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Review

# Voltage clamp fluorometry: Combining fluorescence and electrophysiological methods to examine the structure–function of the $Na^+/K^+$-ATPase

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ABSTRACT

This paper summarizes our recent work investigating the conformational dynamics and structural arrangement of the $Na^+/K^+$-ATPase using voltage clamp fluorometry as well as the latest biochemical, biophysical and structural results from other laboratories. Our research has been focused on combining site-specific fluorophore labeling on the alpha, beta and/or gamma subunit with electrophysiological studies to investigate partial reactions of the ion pump by monitoring changes in fluorescence intensity following voltage pulses and/or solution exchange. As a consequence of these studies, we have been able to identify a residue on the beta subunit, which following labeling with tetramethylrhodamine-6-maleimide can be used as a reporter group to monitor the conformational state of the holoenzyme. Furthermore, we have been able to delineate distance constraints between the alpha, beta and gamma subunits and to examine the relative movements of these proteins during ion transport. Concurrent to this research, significant advancements have been made in understanding the molecular mechanism of the $Na^+/K^+$-ATPase. Thus, our research will be compared with the results from other groups and future experimental directions will be proposed.


## 1. Introduction

The $Na^+/K^+$-ATPase was first identified in 1957 and for this seminal discovery Jens Christian Skou was awarded the Nobel Prize in Chemistry in 1997 [1]. The reaction cycle of the $Na^+/K^+$-ATPase is described by the Albers–Post scheme (Fig. 1). In this scheme, the enzyme can reside in two principle conformations, $E_1$ and $E_2$ where, starting in $E_1$, three $Na^+$ ions bind on the cytoplasmic side for transport across the plasma membrane while two $K^+$ ions bind to extracellular sites when the protein is in the $E_2$ conformation for translocation into the cell. The overall reaction is electrogenic as there is a net movement of charge, three $Na^+$ ions for two $K^+$ ions. Furthermore, the main electrogenic events are the extracellular release/rebinding steps which are energetically coupled to the $E_1P$–$E_2P$ conformational change [2,3].

The ion pump, which is expressed on the plasma membrane of most eukaryotic cells, is comprised of two obligatory subunits, alpha and beta, as well as one member of the FXYD family of proteins which is not required for function. The enzyme is a member of the P-type ATPase family, so called because each member of this family is phosphorylated at a highly conserved aspartate residue within the consensus sequence, DKTGTLT, upon ATP hydrolysis [4]. The alpha subunit comprises approximately 1000 amino acids and contains ligand ($Na^+$ and $K^+$), substrate (ATP) and inhibitor (digitoxin and ouabain) binding sites [5]. This subunit has ten transmembrane domains and three large cytosolic domains: the nucleotide binding site (N) which contains the ATP binding site, the activator domain (A) which acts as the activator of the gates that regulate binding and release of the transported cations and the phosphorylation domain (P) which contains the residue which is phosphorylated. Each of these domains undergoes a significant conformational change during the catalytic cycle which has been elegantly elucidated with a series of crystal structures of the $Ca^{2+}$-ATPase in both the $E_1$ and $E_2$ conformational states [6,7]. This conformational change is translated to the transmembrane domains during ion translocation and it has been demonstrated that the M5–M6 loop is involved in a functional rearrangement during the pumping cycle [8]. Finally, it should be noted that there are four isoforms of the alpha subunit in humans. The α1 isoform is expressed in the kidney and lung, while the α2 is the predominant isoform in skeletal muscle. The third isoform (α3) is present in brain and heart and the α4 isoform has been implicated in sperm motility.

In addition to the alpha subunit, the beta subunit is also required for function. This protein is approximately 300 amino acid long and contains multiple glycosylation sites as well as disulfide bridges which are highly resistant to reduction [9,10]. Interestingly, the beta subunit is required for proper trafficking of the holoenzyme to the plasma

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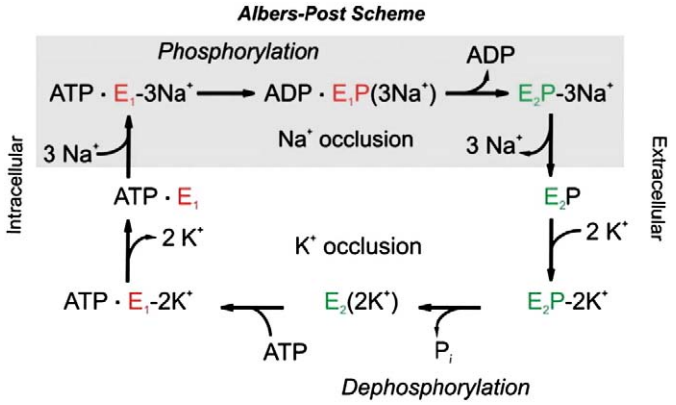


**Fig. 1.** Albers–Post scheme for the $Na^+/K^+$-ATPase reaction cycle. The enzyme can assume two major conformations: $E_1$ (red) where the ion binding sites face the cytoplasm and $E_2$ (green) where access to the cation binding sites is from the extracellular space. In the absence of extracellular $K^+$ and in the presence of $Na^+$, the enzyme is restricted to $Na^+/Na^+$ exchange conditions (grey box).

membrane [11]. That is, following heterologous expression of only the alpha subunit, this protein is localized in sub-cellular organelles and degraded [9]. The beta subunit is also recognized to modulate cation binding affinity [12]. There are three isoforms of the beta subunit (β1, β2 and β3) which are expressed differentially in tissues and it has been established that subtle sequence differences between isoforms result in functional differences [13].

