications. Insect-cell membrane protein (100 µg) was incubated at 37 °C for 1 h in 50 µl incubation buffer (3 mM $MgSO_4$, 1 mM $NaTris_2VO_4$, 1 mM EGTA, 10 mM MOPS-Tris, pH7.2) containing 3.78 µM [^{3}H]ouabain (NEN Life Science Products) and 1.55 µM ouabain, and then was placed on ice for 30 min. The reaction mixtures were filtered through Millipore filters (pore size 0.45 µm), washed 3 times with ice-cold 2 ml washing buffer (10 mM MOPS-Tris, pH7.2), and counted in a scintillation counter. Samples containing 1.4 mM excess ouabain were used to correct for non-specific binding.

Labeling of Intact Cells, Immunoprecipitations, and Immunochemical Analysis--High Five cells (1 x 10^8 cells) were infected for 3 days and were incubated at room temperature for 4 h in 10 ml incubation buffer (150 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH7.4) containing 200 µM MTSEA-Biotin (Toronto Research Chemicals, Inc). At the end of the incubation period, the cell mixture was briefly treated with 2-mercaptoethanol (14 mM) to remove excess reagent and then was washed twice with 50 ml of the incubation buffer. When the 2-mercaptoethanol quench was omitted, the outcome of the labeling reactions was unaltered (data not shown). Cells were harvested and the cell membranes were fractionated on the 5-step sucrose gradient as described above. For immunoprecipitations, 100-300 µg of plasma membrane proteins were solubilized in 500 µl of 2% CHAPS in the incubation buffer for 15 min at room

temperature and the insoluble material was pelleted (600 x g, 6 min). The CHAPS mixture was diluted to 1% and pre-cleared overnight with rabbit pre-immune serum and protein G sepharose beads (Amersham Pharmacia Biotech). The sepharose beads were pelleted (600 x g, 3 min) and the resulting supernatant was incubated with antibody raised against the ATP-binding domain of the α-subunit (Gatto, *et al.*, 1998) and fresh protein G sepharose beads overnight at 4 °C. The sepharose beads were washed twice with 1% CHAPS and eluted with 30 µl sample buffer (equal volumes of 8 M urea, 10% SDS and 125 mM Tris buffer, pH6.8). The protein samples were separated in 7.5% acrylamide gel (Laemmli, 1970), transferred onto nitrocellulose membrane (MSI Micron Separations, Inc.) by electroblotting in 10 mM CAPS (pH11) containing 10% methanol, and immunostained with peroxidase-linked streptavidin (Amersham Pharmacia Biotech). An identical nitrocellulose blot was stained with mouse monoclonal anti-α1 antibody (ABR Bioaffinity Reagent, Inc.) and peroxidase-linked sheep anti-mouse secondary antibody (Amersham Pharmacia Biotech). The signal intensity levels on the blots were analyzed by the computer software NIH Image.

RESULTS

Expression and Enzymatic Characterization of Cysteine Mutants-- Using the C911S/C964A construct as the "null" background, a panel of Na,K-ATPase α mutants was generated. Each mutant contained one cysteine residue in each of the putative extracellular loops (Table 3.1) and was co-expressed with the wild-type sheep β-subunit using infection with baculovirus. The expressed plasma membrane proteins were purified by a 5-step sucrose gradient centrifugation and characterized to confirm functional integrity. As shown in Table 3.2, the mutants displayed specific activities in the range of 0.1-0.6 μmol Pi • mg^{-1} protein • min^{-1}, had equal ouabain-binding and phosphorylation levels, which ranged from 10-100 pmol • mg^{-1} protein, and the turnover number, based on these specific activities and the ligand-binding levels, all were between 7000 and 10000 min^{-1}. In other words, all the expressed mutants showed normal functional activity.

Labeling of Cysteine Mutants--High Five cells expressing the Na,K-ATPase cysteine mutants were incubated with MTSEA-biotin so that a biotin group would be introduced into the α-subunits that contain extracellularly exposed cysteines. After the MTSEA-biotin treatment, the mutant proteins were immunoprecipitated, resolved by SDS-PAGE and the protein biotinylation levels were determined on a blot using peroxidase-linked streptavidin and chemiluminescence. An identical blot was stained with anti-α1 antibody to determine the amount of α-subunit immunoprecipitated. Fig. 3.2A shows the typical α-subunit biotinylation patterns for the expressed proteins and Fig. 3.2B shows the amount of α-subunit on the blot. A plot of the specific activity of the mutants against their respective α-subunit intensities on the blot is shown in Fig. 3.3A. A

strong correlation between the two variables is found in this plot, demonstrating that the difference in the specific activity is due to the difference in the expression level (or the amount of protein reached the plasma membrane). The very low level (if any) of labeling of the null mutant can be readily seen by visual examination of the relative intensity of staining by the streptavidin compared with the α-subunit activity (Figs. 3.2A and 3.2B respectively). To allow for the different expression levels of the mutants, the ratio of the α-subunit biotinylation level to the α-subunit intensity level on the blot was calculated for each mutant. Fig. 3.3B shows such ratios normalized against that of the null construct. The relative biotinylation signals for the P118C, T309C, L793C, L876C and M973C mutants are 4-12 times stronger than that of the null mutant. The ratios demonstrate the significantly greater access of the cysteine residues in these constructs than in the null mutant. To further define the M9M10 extracellular loop, V969C and L976C mutants were constructed. The α-subunit biotinylation and intensity levels for these and the M973C mutant are shown in Figs. 3.4A and 3.4B, respectively. Good expression levels for the three mutants in the M9M10 regions are observed (Fig. 3.4B); however, the labeling data of the V969C and L976C mutants showed that these two residues are not accessible from the extracellular medium (Fig. 3.4A).

DISCUSSION

We have established the membrane topology of the Na,K-ATPase α-subunit by testing the accessibility of cysteine residues introduced in the predicted extracellular loops. Our method rejects all models consisting of less than 10 transmembrane segments and confirms a specific 10 transmembrane segment model (Fig. 3.5). Our strategy is based on the earlier observations that Cys^{911} and Cys^{964} are the only two cysteine residues exposed to non-penetrating cysteine-specific reagents in the extracellular medium (Lutsenko, *et al.*, 1997). In the current study, the C911S and C964A substitutions were made to construct an α-subunit background and the lack of labeling of this mutant confirmed the removal of all exposed sulfhydryl groups (Fig. 3.2), which supports the conclusion of our earlier labeling studies (Lutsenko, *et al.*, 1997). Cysteine was then introduced at residues 118, 309, 793, 876, 969, 973 or 976 of the background construct in each of seven mutants and probed with a membrane-impermeable cysteine-directed reagent.

Our experimental strategy involves the functional characterization of mutants to ensure that the topology of active molecules is determined and uses the same strategy for the topology of the entire α-subunit. It should also be pointed out that this strategy contains both positive and negative controls. Our previous work established the accessibility of Cys^{911} (in M8) and Cys^{964} (in M9) from the extracellular compartment in studies of native canine renal Na,K-ATPase (Lutsenko, *et al.*, 1997). This provides a positive control for our heterologously expressed wild-type enzyme (*lane* 1, Fig. 3.2). The removal of these two cysteine residues provides a negative control where little or no labeling from the extracellular medium is observed (*lane* 2, Fig. 3.2). All of our mutants

are functionally active. The turnover number for the phosphoenzyme intermediates of the mutants is close to the value for the wild-type phosphoenzyme heterologously expressed in High Five cell membranes (Table 3.2). This suggests that their processing and folding are not affected by the substitutions. In contrast, previous topology studies employed α-subunit mutants that were less well characterized (due to endogenous Na,K-ATPase activity) (Yoon & Guidotti, 1994; Canfield, *et al.*, 1996) or non-functional (Xie, *et al.*, 1996; Fiedler & Scheiner-Bobis, 1996) when assumptions about the "normal" orientations of the expressed proteins were made. This is particularly relevant to approaches that use carboxyl-terminal truncations and reporter groups (such as glycosylation status) to establish topology. If truncations are used, usually functional activity is lost so that no reliable test can be made to demonstrate the truncated polypeptide is processed or folded in the same way as the intact native protein.

The amino-terminal third of the Na,K-ATPase α-subunit has been proposed to contain four membrane-spanning regions (Karlish, *et al.*, 1993; Lutsenko & Kaplan, 1994; Yoon & Guidotti, 1994; Canfield, *et al.*, 1996) and the accessibility profiles of the P118C and T309C mutants confirmed the locations of Pro^{118} and Thr^{309} in the respective M1M2 and M3M4 extracellular loops (Figs. 3.2, 3.3). The region around the M1M2 extracellular loop has been identified as a primary determinant of ouabain-binding affinity in several studies (Price & Lingrel, 1988; Price, *et al.*, 1989; Price, *et al.*, 1990; Canessa, *et al.*, 1992). We found that the P118C substitution did not greatly alter the ouabain sensitivity of the mutant (Table 3.2), suggesting that amino acid residue at this position does not make direct contact with the cardiac glycoside. Furthermore, since the

substitution replaces a proline residue, it is unlikely that the precise structure of the M1M2 loop in the region is critical for the ouabain binding interaction.

There has not been a clear consensus in the numbers of membrane-spanning domains in the carboxyl-terminal third of the Na,K-ATPase α-subunit. Indeed the carboxyl-terminal topology of the α-subunit has been the source of most debate. This is doubtless because of the extra mobility and flexibility of the region compared with the amino-terminal region, so that methods which involve disruptive procedures (such as proteolysis and peptide bond cleavage) are prone to generate artifactual observations. We began our investigation in this region by introducing a cysteine at residue 793, which has been predicted to be a part of the M5M6 external loop (Karlish, *et al.*, 1993; Fiedler & Scheiner-Bobis, 1996; Lutsenko, *et al.*, 1997). Previous studies have shown that the M5M6 hairpin, following proteolysis and removal of K^+ ions (at 37 °C), is released from the membrane (Lutsenko, *et al.*, 1995) to the extracellular space (Gatto, *et al.*, 1999a). Such release was not observed in the presence of K^+ ions, suggesting that the M5M6 hairpin in the intact protein is dynamic (possibility moving in a way which is perpendicular to the plane of the membrane) during the conformational shifts of the Na,K-ATPase catalytic cycle. The present study has identified Leu^{793} as one of the residues that are exposed to the extracellular phase in the native protein (Figs. 3.2, 3.3). This provides the first evidence for the extracellular location between the loops M5 and M6 in the native Na,K-ATPase. Studies using a cysteine-specific inhibitor, omeprazole, have previously provided evidence for such a configuration in the gastric H,K-ATPase (Besancon, *et al.*, 1997).

Our demonstration of the exposure of Leu^{793} at the outside surface enabled us to distinguish between two ten transmembrane models recently proposed for the α-subunit. In one study using carboxyl-terminal truncations of the α-subunit expressed in yeast (Fiedler & Scheiner-Bobis, 1996), the authors place Ala^{789} close to the cytoplasmic side and Met^{809} in the extracellular loop between M5 and M6. Their results are compared with another 10 membrane-spanning model proposed earlier by Karlish and coworkers (1993), who placed Ala^{789} close to the extracellular part of M5. Our results support the latter model, since we find Leu^{793} exposed in the extracellular loop which would place Ala^{789} close to the extracellular phase in M5.

Lemas *et al.* (1994) have shown that a 26-sequence peptide (Asn^{889}-Ala^{914}) in the predicted M7M8 extracellular loop interacts directly with an external fragment of the β-subunit. It should be pointed out that their study utilized a co-immunoprecipitation protocol and the functional importance of the association was not determined. Our labeling result with of the L876C mutant provides evidence for the extracellular location of this loop in the intact protein (Figs. 3.2, 3.3). It is interesting to note that previous immunochemical labeling studies using an antibody raised against the Trp^{887}-Arg^{904} sequence have produced conflicting results. In one study, the antibody detected Na,K-ATPase from the extracellular domain after prolonged incubation (Mohraz, *et al.*, 1994). In that study 3 new models were suggested for the transmembrane organization of the Na,K-ATPase α-subunit, consisting of either 8 or 10 transmembrane segments. All three of these models (Models A, B and C in Mohraz, *et al.*, 1994) are rejected by our observations of the extracellular localization of Leu^{793} and Leu^{876}. In another study, the anti- Trp^{887}-Arg^{904} antibody failed to label Na,K-ATPase without cell permeablization by

detergent (Ovchinnikov Yu, *et al.*, 1988). An explanation which might account for the discrepancy would be that this region of the M7M8 loop is not freely accessible, but rather associates with the β-subunit in the extracellular space. Prolonged incubation with the antibody or treatment with detergent disrupted the α/β interaction, thus enabled the binding of the antibody.

In a recent article, Møller *et al* (1997) carried out a very thorough study of the membrane topology of the sarcoplasmic reticulum Ca-ATPase. Their work employed a combination of sequence-specific antibodies and proteolysis methods and they emphasized the extreme caution when using detergents or similar treatments for probing intravesicular locations with the antibodies. The most striking conclusion of their studies was that the extracellular loop between M7 and M8 in the previously accepted model was proposed to be cytosolic. Two different models were then suggested which might account for the cytosolic location of this loop: (i) M7 does not completely protrude across the membrane but folds backs to the cytosol from within the membrane or (ii) the existence of an additional membrane return after M7 but before the usual M8 segment. Our data provide clear evidence for the extracellular location of a residue (Leu^{876}) immediately after M7 and for the extracellular accessibility of Cys^{911} close to the beginning of M8. This establishes that the region Lys^{876}-Cys^{911} may be accessed from the extracellular space. These data contradict the conclusion of Møller *et al*, or else there is a basic difference between the membrane topology of the Na,K-ATPase α-subunit and the Ca-ATPase. Furthermore, Møller *et al* raised the possibility that there may be a "plasticity" in the membrane location of the M8 segment in the Ca-ATPase. They discuss earlier work on the Na,K-ATPase (Lutsenko & Kaplan, 1994; Lutsenko, *et al.*,

1995; Lutsenko, *et al.*, 1997) to suggest the importance of carboxyl-terminal plasticity in ion pumping by P-type ATPases. Although our current data do not address this specific issue, modifications of our approach may in the future be employed to investigate such "plasticity".

The post-tryptic 19-kD membrane fragment of the α-subunit, consisting of residues Asn^{831}-Tyr^{1016}, was first described by Karlish *et al.* (1990). The amino- and carboxyl-termini of this protein fragment have been assigned cytoplasmic locations (Karlish, *et al.*, 1993), suggesting that it should contain an even number of transmembrane segments (at least two). As mentioned above, the detection of the L876C mutant at the extracellular domain provided evidence for the M7M8 transmembrane segments in the 19-kD membrane fragment. The labeling data of the M973C mutant revealed an additional extracellular site after M8 (Figs. 3.2, 3.3), validating the presence of the M9 and M10 transmembrane domains.

In the present study and in contrast to earlier studies (Yoon & Guidotti, 1994; Canfield, *et al.*, 1996; Lee & Guidotti, 1998), we have obtained unequivocal evidence for exposure of the M9M10 loop. Presumably the short length of this loop (Figs. 3.2, 3.3) precluded this finding in earlier studies. In a study by Yoon and Guidotti (1994) using epitope insertion and expression in COS-7 cells, it was concluded that the carboxyl-terminal region contained 4 transmembrane segments. Evidence was lacking for the final two segments after Val^{939}; thus it was concluded that the α-subunit had 8 transmembrane segments. This conclusion was repeated in a more recent work from Lee and Guidotti (1998) who inserted a tag between residues 973 and 974 and determined, using immunofluorescence, that residue 973 was internal. This recent finding is contradicted

by our current observation that Met^{973} is exposed at the extracellular surface, as well as by our earlier suggestion about the membrane location of a segment beginning at Met^{973} based on tryptic digestion studies of membrane-associated peptides (Lutsenko, *et al.*, 1994). Similar conclusions, that the α-subunit contained 8 transmembrane segments, were also reached by Canfield *et al.* (1996) based on their epitope addition studies showing that residues beyond 978 were all intracellular while residue 953 was extracellular. Their study also found that there were no transmembrane segments between 832 (cytoplasmic) and 895 (cytoplasmic), which is at odds with our finding of the extracellular locations of residues Leu^{876} and Met^{973}. It seems likely that all 8 transmembrane segments models can now be disregarded.

The V969C and L976C mutants were generated to further define the M9M10 loop and our data showed that these two residues are not freely accessible from the extracellular medium (Fig. 3.4). These observations establish the very small size of this extracellular loop (it probably only consists of residues Ala^{970}-Pro^{975}) and show that the M9 and M10 helices must be closely apposed. The relative lack of accessibility of V969C and L976C compared with M973C demonstrates that our approach is capable of distinguishing accessibility of residues separated by only three or four residues and may be useful in establishing the locations of the membrane interface in the protein structure. The location of these important membrane-interface regions is difficult to predict from limited structural knowledge and has proven to be difficult to define experimentally using other methods.

Our previous study on the labeling of purified renal Na,K-ATPase with 4-acetamino-4'-maleimidylstilbene-2,2'-disulfonic acid (a charged and non-penetrating

maleimide) and 7-diethyl-amino-3-(4'-maleimidyl)-4-methylcoumarin (a penetrating maleimide) showed that Cys^{964} is accessible from the extracellular space when the enzyme is in a phosphorylated form (Lutsenko, *et al.*, 1997). Upon dephosphorylation and K^+ occlusion, however, Cys^{964} is no longer exposed to the aqueous phase, suggesting the dynamic nature of the M9M10 loop region. It is quite possible that the positions of Val^{969} and Leu^{976} are also sensitive to the protein conformation and that their accessibility alters as the different ligands bind and dissociate. The present studies utilized labeling in intact cells where the protein is turning over and control of protein conformation is limited. Nevertheless, the M973C mutant is consistently labeled and, in future studies where labeling will be performed in isolated membranes, it will be possible to test the issues of conformation-dependent mobility of specific residues.

In summary, we have utilized a Cys-substitution strategy to establish the membrane topology of the α-subunit of the Na,K-ATPase. The α-subunit has ten transmembrane segments. Our approaches can be utilized to test whether or not the accessibility of a particular residue close to the membrane-aqueous interface alters during the reactive cycle.

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TABLE 3.1

Oligonucleotide primers used for site-directed mutagenesis

PCR overlap extension mutagenesis was carried out using primers listed. Underlined bases denote mutations. *Nde*I and *Mlu*I sites were introduced into the wild-type α-subunit cDNA as silent mutations to facilitate cassette mutagenesis.

Mutations	Reactive External Loop	PCR Primers (5'-3')	Subcloning Sites
Silent:			
NdeI site		ggatccacgtccaccatatgctcagacaaaactgg	*Dra*III/*Nsi*I
MluI site		gctgacttggtcatctgcaagacgcgtaggaattccgtcttcc	*BspH*I/*Sac*I
Null mutants:			
C911A		cgtggagttcactgcgcatacggccttcttcg	*BspH*I/*Mlu*I
C911S		cgtggagttcactagtcatacggccttcttcg	*BspH*I/*Mlu*I
C964A		gctttcctgtcctacgcgcctggtatgggtgttgcc	*Mlu*I/*Bsu36*I
Cys mutants:			
P118C	m1m2	ccaagctgctacagaagaggaatgccaaaatgataatctgtaccttgg	*Xho*I/*Xba*I
T309C	m3m4	cctttctctgatccttgagtactgctggttggaggctgtcatcttcc	*Xba*I/*Nde*I
L793C	m5m6	gatatttattattgcaaacattccatgccccctggggacc	*Mun*I/*BspH*I
L876C	m7m8	ctccctattcactgcctaggcatccgag	*BspH*I/*Mlu*I
V969C	m9m10	gccctggaatggggttgtgcactgaggatgtatcccc	*Mlu*I/*Sac*I
M973C	m9m10	ggtgttgctcttcggtgctatcccct	*Mlu*I/*Sac*I
L976C	m9m10	aggatctatccctgcaaacctacct	*Mlu*I/*Sac*I

TABLE 3.2

Characteristics of Na,K-ATPases isolated from dog kidney inner medulla and from membrane preparations of baculovirus-infected High Five cells

Dog kidney enzyme was obtained as described (Jorgensen, 1974; Liang, *et al.*, 1976). Crude membrane preparations from baculovirus-infected High Five cells were fractionated on a 5-step sucrose gradient (Yang, *et al.*, 1997), and the resulting plasma membrane fractions were collected for activity assays. The specific activity (ouabain-sensitive ATPase activity) and ligand binding properties of the mutants were determined as described under "Experimental Procedure". The turnover number, which is the turnover rate of phosphoenzyme per minute, was calculated by taking the ratio of specific activity over phosphorylation number. Each value below represents the means of triplicate determinations of specific activity and ligand binding from at least three different membrane preparations and has a standard error of less than 10%.

	Specific Activity* (nmol Pi • mg^{-1} protein • min^{-1})	**Ligand Binding** (pmol • mg^{-1} protein)		**Turnover Number** (min^{-1})
		Ouabain	phosphorylation	
Dog Kidney	2.15×10^4		2740	7847
Hi5 Expressed:				
wild-type	178	31	28	6357
C911A, C964A	163	18	16	10188
C911S, C964A	452	67	72	6278
P118C (m1m2)	108	10	11	9818
T309C (m3m4)	128	15	16	8000
L793C (m5m6)	108	11	12	9000
L876C (m7m8)	122	15	17	7176
V969C (m9m10)	617	98	86	7174
M973C (m9m10)	403	53	61	6607
L976C (m9m10)	300	36	41	7317

** The Na,K-ATPase activity for the plasma membrane preparations of non-infected High Five insect cells is 0.33 nmol Pi • mg^{-1} protein • min^{-1}*

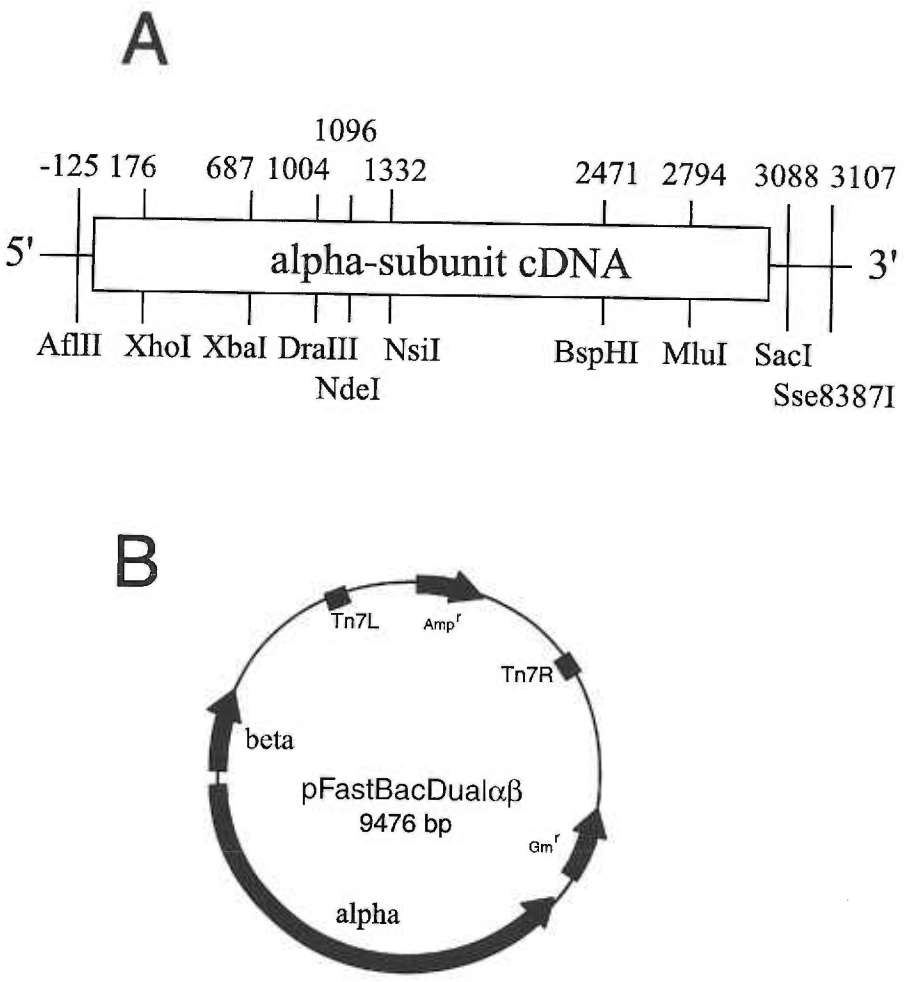


Fig. 3.1. A, restriction enzyme map of the α-subunit. Unique restriction sites in the α-subunit cDNA were used to facilitate cassette mutagenesis. The *NdeI* and *MluI* sites were introduced in this work as silent mutations. **B**, diagram of the donor plasmid pFASTBACDUALαβ. The α-subunit cDNA was subcloned under the p10 promoter and the β-subunit cDNA was subcloned under the polyhedrin promoter. Both promoters are baculovirus-specific and are expressed at the very late stage (>24 hr) of insect cell infection.

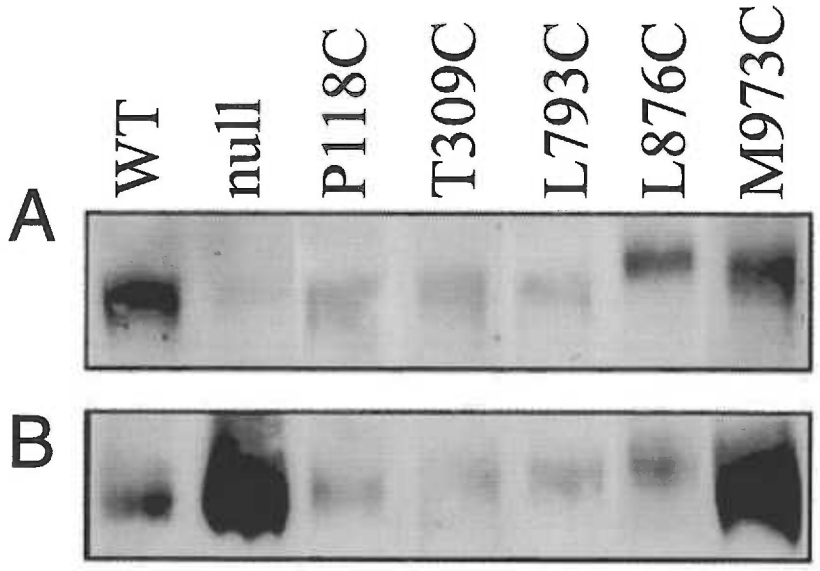
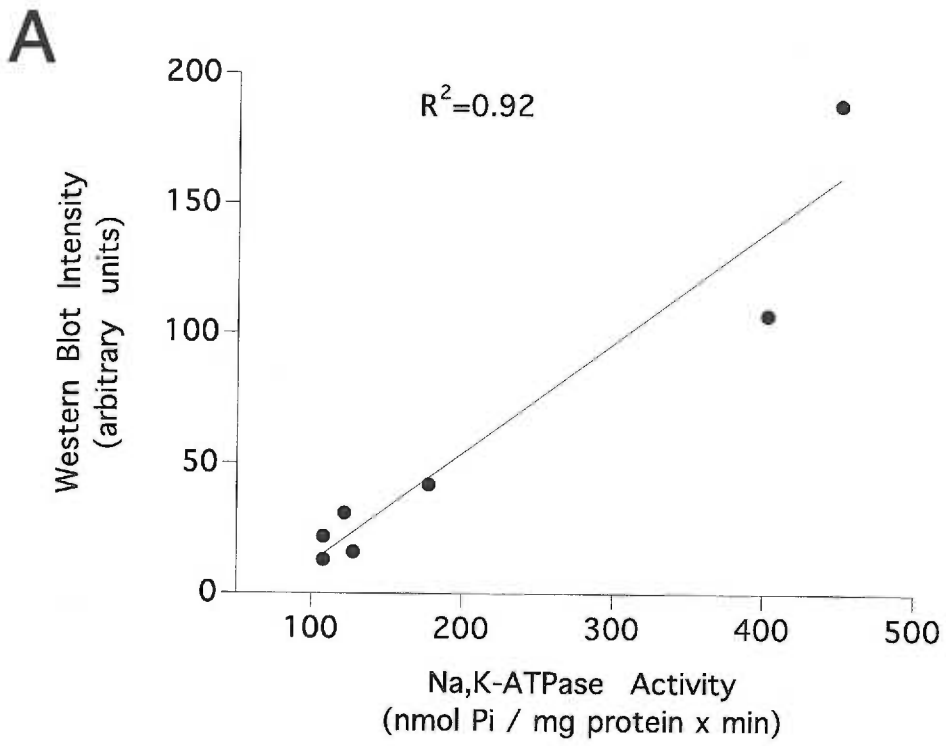


Fig. 3.2. Labeling of introduced cysteine residues in the putative extracellular loops of the α-subunit. High Five cells expressing the Na,K-ATPase cysteine mutants were incubated with 200 μM MTSEA-biotin. After sucrose gradient fractionation, Na,K-ATPase in the plasma membrane fraction was immuno-precipitated, resolved by SDS-PAGE and transferred to nitrocellulose membrane. **A**, biotin incorporation into the α-subunit was detected with peroxidase-linked streptavidin. **B**, an identical blot was probed with a monoclonal anti-α1 antibody. Data shown here represent results of three independent labeling studies. *WT*, wild type.



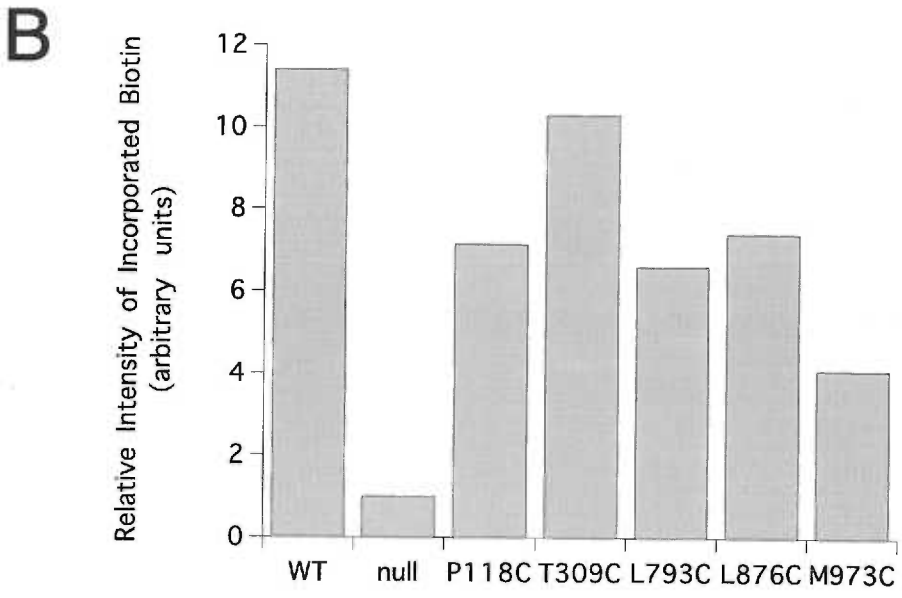


Fig. 3.3. A, the α-subunit intensity levels in Fig. 3.2B were quantified with the computer software NIH Image and were plotted against the Na,K-ATPase activities of the respective mutants. A linear fit of the two sets of variables yielded an R-square value of 0.92. **B**, the ratio of the biotin incorporation level over the α-subunit expression level was calculated for each mutant and was normalized against such ratio of the null construct. *WT*, wild type.

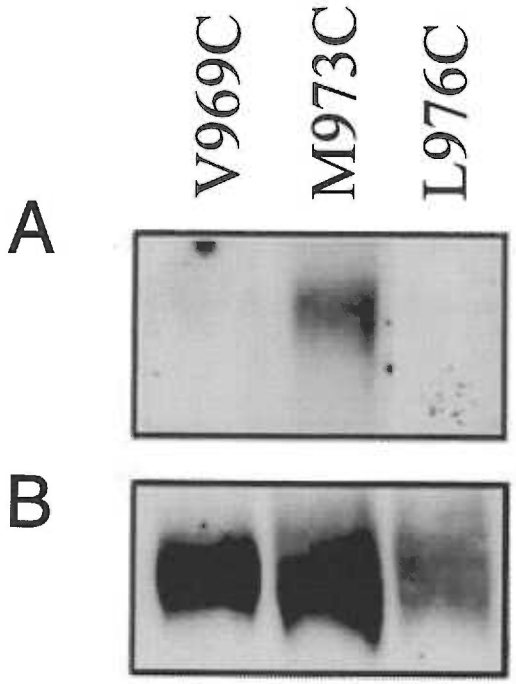


Fig. 3.4. Labeling of introduced cysteine residues in the M9M10 extracellular loop of the α-subunit. High Five cells expressing the V969C, M973C or L976C constructs were incubated with 200 μM MTSEA-biotin. After sucrose gradient fractionation, the expressed mutants were immunoprecipitated, resolved by SDS-PAGE and transferred to nitrocellulose membrane. **A**, biotin incorporation into the α-subunit, as detected with peroxidase-linked streptavidin. **B**, an identical blot was stained with a monoclonal anti-α1 antibody to detect immunoprecipitated α-subunits. Data shown here represent results of three independent labeling studies.

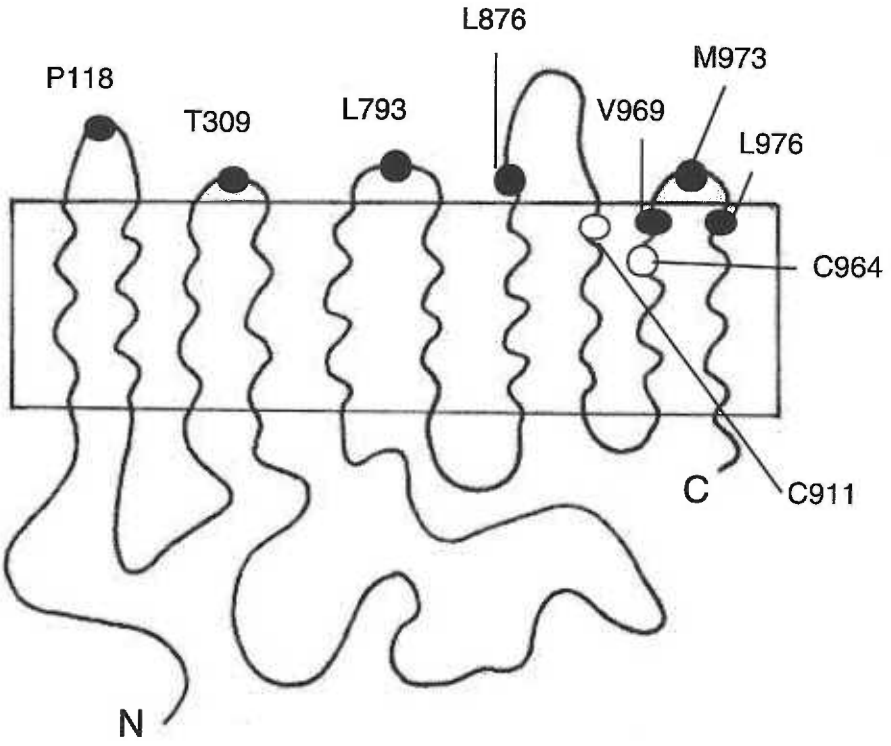


Fig. 3.5. Predicted transmembrane topology model of the Na,K-ATPase α-subunit. Cys911 and Cys964 in the wild-type protein are represented as *open circles*. *Closed circles* denote residues that were changed into cysteines in this study. We propose the following boundaries for the extracellular loops of the α-subunit: M1M2 loop, Ile110-Leu123; M3M4 loop, Leu306-Ala313; M5M6 loop, Ile791-Thr797; M7M8 loop, Ala867-Phe909; M9M10, Ala970-Pro975.

CHAPTER FOUR

EXPRESSION OF AN ACTIVE NA,K-ATPASE WITH AN α-SUBUNIT LACKING ALL TWENTY-THREE NATIVE CYSTEINE RESIDUES

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The abbreviations used are: MIANS, 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid; ER, endoplasmic reticulum; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

SUMMARY

We have constructed a mutant Na,K-ATPase α1-subunit with all native cysteine residues replaced. Using the baculovirus system, this cysteine-less α1-subunit and wild-type β1-subunit were expressed in High Five cells. After 3 days of infection, cells were fractionated, and endoplasmic reticulum, Golgi apparatus and plasma membranes isolated. The molecular activity of the cysteine-less mutant in the plasma membranes was close to the wild-type protein (8223 min^{-1} vs. 6655 min^{-1}). Cation and ATP activation of Na,K-ATPase activities revealed that replacing all 23 cysteines resulted in only a 50% reduction of the K_m for Na^+, a two-fold increase in K_m for K^+, and no changes in K_m for ATP. The distribution of α-subunits among the membranes showed a high percentage of cysteine-less protein in the endoplasmic reticulum and golgi apparatus compared to the wild-type protein. Furthermore, the cellular stability of the αβ assembly appeared reduced in the cysteine-less mutant. Cells harvested after more than 3 days of infection showed extensive degradation of the cysteine-less α-subunit, which is not observed with the wild-type enzyme. Thus the Na,K-ATPase contains no cysteine residues that are critical for function, but the folding and/or assembly pathway of this enzyme is affected by total cysteine substitution.

INTRODUCTION

Na,K-ATPase (E.C. 3.6.1.3) is a heterodimeric membrane protein that utilizes the energy of hydrolysis of one ATP molecule to transport three sodium ions and two potassium ions against their electrochemical potential gradients. This enzyme is present in the plasma membrane of most eukaryotic cell and is a member of the P-type ATPase family (Lutsenko & Kaplan, 1995) which form a phosphorylated enzyme intermediate during the reaction cycle. The Na,K-ATPase is composed of a catalytic α-subunit (110 kDa) and a glycosylated β-subunit (~55 kDa); both subunits are required for function. Various isoforms of each of the subunits have been cloned from different species and tissues, and their enzymatic activities have been characterized (Sweadner, 1989; Lingrel & Kuntzweiler, 1994; Woo, *et al.*, 1999; Blanco, *et al.*, 1999).

The most commonly accepted model for the reaction mechanism of the Na,K-ATPase involves the sequential formation of two acyl-phosphate intermediates. The first intermediate, E_1P, forms on the transfer of the terminal phosphate of ATP to Asp^{369} in a sodium-dependent fashion. This form of phospho-enzyme intermediate is ADP-sensitive and K^+-insensitive. When the sodium ions leave the protein, E_1P is converted to E_2P, which is a potassium-sensitive and ADP-insensitive intermediate. This is the basis of the Albers-Post model (Post, *et al.*, 1975) for the reaction mechanism of the Na,K-ATPase, as well as for most P_2-type ATPases (Lutsenko & Kaplan, 1995). Early important observations using *N*-ethylmaleimide delineated these phospho-enzyme forms and showed that a population of sulfhydryl residues, when modified, blocked the E_1P to E_2P transition and inhibited ATPase activity (Fahn, *et al.*, 1966). These observations were among the first to demonstrate that cysteine residues may play a key role in the Na,K-

ATPase enzymatic cycle. Later works have identified cysteine residues that may be involved in ouabain binding (Canessa, *et al.*, 1992; Schultheis & Lingrel, 1993), ATP hydrolysis (Pedemonte & Kaplan, 1990; Gatto, *et al.*, 1999b), and αβ association (Wang & Farley, 1998). In addition, several sulfhydryl-reactive reagents have been shown to inactivate the Na,K-ATPase (Patzelt-Wenczler & Schoner, 1981; Esmann, 1982; Kaplan & Mone, 1985; Anner, *et al.*, 1990). However, the modified cysteine residues were not identified, and the functional significance of these residues is unknown. We have shown recently that a Cys-specific maleimide, MIANS, inactivates the Na,K-ATPase activity by selectively modifying Cys^{577} and that inactivation is prevented by preincubation with ATP (Gatto, *et al.*, 1999b). Although data from our labeling studies demonstrated that Cys^{577} is located in the ATP-binding domain of the Na,K-ATPase α-subunit, it is not clear whether the sulfhydryl side chain of this residue is directly involved in ATP binding.

In a very recent work, Shi *et al.* (2000) constructed a panel of ouabain-resistant sheep α1-subunit mutants, in each of which one or several of the 23 native cysteine residues were substituted. The α-subunits that contained the single mutations were expressed in HeLa cells, and all but two mutants containing the C242A or C242S substitution were able to support cell growth under ouabain-selective pressure. They concluded in that study that the expression of a functional cysteine-less α-subunit would not be possible because Cys^{242} may play an important role in enzyme function. However, they did not rule out the possibility that Cys^{242} is not essential for function but rather plays a role in protein folding or trafficking and that the expression system used in their study does not result in plasma membrane localization of this particular mutant protein. Of course, the ability to individually substitute single cysteines and produce functional

Na,K-ATPase mutants, each with small changes in enzymatic properties, does not mean that a Na,K-ATPase mutant with all 23 cysteines simultaneously substituted would retain activity.

In the present work we have constructed a form of the sheep renal Na,K-ATPase α1-subunit in which all 23 of its native cysteine residues (Fig. 4.1) are removed. This cysteine-less α-subunit and the wild-type β1-subunit were heterologously expressed in insect cells using a baculovirus expression system. This expression system, first employed in studies of Na,K-ATPase by Mercer and co-workers (DeTomaso, *et al.*, 1993) and recently by us to define the membrane topology of the Na,K-ATPase α-subunit (Hu & Kaplan, 2000), provides the opportunity to study expressed Na,K-ATPases in an environment containing little or no endogenous Na,K-ATPase activity (DeTomaso, *et al.*, 1993; Hu & Kaplan, 2000). We have examined the properties of this novel Na,K-ATPase mutant lacking all the α-subunit cysteines and find that the Na^+, K^+ and ATP concentration dependence and turnover number of the phosphoenzyme are only slightly different from the native enzyme. The availability of a cysteine-less α-subunit provides the groundwork for future structure-function studies that utilize introduced cysteines and sulfhydryl chemistry.

EXPERIMENTAL PROCEDURES

Plasmids and Construction of Mutants--A sheep α1-subunit cDNA was cloned into the pOCUS-2 vector (Novagen) as a *Not*I and *Sse*8387I fragment. The 23 native cysteine residues in the α-subunit (Fig. 4.1) were then substituted with alanines or serines via PCR overlap extension mutagenesis (Higuchi, *et al.*, 1988; Ho, *et al.*, 1989) using primers listed in Table 4.1. Recombinant baculovirus containing the sheep β1-subunit and the wild-type or cysteine-less α1-subunit cDNA was produced by following the protocols described previously (Hu & Kaplan, 2000). The genomic DNA of the recombinant baculoviruses were isolated by using the Easy-DNA Kit (Invitrogen) and were sequenced to ensure the appropriate cysteine mutations in the α-subunit.

Protein Expression and Purification--Log-phase high-viability High Five cells were infected with the recombinant baculoviruses for 3 or 5 days and were harvested for protein purification (Hu & Kaplan, 2000). The harvested High Five cells (~4 x 10^8 cells) were disrupted, cell membranes were fractionated on a five-step sucrose gradient, and the ER, Golgi apparatus and plasma membrane fractions were collected as described (Hu & Kaplan, 2000).

ATPase Assays--Na,K-ATPase assays of High Five membrane proteins were carried out as described (Hu & Kaplan, 2000) . For MIANS inactivation, cell membranes were incubated with 60 μM MIANS (Molecular Probes) at room temperature (~22 °C) for 15 min and then with 1 mM 2-mercaptoethanol for 1 min prior to the ATPase assay. For determining K^+ activation, the KCl concentration was varied from 0 to 30 mM. For Na^+ activation, the NaCl concentration was varied from 0 to 125 mM, and 10 mM potassium azide (American Azide Corp, Cedar City, UT), instead of the sodium azide,

was used. For ATP activation, the ATP concentration was varied from 0 to 1.2 mM. Curve fitting of the ligand-activation data was performed using the computer software Sigma Plot as described previously (Blanco, *et al.*, 1995).

Phosphorylation and Ouabain Binding--Phosphorylation with [^{32}P]ATP was carried out in a 50 µl medium containing: 100 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH7.0, and 50 µg of membrane protein. The mixture was placed on ice for 1 hr and the reaction was initiated by the addition of ATP ([γ-^{32}P]ATP, NEN Life Science Products, and 7.27 µM Tris-ATP) on ice for 60 s. The phosphorylation was quenched with 750 µl of ice-cold 5% (v/v) trichloroacetic acid containing 0.5 mM Tris-ATP and 1.5 mM Tris-phosphate, and the samples were filtered through Millipore filters (pore size 0.45 µm), washed 3 times with 2 ml quenching buffer and counted in a scintillation counter. Phosphorylation of the Na,K-ATPase enzyme was calculated from the difference between [^{32}P]phosphate incorporation in the medium above and that measured in a medium containing 100 mM KCl instead of NaCl. Equilibrium [^{3}H]ouabain binding was carried out as previously described (Hu & Kaplan, 2000).

Immunochemical Analysis--Protein samples were separated in 7.5% acrylamide gels (Laemmli, 1970) and transferred onto nitrocellulose membranes (Osmonics, Inc.) by electroblotting in 10 mM of CAPS, pH11, containing 10% methanol. The nitrocellulose blots were stained with a mouse monoclonal anti-α1 or anti-β antibody (Affinity BioReagent, Inc.) and peroxidase-linked sheep anti-mouse secondary antibody (Amersham Pharmacia Biotech) and were developed with an enhanced chemiluminescent substrate (Pierce).

RESULTS

Expression and Enzymatic Characterization of Expressed Na,K-ATPases--The wild-type and cysteine-less versions of the sheep Na,K-ATPase α1-subunits were co-expressed with the wild-type β1-subunit in baculovirus-infected High Five cells (Hu & Kaplan, 2000) for 3 or 5 days, and the ER, Golgi apparatus and plasma membrane fractions of the infected cells were isolated by sucrose gradient fractionation. The plasma membrane fractions of the 3-day infected High Five cells contained the highest Na,K-ATPase activities (reported in Table 4.2), and were used to measure the ligand-binding activities and cation activation.

The ATP phosphorylation levels of the wild-type and cysteine-less versions of the Na,K-ATPases were measured and the molecular activities for the two enzymes were calculated. As can be seen in Table 4.2, although the specific activity of the cysteine-less mutant is less than the wild-type protein, both forms have equivalent molecular activity. In other words, in spite of the lower copy number of the cysteine-less mutant in the plasma membrane, each α(cysteine-less)β complex is as active as the wild-type protein. The [^{3}H]ouabain-binding activities of the expressed proteins were also determined, which were 18.50±1.00 pmol • mg^{-1} protein for the wild-type enzyme and 4.36±0.35 pmol • mg^{-1} protein for the cysteine-less mutant enzyme. This is a further indication of the lower expression level of the cysteine-less protein and these estimates agree well with those of the phosphorylation levels.

The ouabain-sensitive Na,K-ATPase activities of the expressed proteins were measured in media containing various concentrations of Na^+ and K^+, and the corresponding curves for representative experiments are shown in Figs. 4.2 and 4.3,

respectively. The kinetic parameters of the cation activation are described in Table 4.3, which shows that the cysteine-less mutant displayed a higher apparent affinity for Na^+ ions and a lower apparent affinity for K^+ ions when compared with the expressed wild-type enzyme. However, these differences were only about 2-fold in each case and occurred without any changes in the Hill Coefficient for ion activation. Similarly, we compared the ATP concentration dependence of the Na,K-ATPases activities of the expressed wild-type and cysteine-less enzymes. Typical results are summarized in Table 4.3. The half-maximal concentration for ATP were 0.23 mM and 0.17 mM for the wild-type and cysteine-less Na,K-ATPases, respectively.

Sensitivity to Sulfhydryl Modification--The wild-type and cysteine-less versions of the Na,K-ATPases were incubated with MIANS, a cysteine-modifying reagent previously shown to inactivate the wild-type enzyme isolated from renal tissue (Gatto, *et al.*, 1999b). Typical results are represented in Fig. 4.4, which shows that, while MIANS inactivated the ATPase activity of the wild-type enzyme, the ATPase activity of the cysteine-less enzyme was not affected.

Cellular Stability of the Expressed Na,K-ATPases--After 3 days of infection, the ER and Golgi apparatus membrane fractions contained most of the expressed cysteine-less α-subunits (Fig. 4.5A, compare *lanes 4,5* with *lane 6*), whereas with the expressed wild-type protein a greater proportion is found in the plasma membrane (Fig. 4.5A, *lanes 1-3*). The ATPase activity for the wild-type α-subunits remained constant from 3 to 5 days post-infection (10.7 μmol Pi • mg^{-1} protein • h^{-1} in the plasma membrane) and Western analyses showed that these proteins are not degraded (Fig. 4.5B, *lanes 1-3*). The cysteine-less α-subunit, on the other hand, showed lower ATPase activity 5 days post-

infection (2.18 μmol Pi • mg^{-1} protein • h^{-1} in the plasma membrane compare with 3.34 μmol Pi • mg^{-1} protein • h^{-1} for 3-day infection) and Western blots of the membrane fractions showed that the cysteine-less proteins are highly degraded (Fig. 4.5B, *lanes 4-6*). Comparable amounts of the β-subunits were detected by Western analysis in all membrane fractions and did not appear to be degraded (data not shown).

DISCUSSION

In the present work we have constructed a version of the sheep renal Na,K-ATPase in which all 23 of the native cysteine residues in the α1-subunit have been replaced by alanines or serines. This cysteine-less α-subunit and the sheep β1-subunit were heterologously expressed in baculovirus-infected insect cells, and the resulting cell plasma membrane fractions were isolated for functional characterization. We obtain evidence demonstrating that the removal of all 23 cysteines from the native α-subunit does not compromise enzymatic activity.

Enzymatic Activities of the Expressed Proteins--Our data show that the expression level of the wild-type Na,K-ATPase is higher than that of the cysteine-less protein (Fig. 4.5) and this difference is reflected in their ouabain-sensitive ATPase activities (Table 4.2). However, the molecular activities of these two Na,K-ATPases, which are independent of the expression levels, are essentially the same within the experimental errors (Table 4.2). The expressed Na,K-ATPases were further characterized by activation with cations and ATP, and the results are summarized in Table 4.3. The cysteine-less version of the Na,K-ATPase, when compared with the expressed wild-type protein, displays a higher apparent affinity for Na^+ ions and a lower apparent affinity for K^+ ions. This may come about from a small change in a rate constant that results in a shift in E_1/E_2 equilibria where E_1 forms are favored over E_2 forms in the cysteine-less mutant. In any case, the cation affinities for the two proteins do not differ greatly (Na^+ ions: 16.38 mM for wild-type, 8.57 mM for cysteine-less; K^+ ions: 3.40 mM for wild-type, 7.24 mM for cysteine-less) and agree well with the kinetic parameters of the rat α1β1 Na,K-ATPase also expressed in insect cells (16.4 mM for Na^+, 1.9 mM for K^+) (see Blanco, *et al.*,

1995). Furthermore, the K_m values for ATP in the overall Na,K-ATPase activity are only slightly altered (from 0.23 to 0.17 mM) when all 23 cysteine residues of the wild-type enzyme are replaced (Table 4.3). Together, these results suggest that the sulfhydryl-bearing residues of the Na,K-ATPase α-subunit do not play an important role in ligand binding or ATP hydrolysis.

Cellular Distribution of the Expressed Na,K-ATPases--Our western analyses show that most of the cysteine-less α-subunits are localized in the ER and Golgi apparatus membrane fractions (Fig 4.5A, *lanes 4-6*) unlike the wild-type enzyme, which is predominantly located in the plasma membrane (Fig 4.5A, *lane 3*). Because the cysteine-less mutant has the highest specific activity in the plasma membrane, this suggests that a large portion of the α-subunits may be incorrectly folded or assembled in the ER and Golgi fractions. This lack of correct folding or assembly also apparently influences stability or protease susceptibility, because protein degradation is clearly seen in the cysteine-less α-subunits (Fig. 4.5B, *lanes 4-6*) but not in the expressed wild-type proteins (Fig. 4.5B, *lanes 1-3*). These observations suggest to us that while the lack of cysteine residues in the α-subunit has no major effects on function, one or several of the 23 native cysteines may affect the rates of protein folding and/or assembly. The greater protease susceptibility of incorrectly folded or immature α-subunit has been reported previously (Geering, *et al.*, 1987; Beggah, *et al.*, 1996).

The Role of Cysteine Residues--The results obtained in the present studies allow several important conclusions regarding the functions of the 23 native cysteines in the Na,K-ATPase α-subunit. First, it is clear that none of the cysteines is essential for the structural integrity of the Na,K-ATPase. Our total cysteine-less α-subunit was expressed

and reached the plasma membranes of baculovirus-infected insect cells (Fig. 4.5A, *lane 6*) and showed molecular activity that is similar to the wild-type (Table 4.2).

Second, it seems likely that previous studies that have shown inactivation or modification of the properties of the Na,K-ATPase following selective modification of sulfhydryls are due to the introduction of bulky moieties into the cysteine side-chains and not due to the modification of the cysteine side chain *per se*. We have previously shown using chemical modification that Cys^{577} is located in the ATP-binding domain (Gatto, *et al.*, 1999b), *i.e.* modification of this cysteine inhibits activity and inhibition is prevented by ATP. Data presented here suggest that the sulfhydryl side chain of Cys^{577} is not directly involved in substrate binding. Several other Cys-directed reagents have also been used to inhibit and study the structure-function relationships of the Na,K-ATPase (Patzelt-Wenczler & Schoner, 1981; Esmann, 1982; Kaplan & Mone, 1985; Anner, *et al.*, 1990). Although the locations of these modified cysteines have not been identified, our data show that none of these residues would be critical for function. Similar observations have been made previously with the *Neurospora crassa* H^+-ATPase, another P_2-ATPase. Enzyme inactivation with *N*-ethylmaleimide led to the suggestions of important cysteine residues in the ATP-binding or catalytic domain of this protein. However, site-directed mutagenesis and analyses of the expressed mutants revealed that cysteines play no essential role in the enzymatic mechanism of the H^+-ATPase (Petrov & Slayman, 1995; Mahanty & Scarborough, 1996).

The third conclusion that can be drawn from our experiments is that Cys^{242} plays no functional role in the Na,K-ATPase α-subunit. As described earlier (see Introduction), Shi and co-workers have constructed a panel of ouabain-resistant sheep α1-subunit

mutants in which one to three of the 23 native cysteine residues were substituted (Shi, *et al.*, 2000). The mutants were expressed in HeLa cells, and all but the Cys^{242}-substituted mutants were able to support cell growth under ouabain selective pressure. Although Cys^{242} does not appear to be located within the catalytic domains, it was concluded in their study that Cys^{242} is important for function, and that construction of a cysteine-less α-subunit would not be possible. Their conclusions are at odds with our findings, which clearly show that a total cysteine-less α-subunit is expressed and fully functional. One explanation to account for this would be that Cys^{242} plays a role in protein folding or trafficking and that the system used in that study does not result in plasma membrane localization of this particular mutant protein. Our cysteine-less α-subunit, though fully functional, does appear to have altered kinetic properties in protein folding and/or assembly (Fig. 4.5, *lanes 4-6*). Using our system, it would be interesting to study the trafficking pattern and degradation of a protein in which Cys^{242} is introduced back into the cysteine-less background.

Site-directed mutagenesis is a powerful tool that has be used in structure-function studies of membrane proteins by identifying individual residues important for structure and activity. The construction of a cysteine-less membrane protein, such as H^+-ATPase (Petrov & Slayman, 1995), lactose permease (van Iwaarden, *et al.*, 1991), erythrocyte plasma membrane anion exchange protein (Casey, *et al.*, 1995), and rhodopsin (Karnik, *et al.*, 1988) has been reported and utilized to study the structure-function relationships of these important proteins. The total replacement of cysteine residues in the Na,K-ATPase α-subunit has enabled us to eliminate any important role for cysteine residues in the α-subunit of the Na,K-ATPase. We have shown here for the first time that the 23 native

cysteine residues in the Na,K-ATPase α-subunit have no essential role in the protein and that these residues can all be simultaneously replaced without any deleterious effect on enzyme function. The successful expression of a cysteine-less Na,K-ATPase α-subunit should be useful for future structure-function studies where individual cysteine residues will be incorporated into different parts of the protein so that spectroscopic methods can be employed to obtain dynamic structure data.

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TABLE 4.1

Oligonucleotide primers used for site-directed mutagenesis

PCR overlap extension mutagenesis was carried out with primers listed to replace the native cysteine residues with alanine or serine residues in the Na,K-ATPase α-subunit. Base changes are denoted by lower case letters. Underlined bases represent the corresponding serine or alanine codons. Lower case letters that are not underlined introduce silent mutations and restriction sites for mutant screening.

Mutations	PCR Primers (5'-3')
C86S	GGGTCAAGTTCTcgCGACAGCTGTTTGGG
C104S	GGATTGGAGCAGTTCTaaGCTTCCTGGCCTATGGC
C138S	GCCGTCGTCATCATAACCGGaaGcTTCTCCTACTATCAAG
C204S	GCGAATGGCTcCAAGGTcGAcAACTCCTCACTCACG
C242S	CAACCAACTcTGTTGAAGGCACTGC
C336A	TGGCCACCGTCACGGTGgcgCTGACCCTGACTGCC
C349S	GGAAGAACTcCTTAGTGAAGAACCTGG
C367S	CCACGTCCACgATaTcCTCAGACAAAACTGG
C421S	GTCCAGAATTGCAGGcCTTTcTAACAGaGCCGTGTTTC
C452S	GTCTGCGCTTCTGAAaTcgATaGAGGTGTGCTGC
C456S, C457S	GAAaTcgATaGAGGTGaGCTcCGGTTCTGTGAAGG
C511S	GATCCTGGATCGCTcgACtTCCATCC
C549S	CGTGTGCTGGGTTTCaGCCACTTGATGCTG
C577S	CTGTTGATAATCTaaGCTTCGTGGGGCTCATC
C599S	GATGCTGTGGGCAAATcgCGAAGTGCTGGAATC
C656S	CAGGGATGCCAGGGCtaGCGTGGTGCATGGAAG
C698S	CATCATTGTGGAAGGaTcCCAGAGACAGGGTG
C802A	GACCGTCACCATCCTCgcgATAGACTTGGGAAC
C911S	TCGTGGAATTCACCTctCAtACGGCCTTCTTT
C930A	GTGGGCTGACTTGGTCATCgcgAAGACgcGtAGG
C964A	CTTTCCTTTCCTACgcgCCTGGtATGGGTGTTGC
C983S	TACCTGGTGGTTCTcTGCaTTCCCCTACTCG

TABLE 4.2

Characteristics of Na,K-ATPases isolated from plasma membrane preparations of baculovirus-infected High Five cells

Na,K-ATPase and ATP phosphorylation assays were performed as described under "Experimental Procedures." The molecular activity, which is the turnover rate of phospho-enzyme per minute, was calculated by taking the ratio of specific activity over phosphorylation number. Each value below represents the means ± standard deviations of triplicate determinations from at least three different membrane preparations.

	Na,K-ATPase Activity (μmol Pi • mg^{-1} protein • hr^{-1})	**ATP Phosphorylation** (pmol • mg^{-1} protein)	**Molecular Activity** (min^{-1})
Wild Type	11.02±0.35	27.6±2.16	6655±562
Cys-Less	3.34±0.53	6.77±0.50	8223±1439

TABLE 4.3

Apparent affinities and Hill Coefficients for Na^+, K^+ and ATP activation of Na,K-ATPases isolated from plasma membrane preparations of baculovirus-infected High Five cells

Na,K-ATPase assays were performed in the presence of various Na^+, K^+ or ATP concentrations as described under "Experimental Procedures." The apparent affinities (K_m) and Hill Coefficients (n_H) were calculated according to the methods of Blanco *et al* (1995). Each value below represents the means ± standard deviations of duplicate determinations from two to four different membrane preparations.

	Na^+ Activation		K^+ Activation		ATP Activation
	K_m (mM)	n_H	K_m (mM)	n_H	K_m (mM)
Wild Type	16.38±1.64	2.04±0.08	3.04±0.15	1.59±0.01	0.23±0.04
Cys-Less	8.57±1.97	1.66±0.43	7.24±0.64	1.47±0.26	0.17±0.02

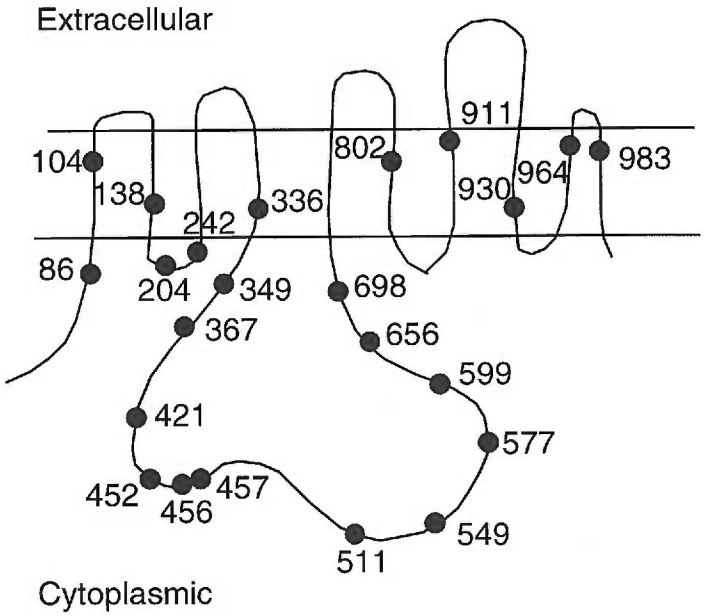

Fig. 4.1. Relative locations of cysteines on the topographic map of the Na,K-ATPase α-subunit.

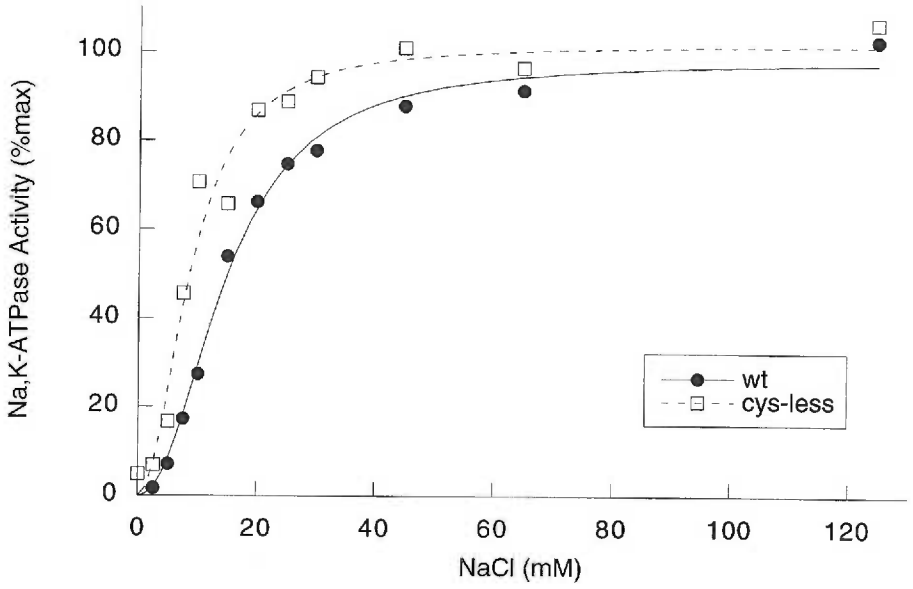

Fig. 4.2. Na activation of Na,K-ATPases expressed in High Five insect cells. Na,K-ATPase activity of High Five plasma membranes expressing the wild-type (*wt*) sheep α1β1 (*closed circle*) or α1(cysteine-less)β1 (*open square*) was determined in assay medium containing 0-125 mM NaCl as described under "Experimental Procedures." Each value represents the means of duplicate determinations from at least three different membrane preparations.

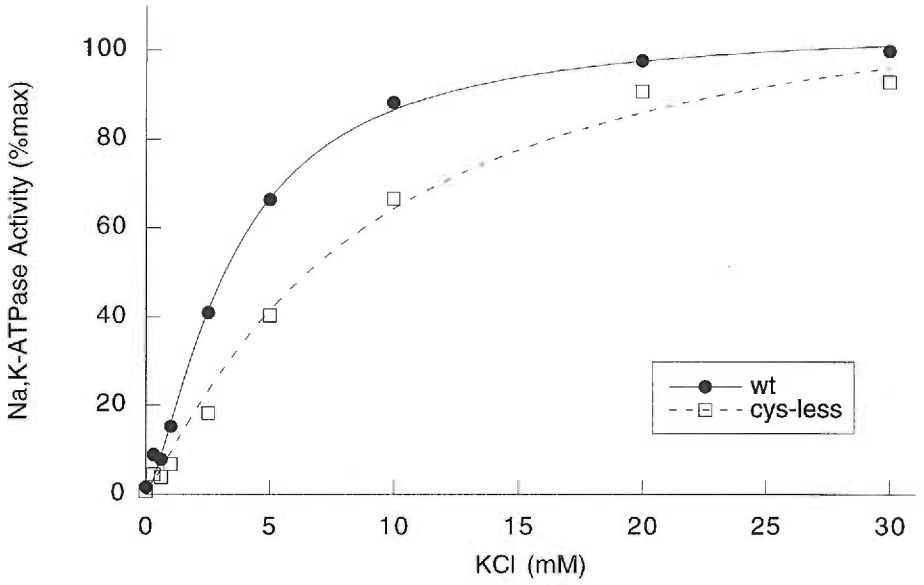

Fig. 4.3. K activation of Na,K-ATPases expressed in High Five insect cells. Na,K-ATPase activity of High Five plasma membrane expressing the wild-type (*wt*) sheep α1β1 (*closed circle*) or α1(cysteine-less)β1 (*open square*) was determined in assay medium containing 0-30 mM KCl as described under "Experimental Procedures." Each value represents the means of duplicate determinations from at least three different membrane preparations.

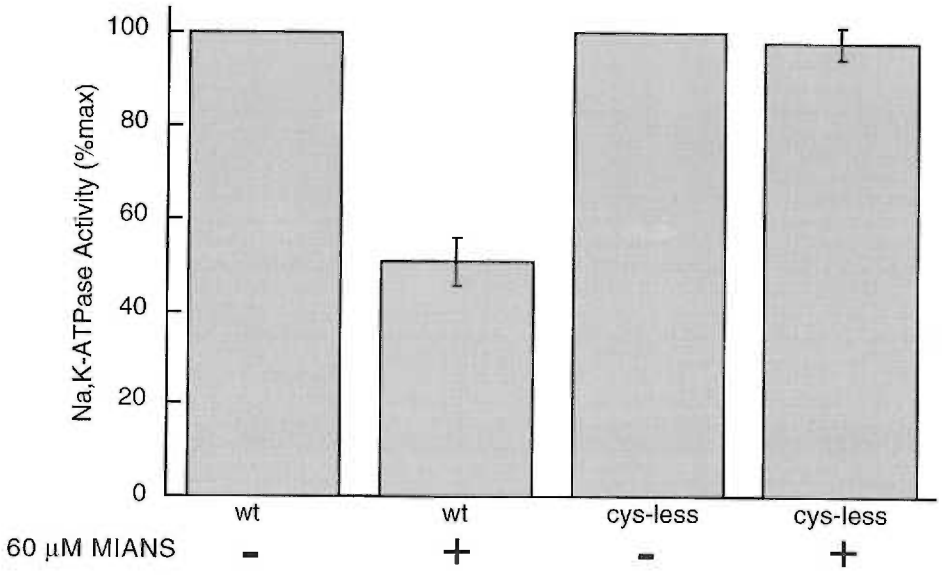

Fig. 4.4. MIANS inactivation of Na,K-ATPases expressed in High Five insect cells. High Five plasma membranes expressing the wild-type (*wt*) sheep α1β1 or α1(cysteine-less)β1 were incubated with a cysteine-specific reagent MIANS at room temperature for 15 min, and their ouabain-sensitive ATPase activities were determined as described under "Experimental Procedures." Each value represents the means ± standard deviations of triplicate determinations from at least three different membrane preparations.

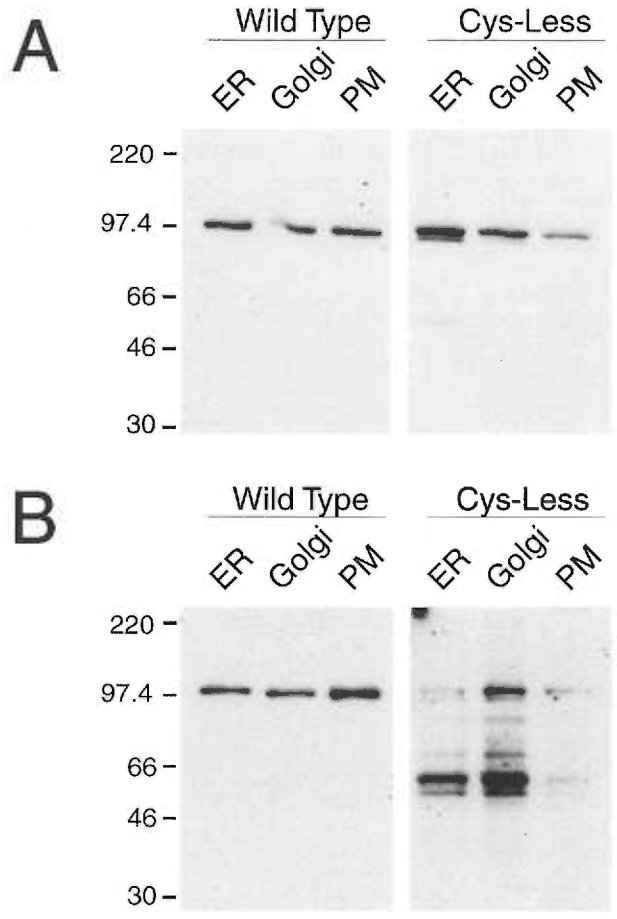

Fig. 4.5. Immunoblots of expressed Na,K-ATPases in High Five insect cells. Membrane fractions expressing the wild-type (10 μg) or cysteine-less (25 μg) form of the Na,K-ATPase were resolved on a 7.5% acrylamide gel, transferred to nitrocellulose and probed with a monoclonal anti-α1 antibody. **A**, cells were harvested 3-day after infection. **B**, cells were infected for 5 days.

CHAPTER FIVE

GENERAL DISCUSSION AND FUTURE DIRECTIONS

One aim of this thesis was to develop and optimize a suitable expression system for investigating the structure-function relationships of the Na,K-ATPases. Using a baculovirus expression system, we were able to obtain functional Na,K-ATPase molecules in a eukaryotic environment containing little or no endogenous background, and the specific activity of our expressed proteins is among the highest reported in any system. As discussed in chapter two, we have further improved this baculovirus system by engineering a viral construct that directs the coexpression of the sodium pump and green fluorescent protein in the infected cells. In our hands, the expression of the GFP does not seem to have any negative effects on the expression of the Na,K-ATPase. This novel viral construct enabled us to monitor cell infections with fluorescent microscopy and to more accurately and efficiently determine the titer of our viral stocks. Having the titer information is important in infection studies where a specific virus-to-cell ratio is needed for (i) producing high-quality viral stocks and (ii) expressing high levels of the desired proteins.

Another aim of this thesis was to demonstrate that the combination of site-directed mutagenesis and chemical modification methods provides structure-function information that was hitherto unobtainable using other methods. As discussed in chapter three, using our baculovirus system, a panel of Na,K-ATPase mutants each with a unique cysteine residue in the putative extracellular loops was expressed in insect cells. The chemical accessibility of these cysteines had allowed us to characterize the topogenic properties of these functional Na,K-ATPases and dissect this protein's transmembrane secondary structure. We were able to reject several proposed models in previous studies and to establish membrane boundaries of the α-subunit.

In chapter four, we described the construction of a Na,K-ATPase α-subunit with all its 23 native cysteine residues replaced. This cysteine-less protein is active and displays similar ligand binding affinities as the wild-type enzyme, suggesting that none of the native cysteines is essential for function. The results of our studies, as well as the potential applications of our expression system, are discussed below.

HETEROLOGOUS EXPRESSION OF NA,K-ATPASE IN INSECT CELLS

Most mutagenesis studies of the sodium pump until now have relied on established mammalian expression systems to produce the desired proteins. Although applications of this type of system have been described, the presence of endogenous Na,K-ATPase can greatly complicate the interpretation of the results of site-directed chemical labeling experiments. One way to avoid the problem of the endogenous protein is to use an expression system with little or no detectable Na,K-ATPase activity. Yeast cells are completely devoid of Na,K-ATPase, however, the abundance of endogenous H-ATPases may contribute to a high background signal in measurements. Insects are reported to contain detectable Na,K-ATPase activities only in the nervous system. Mercer and co-workers have expressed the sodium pump in the sf9 cell line using a baculovirus expression system. In their initial studies, the highest specific activity of the expressed proteins was 5 μmol P*i* · mg^{-1} protein · h^{-1} (DeTomaso, *et al.*, 1993). We explored an alternative baculovirus expression system for our studies. This system offers a quicker and easier way for selecting the desired recombinant baculovirus because insertions of the α- and β-subunit cDNAs into the baculovirus take place in *E. coli* cells instead of the homologous recombination in insect cells like the classical method

employed by Mercer and coworkers (Chapter 2). Since only the recombinant viral DNAs are used to transfect insect cells, all of the baculoviruses produced in our system are recombinant. Several rounds of plaque purification are no longer needed to rid the non-recombinant native virus, and we estimate that our system could save more than 2 weeks of laboratory time per mutant production than the classical system. We do not detect endogenous Na,K-ATPase in the insect cell lines and the highest specific activity of our expressed proteins is 37 μmol P*i* · mg^{-1} protein · h^{-1} (Hu & Kaplan, 2000), which is perhaps the best among any system reported (Pedersen, *et al.*, 1996).

Optimization studies of our expression system included comparing the protein expression levels in the infected sf9 and High Five insect cells. My initial study showed that the Na,K-ATPase activity in the infected High Five cells was ~7 fold higher than the infected sf9 cells, and the subsequent infection studies consistently yielded higher protein expression levels in the High Five over the sf9 cell lines. High Five cells are reported to exhibit up to one log higher expression levels of secreted proteins compared to sf9 cells (O'Reilly, *et al.*, 1994), and our studies show that this trend is observed with membrane protein expressions. However, in contrast with the wild-type sodium pump and the topology mutants (Chapter 3), the expression levels of our Cys-less protein are higher in the sf9 cells than the High Five cells (~2-3 fold higher). As discussed in Chapter 4, a large portion of the Cys-less α-subunits is incorrectly folded or assembled and is degraded in the ER and Golgi fractions of the infected insect cells because one or several of the native cysteines may be important for protein folding and/or trafficking. It is possible that since the sf9 cell line appeared to have a slower protein production machinery (sf9 replicates more slowly and is less efficient in producing foreign proteins

than the High Five), this cell line may be able to better accommodate the synthesis of the Cys-less pump that has impaired protein folding kinetic properties.

Recently, I cloned a green fluorescent protein (GFP) gene into a recombinant baculovirus that directs the expression of the Na,K-ATPase α- and β-subunits in insect cells. The co-expression of GFP enables us to monitor infection with fluorescence microscopy and facilitated efficient titer determination without the use of costly enzyme substrates or antibodies. Our study should be useful to researchers who utilize the baculovirus system for expression studies as the Na,K-ATPase genes can be easily excised to accommodate other genes of interest.

THE MEMBRANE TOPOLOGY OF THE NA,K-ATPASE α-SUBUNIT

We have determined the membrane topology of the Na,K-ATPase α-subunit using a combination of introduced cysteines and sulfhydryl accessibility methods. The Na,K-ATPase α-subunit contains ten transmembrane domains. Prior to our study, most methods used to establish the membrane topology of the Na,K-ATPase suffered from inherent limitations and produced conflicting results. In the protease accessibility method, it was not clear if after the initial cleavage step the protein would still maintain its native state. The partially digested protein may unfold and expose sites that are normally inaccessible in the intact protein. In more recent cleavage methods, based on metal-ion catalyzed cleavages (Shimon, *et al.*, 1998), assumptions are made that the cleavage event occurs close to the metal binding sites, and the locations of the bound metals are also unknown. With this and other cleavage approaches, interpretations based on minor fragmentation products need further confirmation by other methods.

In the immunochemical methods, antibodies produced against different regions of the protein were used to test the accessibility of the epitopes in intact cells. If an antibody failed to label the protein in the intact cells, the labeling experiment was repeated with detergent solubilized cell membranes to demonstrate that the respective epitope is located within the transmembrane or intracellular region. This method must assume that the epitope is not buried in the extracellular domain of the native protein (*i.e.* by folding or protein-protein interaction) and that the detergents do not alter the overall protein structure. It should be mentioned that previous labeling studies using an antibody raised against the Trp^{887}-Arg^{904} sequence of the α-subunit have produced conflicting results. In one study, the antibody detected Na,K-ATPase from the extracellular domain after prolonged incubation (Mohraz, *et al.*, 1994). Whereas in another study, the anti- Trp^{887}-Arg^{904} antibody failed to label the protein without cell permeablization by detergent (Ovchinnikov Yu, *et al.*, 1988). This region of the α-subunit has been proposed as a site of contact for the β-subunit (Lemas, *et al.*, 1994), and the discrepancy observed in the studies could be caused by protein-protein interactions of the two subunits. It seems likely that the use of detergents and bulky antibodies in the immunochemical methods is prone to provide misleading information with a protein such as the Na,K-ATPase where abundant evidence exists demonstrating the mobility or flexibility of its carboxyl-terminal regions.

In the chimeric protein method, various segments of the Na,K-ATPase were attached to reporter proteins, such as β-galactosidase, so that the topogenic properties of the fusion regions could be studied (Xie, *et al.*, 1996; Fiedler & Scheiner-Bobis, 1996). However, because the chimeric molecules did not have Na,K-ATPase activity to affirm

structural integrity, this method assumed that the membrane orientations of the truncated Na,K-ATPases faithfully reflected those of the native protein.

In our method discussed in chapter three, the topology studies were carried out with fully functional Na,K-ATPases in their native state. The extracellular labeling of the wild-type protein (our positive control) , but not our background null mutant (our negative control), agrees with our previous findings that Cys^{911} and Cys^{964} are the only two accessible cysteines in the Na,K-ATPase α-subunit (Lutsenko, *et al.*, 1997). Unique cysteines were introduced into the null mutant at positions 118, 309, 793, 876, and 973, and the positive labeling results of these molecules supported a specific topological model for the Na,K-ATPase α-subunit, which was presented in Fig. 3.5. In addition to the M973C mutant, cysteines were introduced at positions 969 and 976. The lack of labeling of these two mutants demonstrates that our method is capable of distinguishing accessibility of residues separated by only three or four residues and may be useful in establishing the locations of the membrane interface in the protein structure.

Shortly after the publication of our topology study, a crystal structure of the sarcoplasmic reticulum Ca-ATPase was solved at 2.6 Å by Toyoshima and coworkers (2000). The Ca-ATPase (calcium pump) uses the energy of hydrolyzing ATP molecules to carry out the active transport of calcium ions and is a member of the P-type ATPase family. Unlike the heterodimeric P-type ATPases such as Na,K-ATPase and the H,K-ATPase, the Ca-ATPase has only one subunit. According to the crystal structure, the calcium pump, composed of 994 amino acid residues, contains 10 transmembrane α-helices. The lengths and inclinations of the helices vary substantially, and their relative positions are depicted in Fig 5.1. Two calcium-binding sites, stabilized by hydrogen

bond networks, were detected in the transmembrane regions of the protein. Site I is located between the space of the M5 and M6 helices with contribution from M8 at a rather distal position, whereas site II is 5.7 Å away from the site I and is coordinated by residues from M4 and M6 (Toyoshima, *et al.*, 2000).

It is interesting to note that previous biochemical and mutagenesis studies on the Na,K-ATPase had also shown that amino acid residues in M4, M5, M6 and M8 may be involved in cation coordination (see Table 1.1). Sequence alignments of the sodium pump α-subunit and the calcium pump revealed that several of the calcium-coordinating residues are conserved in the sodium pump (Fig. 5.2). For example, the side-chain oxygen atoms of Asn^{768}, Glu^{771} (M5), Asn^{796}, Asp^{800} (M6), and Glu^{908} (M8) of the calcium pump contribute to the hydrogen bond networks of the cation-binding sites. Mutating the corresponding residues in the sodium pump (Asn^{776}, Glu^{779}, Asp^{804}, Asp^{808}, and Asp^{926}, respectively) altered the sodium and/or potassium affinity or produced inactive proteins (Table 1.1). Additionally, the M4 helical structure of the calcium pump is unwound in the middle of the membrane because of Pro^{308}, and this structure allows the main-chain carbonyl oxygen atoms of Val^{304}, Ala^{305} and Ile^{307} as well as the side-chain oxygen atoms of Glu^{309} to stabilize the bonding network of the second calcium-binding site. This type of helical structure appears to be important also for the Na,K-ATPase because mutating the corresponding proline (Pro^{326}) in M4 reduced the cation affinities of the sodium pump (Vilsen, 1992). Furthermore, the ion-coordinating residues in M4 of the Ca-ATPase are conserved in the Na,K-ATPase (Val^{322}, Ala^{323}, Val^{325}, and Glu^{327} respectively). Together, these data suggest that transmembrane segments M4, M5, M6 and M8 of the Na,K-ATPase are the cation-coordinating "core" helices of the protein and may share a similar

spatial arrangement as the Ca-ATPase. Indeed, we have previously demonstrated the extracellular release of the M5M6 hairpin of the Na,K-ATPase in the absence of potassium ions (Lutsenko, *et al.*, 1995; Gatto, *et al.*, 1999a). This is another indication that the core helices of the sodium pump make limited contacts with the lipid bilayer and are stabilized in the membrane by interacting with cations and other transmembrane domains.

Based on the crystal structure of the Ca-ATPase, we have constructed a projected three-dimensional model of the Na,K-ATPase α-subunit (Fig. 5.3). The sheep Na,K-ATPase α1-subunit and the rabbit sarcoplasmic reticulum Ca-ATPase share ~48% sequence similarity, most of which are in the M1-M6 regions (Fig. 5.2). However, large gaps exist in the ATP-binding and the M7 domains, and deletions are made (~18% of the protein) around these regions to accommodate the fitted model. The relative cysteine positions of our topology mutants are highlighted in the model and are presented in Fig. 5.4. With the exception of the residue in the M7M8 loop, all of our introduced cysteines are located in the extracellular loops and are highly accessible. Cysteine at position 876 seems to be located at the carboxyl end of the M7 helix, and its accessibility is not readily discernible. As discussed in chapter three, we had intentionally placed this cysteine residue close to M7 because of the proposed interactions between the M7M8 loop and the β-subunit (Lemas, *et al.*, 1994). It should be pointed out that the α-helical structure of several transmembrane segments, including M7, extends out of the membrane region of the calcium pump (Toyoshima, *et al.*, 2000). Therefore, it is likely that Cys^{876} and several other residues located at the ends of the helices are exposed to the aqueous phase. Additionally, the readers must be reminded that M7 had undergone extensive sequence

deletions during the construction of our model. The M7M8 loop contains ~35 residues in the native Ca-ATPase structure (Toyoshima, *et al.*, 2000) and can easily include Cys^{876} in the sodium pump structure when a different set of criteria is used during our modeling process.

Functional Expression of a Cysteine-Less Na,K-ATPase α-Subunit

As described in chapter four, we have constructed a version of the sheep renal Na,K-ATPase in which all 23 of the native cysteine residues in the α1-subunit have been replaced by alanines or serines. This cysteine-less α-subunit and the sheep β1-subunit were heterologously expressed in baculovirus-infected insect cells, and the resulting cell plasma membrane fractions were isolated for functional characterization. We obtain evidence demonstrating that the removal of all 23 cysteines from the native α-subunit does not compromise enzymatic activity.

Early important observations using *N*-ethylmaleimide showed that a population of sulfhydryl residues, when modified, blocked the E_1P to E_2P transition of the sodium pump and inhibited ATPase activity (Fahn, *et al.*, 1966). These observations were among the first to demonstrate that cysteine residues may play a key role in the Na,K-ATPase enzymatic cycle. Several other sulfhydryl-reactive reagents have been used to inactivate the sodium pump (Patzelt-Wenczler & Schoner, 1981; Esmann, 1982; Kaplan & Mone, 1985; Anner, *et al.*, 1990), and we have shown recently that a Cys-specific maleimide, MIANS, inactivates the Na,K-ATPase by selectively modifying Cys^{577} (Gatto, *et al.*, 1999b). Although data from our labeling studies demonstrated that Cys^{577} is located in the ATP-binding domain of the Na,K-ATPase α-subunit, the functional significance of

this cysteine residue was not identified. Indeed, with modification studies, it is not clear if the residues of interest are responsible for function. The sulfhydryl side chain of Cys^{577} could be directly involved in ATP binding. Alternatively, Cys^{577} may serve no functional role and the bulky maleimide reagent introduced at the catalytic domain could be the cause of inactivation. In our expression study, the cysteine-less version of the α-subunit is functional and is targeted to the plasma membranes of the baculovirus-infected insect cells. This protein's ligand-binding activities as well as the turnover of the phosphoenzyme intermediate were close to wild-type enzyme, demonstrating that none of the cysteines in the α-subunit is essential for structure and/or function.

The successful expression of the cysteine-less Na,K-ATPase α-subunit provides additional opportunities for structure-function studies of the protein. We are now in a position where cysteines can be introduced back into designated regions of the sodium pump to carry out a variety of biochemical and biophysical experiments. Additionally, in our topology study discussed in chapter three, accessibility tests were performed on intact cells because there are several native cysteine residues in the cytoplasmic region of the sodium pump. With cysteine mutants constructed on the Cys-less background, on the other hand, future labeling studies can be performed on purified membrane fragments to simplify and enhance the modification process.

FUTURE DIRECTIONS

As mentioned in chapter one, experimental results from studies to date suggest that multiple extracellular and transmembrane regions are involved in determining ouabain sensitivity. However, the actual ouabain-binding sites remain to be elucidated.

A recent NMR study has provided evidence suggesting that the rhamonose sugar and steroid moieties of ouabain interact with different regions of the sodium pump (Middleton, *et al.*, 2000). The study showed that the steroid group is more constrained than the sugar group when bound to the protein, possibly due to the surface hydrogen-bond network around the steroid region. Based on this and previous mutagenesis findings, as well as the helical arrangement of the Ca-ATPase, they proposed a structure of ouabain-bound sodium pump, shown in Fig. 5.5. However, this model needs to be validated by other methods due of the lack of direct evidence in the NMR study.

Anthroylouabain is a fluorescent derivative of ouabain that exhibits similar protein-binding characteristics as the native cardiac glycoside (Fortes, 1977) and has been used to characterize the catalytic mechanism of the sodium pump (Fortes & Lee, 1984). This molecule, shown in Fig. 5.6, is synthesized by attaching a fluorescent anthroyl group to the sugar moiety of ouabain. We propose the following experiment using anthroylouabain to investigate the ouabain-binding sites of the sodium pump. First, unique cysteines are introduced at positions 118, 309, 793, 876, and 973 of the cysteine-less Na,K-ATPase α-subunit. We have shown in the topology study that residues at these positions are located at each of the extracellular regions of the protein and are accessible from the aqueous phase (Hu & Kaplan, 2000). The expressed mutants will then be labeled with a Cys-directed fluorescent quencher, 4-maleimido-TEMPO (4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) (Fig. 5.7), and the fluorescent intensities of the bound anthroylouabain will be measured and compared with those bound to the unmodified mutant proteins. Using the TEMPO molecule as a fluorescent quencher has been demonstrated in previous biophysical studies of membrane proteins

(Rasmussen, *et al.*, 2001). If the sugar moiety of the bound ouabain were near the M1M2 loop, as proposed in the NMR study, the TEMPO group introduced at Cys^{118} would decrease the fluorescent intensity of the anthroyl group. On the other hand, if the spatial arrangement of the bound-ouabain is different than the proposed model, the quenching should be observed with the TEMPO group placed elsewhere.

The choice of fluorescent quenchers is not restricted to the TEMPO reagent in the proposed study. There are several commercially available Cys-directed fluorescent probes whose maximum absorption wavelength overlaps the emission wavelength of the anthroylouabain (see Molecular Probes catalog for examples). These fluorescent probes, with different sizes and photochemical properties, should also be useful in energy transfer studies to further define the ouabain binding regions of the Na,K-ATPase.

Currently, the five cysteine-mutants proposed above have been expressed in the baculovirus-infected sf9 cells. Preliminary characterization of these mutants yielded ouabain-sensitive ATPase activities of 3-6 μmol P*i* · mg^{-1} protein · h^{-1}. Further tests will be performed to affirm their structural and functional integrity. Additionally, we have started producing antibodies against 4-maleimido-TEMPO in rabbit, which will be useful in evaluating the labeling status of our mutant proteins.

Using the cysteine-less protein as the background, we have also constructed several other mutants with single or double cysteines at the various positions the α-subunit. With the recent advances in spectroscopic technology, our strategy is to label these cysteine mutants with fluorescent and/or environmentally sensitive probes so that their movements and interactions during the catalytic cycle can be monitored. For example, the use of 4-maleimido-TEMPO is not limited to the fluorescent quenching

studies. Mutants labeled with this reagent can also be analyzed by electron spin resonance spectroscopy (ESR). ESR detects the presence of unpaired electrons, and the introduction of a stable nitroxide free radical creates a molecular probe that conveys information about the structure and motional dynamics at its site of attachment. This approach has generated extensive information about other membrane proteins, such as rhodopsin (Farahbakhsh, *et al.*, 1993; Farrens, *et al.*, 1996).

It is anticipated that a high-quality crystal structure of the Na,K-ATPase will be solved within the next 5-10 years. Although having this information may enable us to draw more accurate hypotheses on the structure-function relationships of the protein, additional methods are needed to investigate the mechanism of protein catalysis. Our Cys-replacement approach is capable of providing dynamic information of the protein and will be an important companion to the crystallographic method in studying the structure-function relationships of the Na,K-ATPase.

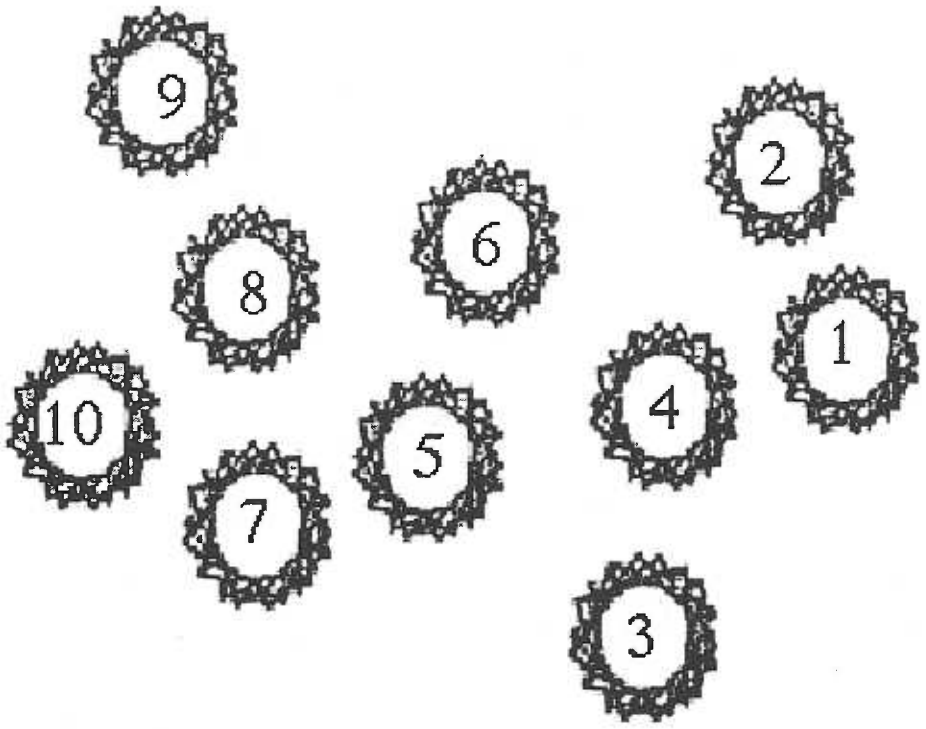

Fig. 5.1. The arrangement of transmembrane helices of the sarcoplasmic reticulum Ca-ATPase, viewed from the cytoplasmic side. (Adapted from Toyoshima *et al.*, 2000)

```
Na,K : 37  HKLSLDELHRKYGTDLNRGLTTARAAEILARDGPNALTPPPTTPEWVKFCRQLFGGFSML 96
           H  S +E    +G     GLT  +    L + G N L        W     Q       +
SrCa : 5   HSKSTEECLAYFGVSETTGLTPDQVKRHLEKYGHNELPAEEGKSLWELVIEQFEDLLVRI 64
           M1                                   M2
Na,K : 97  LWIGAVLCFLAYGIQAATEEEPQNDNLYL-GVVLSAVVIITGCFSYYQEAKSSKIMESFK 155
           L + A + F    + A  EE  +    ++   V+  ++I        +QE  +   +E+ K
SrCa : 65  LLLAACISF----VLAWFEEGEETITAFVEPFVILLILIANAIVGVWQERNAENAIEALK 120

Na,K : 156 NMVPQQALVIRNGEK--MSINAEEVVVGDLVEVKGGDRIPADLRIIS--ANGCKVDNSSL 211
              P+   V R   K    I A ++V GD+VEV  GD++PAD+RI+S  +   +VD S L
SrCa : 121 EYEPEMGKVYRADRKSVQRIKARDIVPGDIVEVAVGDKVPADIRILSIKSTTLRVDQSIL 180

Na,K : 212 TGESEPQTRS----PDFTNENPLETRNIAFFSTNCVEGTARGIVVYTGDRTVMGRIATLA 267
           TGES    +     PD    N  + +N+ F  TN   G A GIV  TG  T +G+I
SrCa : 181 TGESVSVIKHTEPVPDPRAVNQ-DKKNMLFSGTNIAAGKALGIVATTGVSTEIGKIRDQM 239
                                  M3
Na,K : 268 SGLEGGQTPIAAEIEHFIHIITGVAVFLGVSFFILSL------ILEYTWLEAVIFLI--- 318
           +  E  +TP       F    + V   + V+ +++++      +   +W+   I+
SrCa : 240 AATEQDKTPLQQKLDEFGEQLSKVISLICVAVWLINIGHFNDPVHGGSWIRGAIYYFKIA 299
             M4
Na,K : 319 -GIIVANVPEGLLATVTVCLTLTAKRMARKNCLVKNLEAVETLGSTSTICSDKTGTLTQN 377
             + VA +PEGL A  T CL L  +RMA+KN +V++L +VETLG TS ICSDKTGTLT N
SrCa : 300 VALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTN 359

Na,K : 378 RMTVAHMWFDNQIHEADTTENQSGVSFDKTSATW----------------------LALSR 416
           +M+V  M+  +++     + N+    F  T +T+                       + L+
SrCa : 360 QMSVCKMFIIDKVDGDFCSLNE----FSITGSTYAPEGEVLKNDKPIRSGQFDGLVELAT 415

Na,K : 417 IAGLCNRAVFQANQDNLPILKRAVAGDASESALLKCIEV------------------CCG 458
           I  LCN +    N+      K    G+A+E+AL   +E                    C
SrCa : 416 ICALCNDSSLDFNETKGVYEK---VGEATETALTTLVEKMNVFNTEVRNLSKVERANACN 472

Na,K : 459 SVKEMRERYAKIVEIPFNSTNKYQLSIH----KNANAGEPRHLLVMKGAPERILDRCSSI 514
           SV  +R+   K   + F S ++  +S++    K++ A     + V KGAPE ++DRC+ +
SrCa : 473 SV--IRQLMKKEFTLEF-SRDRKSMSVYCSPAKSSRAAVGNKMFV-KGAPEGVIDRCNYV 528

Na,K : 515 LIHGKEQPLDEELKDAFQNAYLELGGLGERVLGFCHLMLPDEQFPEGFQFDTDDVNFPVD 574
             +     P+   +K+   +     G          C  +   +  P+  +   DD +  ++
SrCa : 529 RVGTTRVPMTGPVKEKILSVIKEWGTGRDTLR--CLALATRDTPPKREEMVLDDSSRFME 586

Na,K : 575 ---NLCFVGLISMIDPPRAAVPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGN 631
              +L FVG++ M+DPPR  V  ++  CR AGI+VIM+TGD+  TA AI + +GI  E N
SrCa : 587 YETDLTFVGVVGMLDPPRKEVMGSIQLCRDAGIRVIMITGDNKGTAIAICRRIGIFGE-N 645

Na,K : 632 ETVEDIAAR----LNIPVSQVNPRDARACVVHGSDLKDMTPEQLDDILKYHTEIVFARTS 687
           E V D A       ++P+++      RAC                          FAR
SrCa : 646 EEVADRAYTGREFDDLPLAEQREACRRAC----------------------CFARVE 680

Na,K : 688 PQQKLIIVEGCQRQGAIVAVTGDGVNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDN 747
           P  K  IVE  Q    I A+TGDGVND+PALKKA+IG+AMG +G+ V+K A++M+L DDN
SrCa : 681 PSHKSKIVEYLQSYDEITAMTGDGVNDAPALKKAEIGIAMG-SGTAVAKTASEMVLADDN 739
                                                M5
Na,K : 748 FASIVTGVEEGRLIFDNLKKSIAYTLTSNIPEITPFLIFIIANIPLPLGTVTILCIDLGT 807
           F++IV  VEEGR I++N+K+ I Y ++SN+ E+    +      +P  L  V +L ++L T
SrCa : 740 FSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAALGLPEALIPVQLLWVNLVT 799
                  M6                                  M7
Na,K : 808 DMVPAISLAYEQAESDIMKRQPRNPQTDKLVNERLISMAYGQIGMIQALGGFFTYFVIMA 867
           D +PA +L +   + DIM R PR+P+ + L    L                FF Y   MA
SrCa : 800 DGLPATALGFNPPDLDIMDRPPRSPK-EPLISGWL----------------FFRY---MA 839
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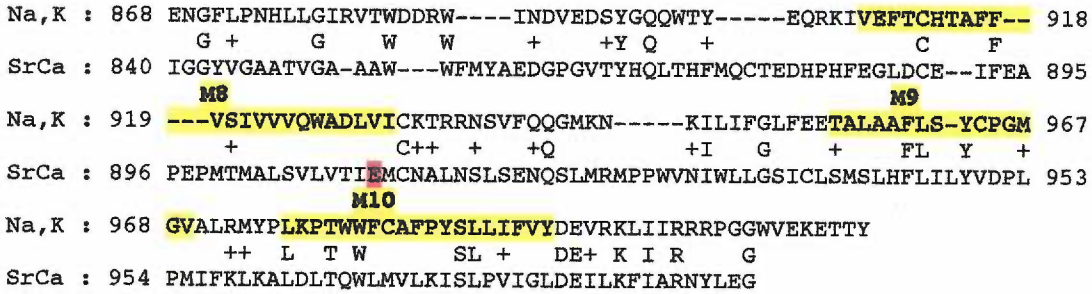
Na,K : 868 ENGFLPNHLLGIRVTWDDRW----INDVEDSYGQQWTY-----EQRKIVEFTCHTAFF-- 918
G + G W W + +Y Q + C F
SrCa : 840 IGGYVGAATVGA-AAW---WFMYAEDGPGVTYHQLTHFMQCTEDHPHFEGLDCE--IFEA 895
M8
M9
Na,K : 919 ---VSIVVVQWADLVICKTRRNSVFQQGMKN-----KILIFGLFEETALAAFLS-YCPGM 967
+ C++ + +Q +I G + FL Y +
SrCa : 896 PEPMTMALSVLVTIEMCNALNSLSENQSLMRMPPWVNIWLLGSICLSMSLHFLILYVDPL 953
M10
Na,K : 968 GVALRMYPLKPTWWFCAFPYSLLIFVYDEVRKLIIRRRPGGWVEKETTY
++ L T W SL + DE+ K I R G
SrCa : 954 PMIFKLKALDLTQWLMVLKISLPVIGLDEILKFIARNYLEG

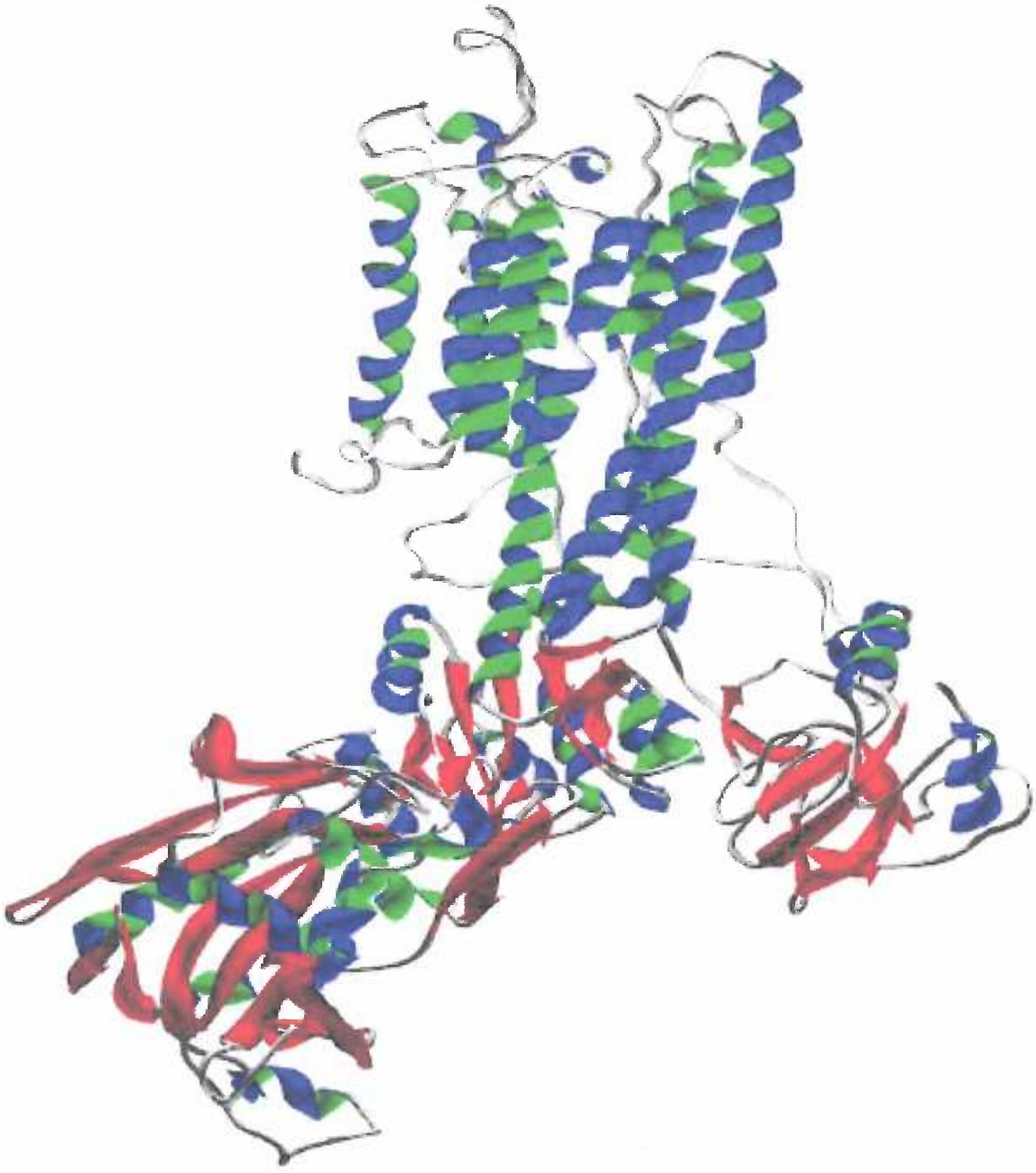

Fig. 5.3. A projected three-dimensional model of the Na,K-ATPase α-subunit based on the structure of the sarcoplasmic reticulum Ca-ATPase of Toyoshima (2000).

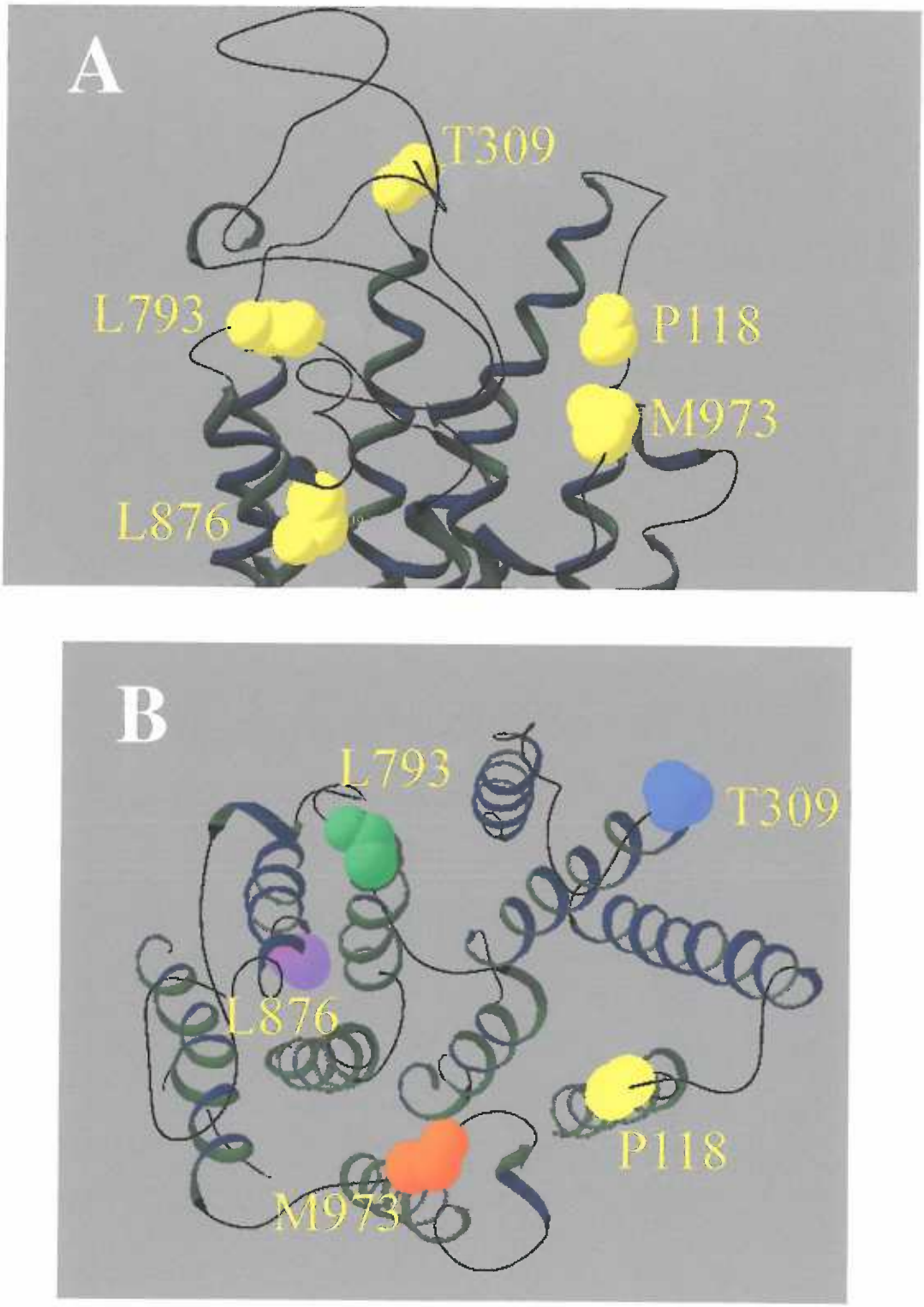

Fig. 5.4. Relative positions of introduced cysteines in our topology study of the Na,K-ATPase α-subunit, viewed from two different angles of the proposed model in Fig. 5.3. **A**, a side view. **B**, viewed from the extracellular domain.

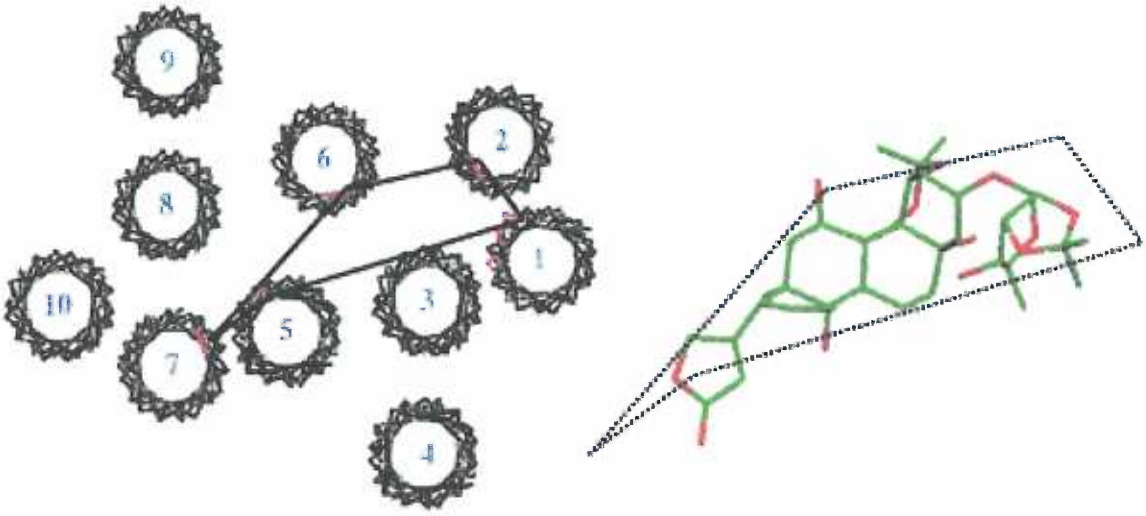

Fig. 5.5. Proposed ouabain-bound structure of Na,K-ATPase based on the solid-state NMR studies of Middleton, *et al.* (2000). (reprint without permission)

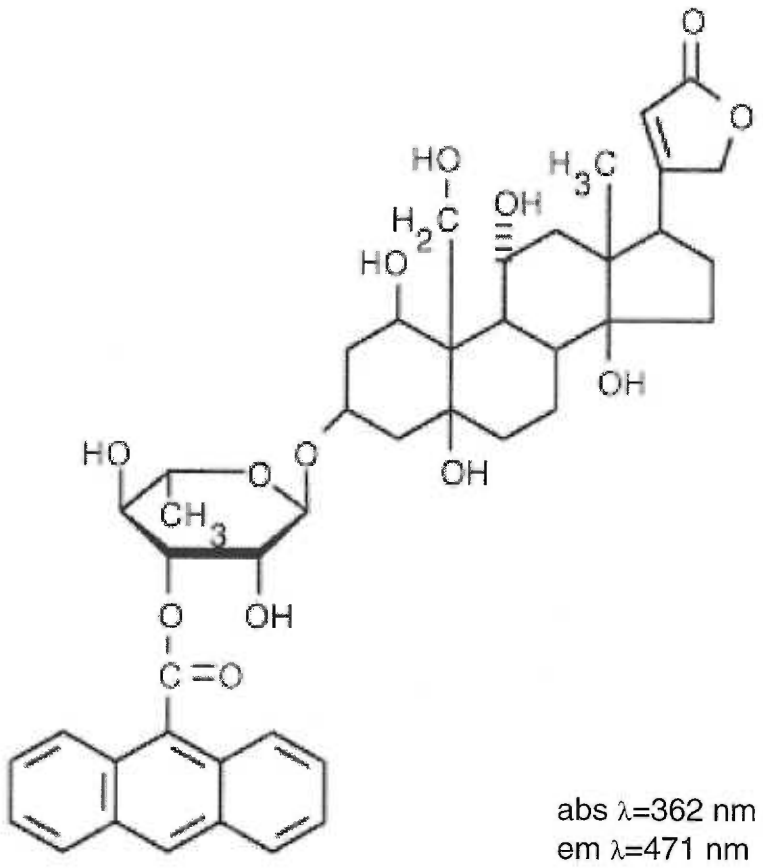

Fig. 5.6. Chemical structure of anthroylouabain.

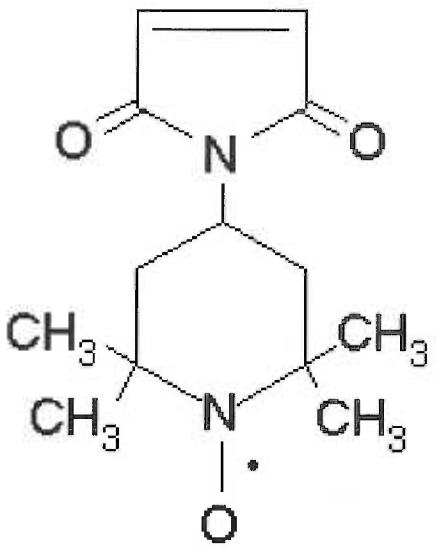

Fig. 5.7. Chemical structure of 4-maleimido-TEMPO

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APPENDIX I

PRE-STEADY-STATE CHARGE TRANSLOCATION KINETICS OF NA,K-ATPASE EXPRESSED IN BACULOVIRUS-INFECTED INSECT CELLS

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The abbreviations used are: BLM, black lipid membrane; NEP-caged ATP, P3-[1-(2-nitrophenyl)ethyl] ester of ATP.

INTRODUCTION

Na,K-ATPase (sodium pump) is an integral membrane protein found in the cells of all higher eukaryotes. This enzyme transports across the plasma membrane three sodium ions out the cell and two potassium ions into the cell against their potential electrochemical gradient using the hydrolysis of ATP as the driving force. The sodium pump is a member of the P_2-type ATPase family, which includes Ca-ATPase and H,K-ATPase. These enzymes share a similar catalytic cycle that contains a phosphorylated protein intermediate. The most commonly accepted model for the reaction mechanism of the sodium pump involves the sequential formation of two acyl-phosphate intermediates. The first intermediate, E_1P, forms on the transfer of the terminal phosphate of ATP to the enzyme in a sodium-dependent fashion. When the sodium ions leave the protein, E_1P is converted to E_2P, which has the highest potassium-binding affinity in the cycle. This is the basis of the Albers-Post model for the reaction mechanism of Na,K-ATPase.

In the current study, we present time-resolved measurements of charge translocation of sheep renal Na,K-ATPases expressed in baculovirus-infected insect cells. The plasma membranes of the infected cells expressing the wild-type and mutant enzymes were isolated by sucrose gradient fractionation and were adsorbed to planar lipid bilayers for investigation of charge translocation. The Na,K-ATPase was activated with rapid release of ATP from caged-ATP upon illumination with an eximer laser pulse (10 ns, 308 nm). We found that the heterologously expressed wild-type Na,K-ATPase displays similar kinetic properties as the purified native enzymes from eel electric organ and pig kidney. Additionally, the kinetic properties of mutants (S775T, E779Q, and E779A) have been shown previously by steady-state studies to contain altered K_m for

potassium ions. Our data demonstrate that the pre-steady-state charge translocation of these mutants is not altered when compared with the wild-type enzyme. This is the first time that the pre-steady-state measurements have be performed on Na,K-ATPase mutants.

EXPERIMENTAL PROCEDURES

Preparation of the Na,K-ATPases – Site-directed mutagenesis, baculovirus expression, protein purification and characterization were described in detail previously (Hu & Kaplan, 2000). Microsomal membranes containing Na,K-ATPase were prepared by following the procedures of Jorgensen (1974a,b).

Bilayer Measurements – Charge translocation measurements were carried out as described (Geibel, *et. al.*, 2000). Briefly, optically black lipid membranes (BLMs) with an area of 0.01-0.02 cm^2 were formed in a thermostatted Teflon cell. Each of the two compartments of the cell was filled with 1.5 ml of electrolyte containing 130 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 25 mM imidazole at pH 6.2. The membrane forming solution contained 1.5% (w/v) diphytanoylphosphatidylcholine and 0.025% (w/v) octadecylamine dissolved in *n*-decane. The membrane was connected to an external measuring circuit via polyacrylamide gel salt bridges and Ag/AgCl electrodes. The signal was amplified, filtered, and recorded with a digital oscilloscope. A first-order, low-pass filter with a cutoff frequency of 500 Hz was used. Na,K-ATPase membrane fragments (20 µl) and 100 µM NPE-caged ATP (Kaplan, *et. al.*, 1978) were added under stirring to one compartment of the cuvette. The membrane preparations contained 0.5 mM EGTA and were sonicated for 15 s in a bath sonicator prior to the addition. To photolyze the caged ATP, light pulses of XeCl excimer laser (λ = 308 nm) with a duration of 10 ns were attenuated by neutral density filters and focused onto the lipid bilayer membrane at an energy density of ~150 mJ/cm^2.

Analysis of the Electrical Signals – The recorded signals (Fig. 6.2) were fitted with a function based on a general first-order model:

$$y = y_0 + A_1 e^{\frac{-(x-x0)}{t1}} + A_2 e^{\frac{-(x-x0)}{t2}}$$

where the y-axis is the measured current (nA), the x-axis is the time scale (sec), t_i is the relaxation time constant and A_i is the amplitude of the curve.

RESULTS AND DISCUSSIONS

ATPase Activity of the Expressed Proteins – Using the baculovirus system, the wild-type and mutant versions of the sheep renal Na,K-ATPases were expressed in insect High Five cells. The mutant proteins contain the E779Q, E779A or S775T substitution in the α-subunit. After five days of infection, cells were harvested and the cell membranes were fractionated on a five-step sucrose gradient to obtain the plasma membranes. The Na,K-ATPase activities of the expressed proteins were ranged from 7-30 μmol P*i* · mg^{-1} protein · h^{-1} (Table 6.1). Potassium activation of the ATPase activities was also carried out and the results are shown in Table 6.1. The potassium affinity of the E779Q mutant is not altered when compared with the wild-type protein. On the other hand, the E779A mutant displayed a two-fold decrease in affinity and the S775T mutant had a 4-fold lowered affinity for potassium ions.

Table 6.1. Characteristics of Na,K-ATPases isolated from plasma membranes of baculovirus-infected High Five cells

	Na,K-ATPase Activity (μmol P*i* · mg^{-1} protein · h^{-1})	K_m for Potassium (mM)
Wild type	30	4.9
E779Q	20	4.9
E779A	6	9.8
S775T	7	20

Pre-steady-state Charge Translocation of the Expressed Proteins - The plasma membranes of the infected cells expressing the wild-type and mutant enzymes were adsorbed to planar lipid bilayers for investigation of charge translocation . As shown in

Fig. 6.1, the capacitive currents of the BLM and the adsorbed membranes are coupled in the experimental setup. The Na,K-ATPases were synchronously activated with rapid release of ATP from caged-ATP upon illumination with an eximer laser pulse (10 ns, 308 nm), and a transient current was recorded (Fig. 6.2). This signal reflects charge transport during the early, sodium-dependent steps of the reaction cycle, which is depicted as a simplified Albers-Post Model in Fig. 6.3. The recorded signals were fitted with a first-order kinetic model and the relaxation time constants were determined for each proteins, which are shown in Table 6.2.

Table 6.2. Rate Constants of the Na,K-ATPase Mutants

	t_1^{-1} (s^{-1})	t_2^{-1} (s^{-1})
Pig kidney enzyme	227	35.5
Expressed wild type	127	45
E779Q	233	37
E779A	334	60
S775T	148	73

The time constant t_1 describes a rapid step(s) in the reaction cycle, which is the formation of E_2P from E_1ATP, where as t_2 describes the slow step of the formation of E_1ATP from E_1 (Fig. 6.3) (for derivation, see Fendler, *et. al.*, 1993).

The purpose of the current study was to perform time-resolved measurements of charge translocation of Na,K-ATPases expressed in baculovirus-infected insect cells. The preliminary results from our study show that the pre-steady-state kinetic properties of the heterologously expressed wild type Na,K-ATPase are similar to the native enzymes. This is a further indication that the baculovirus system produces functional Na,K-ATPases in their native states. Additionally, the pre-steady-state kinetic properties of the

S775T mutant were not significantly altered, suggesting that the four-fold decrease in its potassium affinity is not due to changes in the rate of E_1P or E_2P formation.

Our study is the first to demonstrate that the pre-steady-state kinetic measurements can be performed on Na,K-ATPase mutants. We hope to further characterize the expressed proteins under different cation and caged ATP concentrations. Additionally, mutants that have been previously determined by steady-state methods to have altered E_1P or E_2P population kinetics will also be studied.

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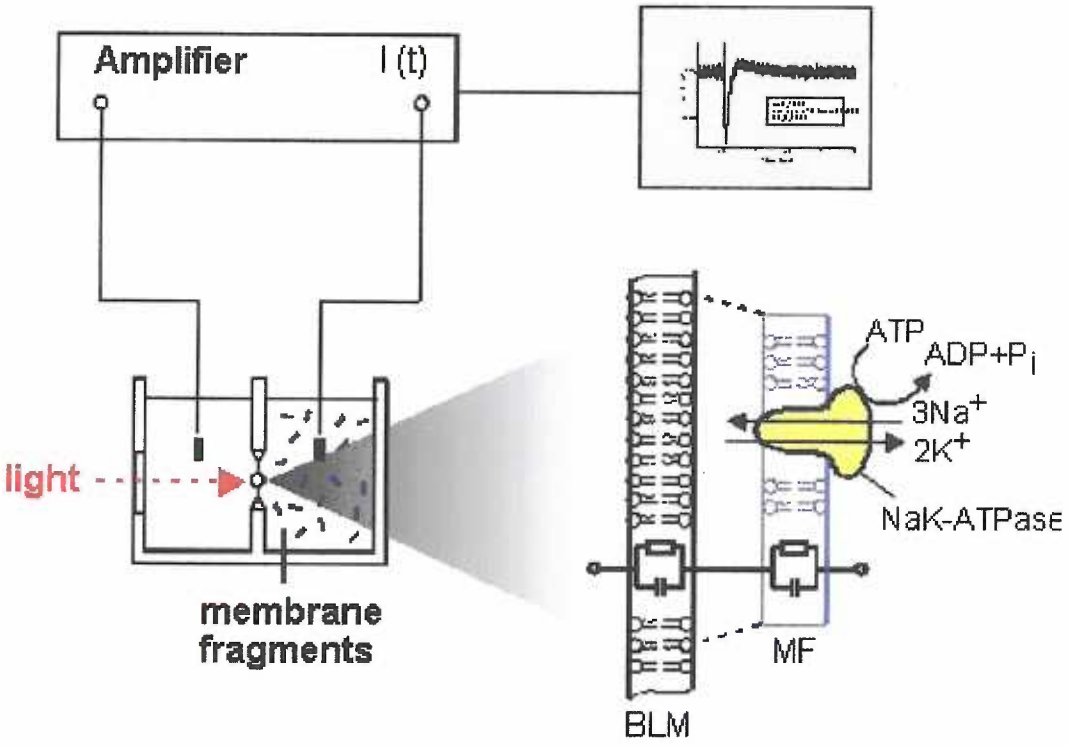

Fig. 6.1. The black lipid membrane setup for measuring the pre-steady-state charge translocation kinetics of Na,K-ATPases.

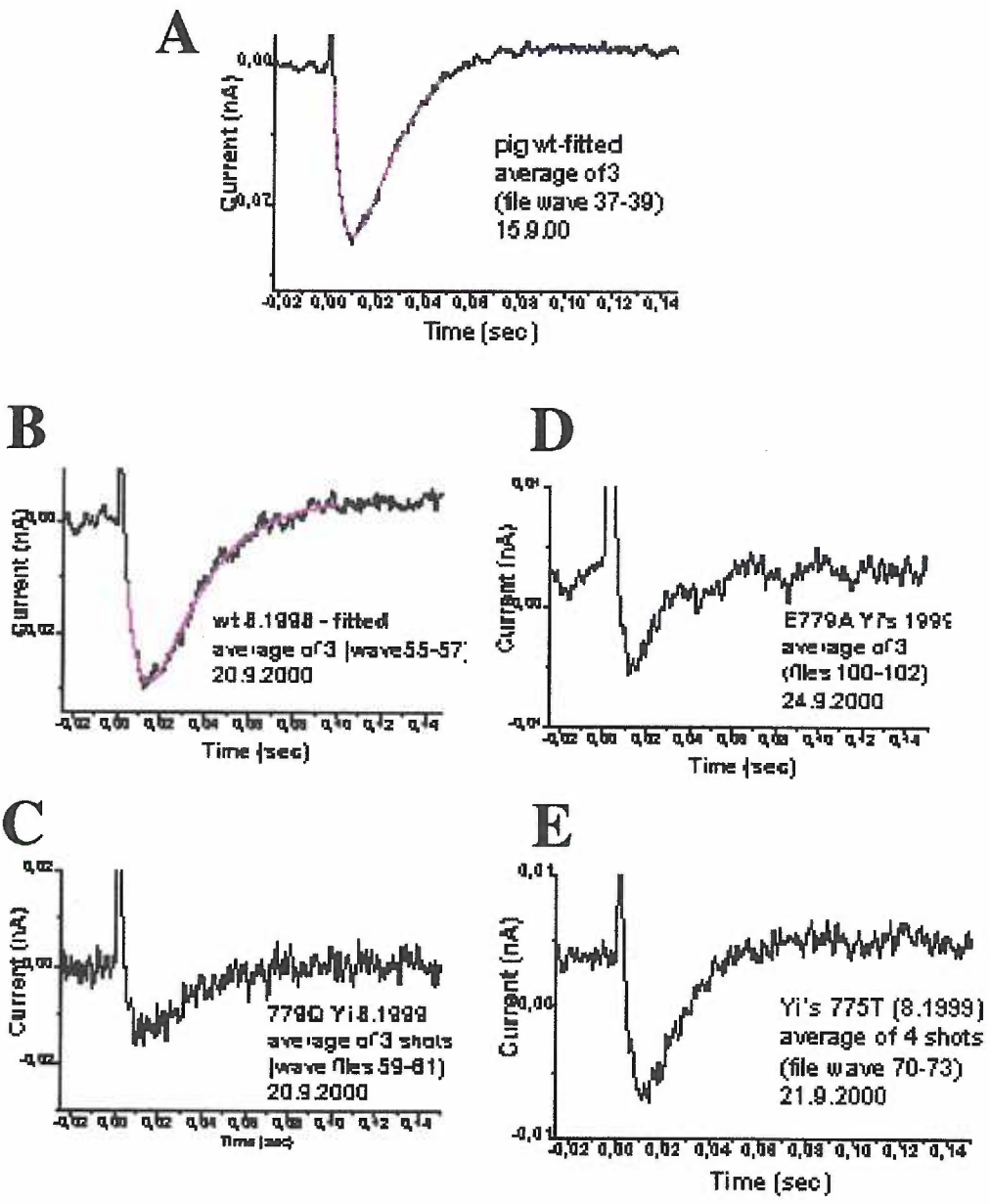

Fig. 6.2. Electrical currents after activation of the **A**, pig kindey Na,K-ATPase; **B**, High Five expressed wild type sodium pump; **C**, E779Q mutant; **D**, E779A mutant; and **E**, S775T mutant. The noisy traces are the electrical recordings, and the smooth ones are the fit.

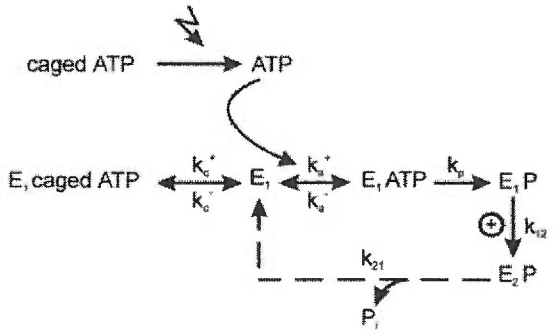

Fig. 6.3. Kinetic model for Na,K-ATPase and its activation, using caged ATP.

APPENDIX II

CONFORMATIONAL COUPLING: THE MOVING PARTS OF AN ION PUMP

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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 released at the other membrane surface which parts of 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 sub-groups reflect changes in the type of transported cation (Lutsenko & 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.

ATP Binding and Hydrolysis

During the period from the mid-1960's 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 & 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 in a specific way, ATP binding. 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. The great advances in cDNA-based protein expression approaches and purification of appropriately tagged proteins has filled out this picture in recent years. It has been possible to express this loop from the Na,K-ATPase in *E. coli*, to purify the polypeptide in mg quantities and demonstrate that the structured polypeptide in solution had the same nucleotide specificity which 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) towards 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 structure 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 & Kaplan, 2000a; Hu & Kaplan, 2000b). 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. 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 coworkers (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 extramembrane parts of the protein, but left intramembrane or membrane-associated segments relatively intact, provided a preparation which 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 (Arguello & Kaplan, 1991). The presence of cations also specifically prevented this inactivation. The single modified residues was subsequently identified as E779 (Arguello & 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 coworkers (Jewell-Motz & Lingrel, 1993; Kuntzwieler, *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 coworkers (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 then, 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 conformation changes at the protein level involved the use of controlled proteolysis of trypsin. In these studies, Jorgensen demonstrated that the proteolytic cleavage sites were altered depending on the presence of either Na or K ions (Jorgensen, 1977). 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 kinds of proteolysis studies led to a more detailed picture of the likely structural changes occurring (Lutsenko & Kaplan, 1994). In this latter work, extensive tryptic digestion was performed and the parts of the protein remaining associated with the membrane were analyzed. Even though 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 carboxyl-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 & 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 coworkers (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 intramembrane 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: (i) it appears that the M5M6 loop is more stable within the membrane when cations are bound than when it is cation-free. (ii) 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. (iii) 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 protein 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 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 helices (especially M5) 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 piston-like motions or via rotations of the transmembrane helices awaits further characterization. A prediction that was made following the studies describing this specific loss of M5M6 from the membrane was that these two helices were probably anchored by protein-protein interaction in the intact protein. It was suggested that M5 and M6 would be internal helices in the two-dimensional arrangement of the transmembrane helices. 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 helices, 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 reactions cycle? Experiments using no-penetrating 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 non-penetrating reagent, suggesting that C964 which is probably close to the extracellular boundary of M9 (Hu & 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:aquous 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 αβ complex for cations (Eakle, *et al.*, 1994; Eakle, *et al.*, 1992; Eakel, *et al.*, 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 3 S-S bridges in the β-subunit extracellular domain also disrupts cation occlusion and enzyme function (Lutsenko & Kaplan, 1992; Lutsenko & Kaplan, 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 α-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 cations or with MgPi (Lutsenko & 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 Pi. 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 inter-domain 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 & 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 M6M6 loop on phosphorylation, but the possibility of alterations in the transmembrane helices 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 change in environment or location. In recent years optical methods using fluorescent or phosphorescence techniques to report such changes have been used with some success n 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 system 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 coworkers (see articles in this issue) and by Farley and colleagues (Ealke, *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 DePont and coworkers 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 & 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 & Kaplan, 2000a). These studies also demonstrated the ability to label with non-penetrating fluorescent probes the heterologously expressed α-subunit of the renal Na,K-ATPase (Hu & 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. Vanadate acts on E_2 form 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. 7.1. As well as 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. 7.2. In these experiments we have compared the Rb activation of ouabain-sensitive ^{86}RB uptake by wild-type (7.2A) or Cys-less (7.2B) Na pump molecules in the sf9 system. The data yields half-maximal values which differ by about 2-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 re-introduced 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 rapid introduction of substrate through photorelease techniques using caged ATP (Kaplan, *et al.*, 1978) or caged Mg reagents (Kaplan & Ellis-Davis, 1988) as has been developed and exploited by Bamberg and coworkers (see earlier in this issue).

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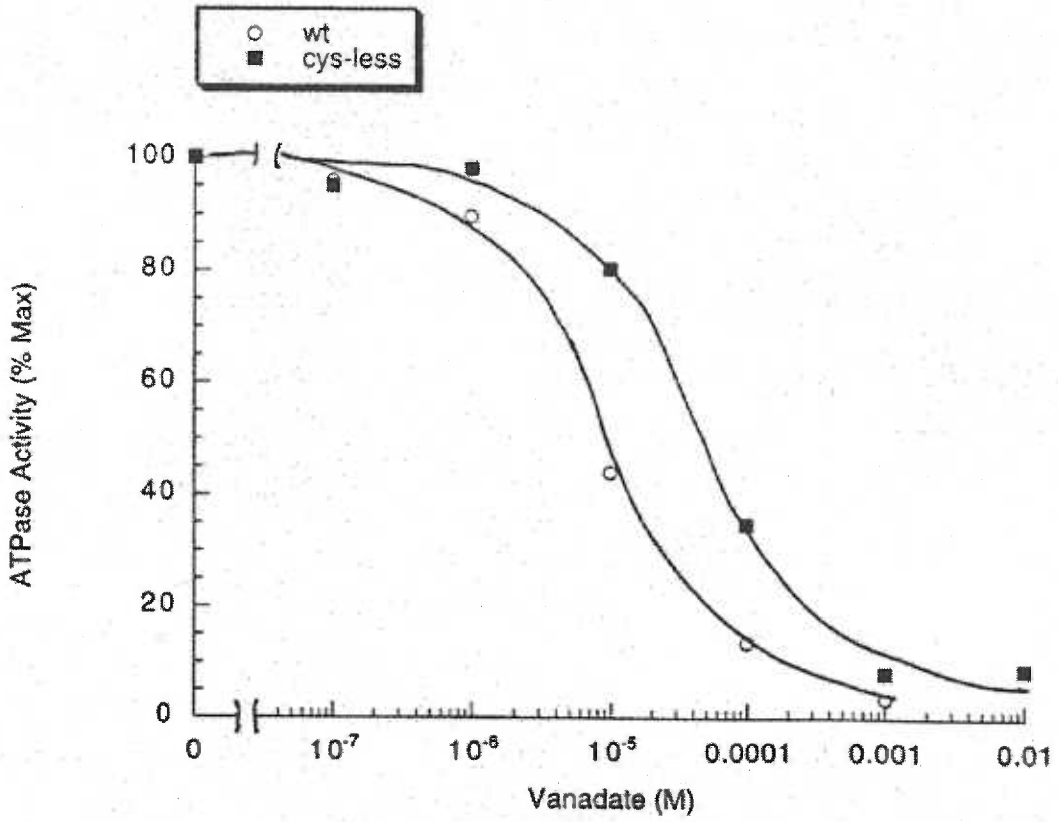
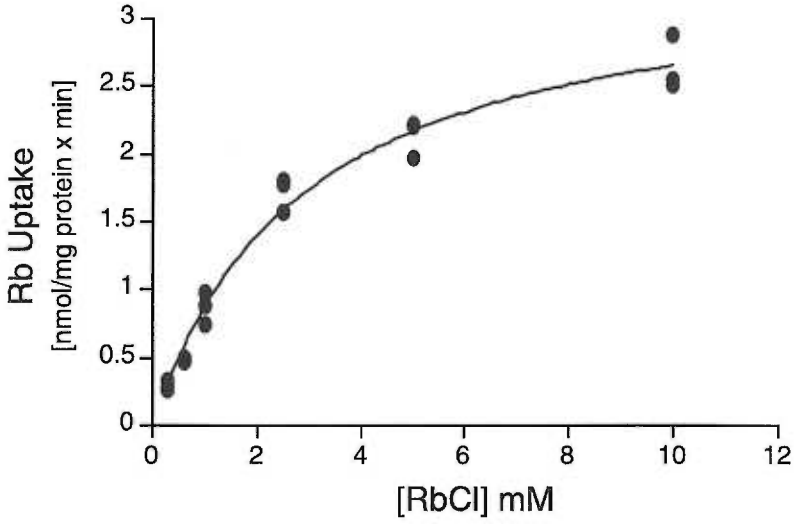


Fig. 7.1. Vanadate inhibition of Na,K-ATPase in insect cells. The Na,K-ATPase activity of purified plasma membranes from sf9 insect cells containing heterologously expressed sheep renal Na pump molecules. The effects of increasing concentrations of vanadate on the activity of wild-type and Cys-less Na,K-ATPase α-subunit are compared.

A



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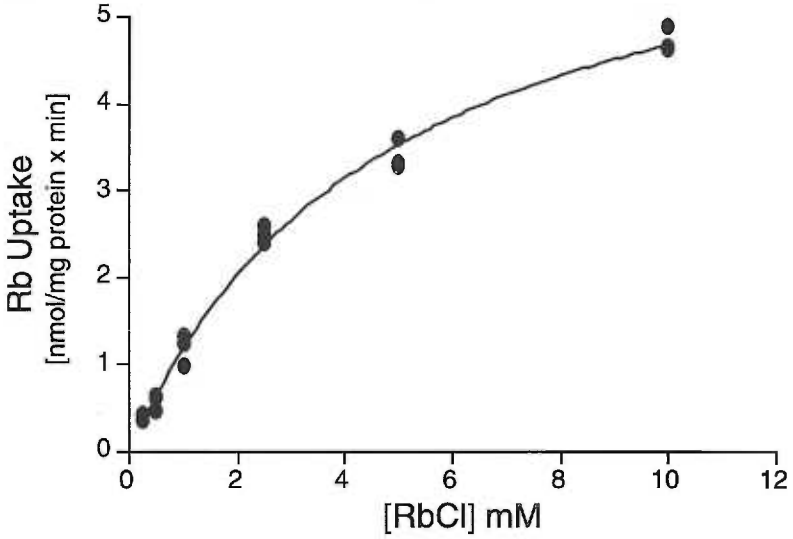

Fig. 7.2. Cation transport of heterologously expressed Na,K-ATPase. ^{86}Rb uptake was measured in sf9 insect cells which had been infected with baculovirus particles containing the cDNA for Na,K-ATPase heterodimers containing either wild type (**A**) or cys-less (**B**) α-subunits. The dependence of the uptake rates on extracellular Rb concentrations are compared. The apparent Km 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.