In contrast to the alpha and beta subunits, the FXYD family of proteins is not required for function. There are seven members of the mammalian FXYD proteins which are less than 100 residues and modulate the apparent affinity for sodium, potassium and/or ATP [14]. As such an important physiological molecule, a wide variety of experimental techniques have been used to investigate the mechanism of this protein over the past 50 years. This includes purifying the enzyme from native species for subsequent biochemical and biophysical studies using fluorescence labeling [15,16]. The past few years have seen significant advances in understanding the mechanism of this enzyme, whether through the use of the experimental techniques described above or novel approaches. Some of the more recent work in these areas will be addressed in the following sections.

## 2. Mechanistic information on the ion pump

Although basic outline of the ion transport has been determined, as described by the Albers–Post scheme, much work has been done to elucidate the fine details of ion transport. Notably, Gadsby and co-workers have used the marine toxin palytoxin to examine the transmembrane pathway of ions through the $Na^+$ pump. Palytoxin binds to the $Na^+/K^+$-ATPase and initiates the formation of a non-selective cation channel. Thus, it has been possible to examine some partial reactions of the ion pump following application of ligands or following the application of thiol-specific reagents upon incorporation of cysteine residues on the ion pump [17–19]. As a function of these experiments, it has been determined that residues which reside in transmembrane domains 1, 2, 4 and 6 are involved in the cation pathway. Computational approaches have been combined with these results to describe the kinetics of phosphorylation and dephosphorylation of the ion pump in the presence of palytoxin [20].

During the past few years, the objective of our research has been to detect site-specific conformational changes in real time under physiological conditions by combining fluorescence and two electrode voltage clamp (voltage clamp fluorometry). In these experiments, two point mutations (Q111R and N122D) are utilized to confer a reduced ouabain sensitivity of the ion pump [21]. This enables selective inhibition of the endogenous $Na^+/K^+$-ATPase as it is expressed in *Xenopus* oocytes versus the heterologously expressed ion pump. Two further point mutations (C911S and C964A) were added in order to remove all free extracellular cysteine residues [22]. None of these mutations have an appreciable effect upon the kinetic parameters of the ion pump. Upon insertion of a cysteine residue at a specific site, it is then possible to site-specifically label the ion pump with tetramethyrhodamine-6-maleimide. It should be noted that the beta subunit contains additional cysteine residues. However, each of these residues forms disulfide bonds which are highly resistant to reduction and are not labeled with the fluorophore [9,10].

We utilized the information that the M5–M6 loop of the alpha subunit undergoes a conformational rearrangement during ion translocation to perform cysteine-scanning mutagenesis of this region followed by fluorophore labeling [8]. From this work, one residue (N790C) was identified which demonstrated changes in fluorescence intensity following changes in extracellular solution and/or membrane potential [23]. These experiments enabled the determination of spatially defined conformational changes of the fully functional $Na^+/K^+$-ATPase in real time and under physiological conditions. Furthermore, it was possible to determine the distribution of the two main conformational states under $Na^+/Na^+$ exchange conditions. Under these conditions the pump is restricted to the $Na^+$ translocating branch of the cycle (see Fig. 1) and shuttles in a voltage dependent manner almost exclusively between the $E_1P$ and $E_2P$ conformations as dephosphorylation under $K^+$-free conditions is very slow. Hyperpolarizing potentials drive the enzyme in a saturating fashion into the $E_1P$ conformation and depolarizing potentials lead to accumulation of $E_2P$. Transient currents can be observed upon voltage jumps which are the product of electrogenic $Na^+$ re-uptake and $Na^+$ release. By measuring the fluorescence intensity and plotting this as a function of membrane potential, it was possible to determine the relative populations of holoenzyme in the $E_1P$ and $E_2P$ conformations. In addition, the kinetics of ion transport could be compared to the kinetics of the conformational changes. However, in these experiments it should be noted that the N790C mutation did affect the kinetic parameters of the ion pump. The value for $V_{0.5}$, which represents the membrane potential where there is an equal proportion of ion pumps in the $E_1P$ and $E_2P$ states, was more negative ($V_{0.5}$ of WT: $\approx -65$ mV and N790C: $-110 \pm 9$ mV) when compared to the wild type ion pump. This data suggests that the $E_2P$ conformation was stabilized when compared to the wild type ion pump. In addition, the transient current kinetics of ion transport were slower (WT: ≈50–200 $s^{-1}$ and N790C: ≈20 $s^{-1}$). Thus, the asparagine to cysteine mutagenesis and subsequent fluorescence labeling of the N790C residue affected the kinetic properties of the ion pump. Therefore, the next objective was to identify a construct which could be used as a reporter group for the ion pump, but where the kinetic parameters were not affected.

We therefore focused on the beta subunit of the ion pump. Following scanning cysteine mutagenesis of the sheep β subunit (isoform 1: $s\beta_1$), three residues were identified (S62C, F64C and K65C) which exhibited significant (greater than 5%) fluorescence changes in response to voltage steps under $K^+$-free conditions or concurrent with changes in extracellular ionic conditions that induce stationary $Na^+/K^+$ exchange currents (Fig. 2A).

The kinetics of the change in fluorescence intensity was distinct for each of these three constructs and therefore we will limit ourselves to the results for one construct, $s\beta_1$-S62C, in this review [24]. In order to compare fluorescence signals to certain partial reactions of the Albers–Post cycle, we performed voltage pulse experiments at high extracellular $Na^+$ concentrations (100 mM) in the absence of $K^+$. As mentioned previously, under these conditions the $Na^+/K^+$-ATPase carries out $Na^+/Na^+$ exchange [3]. We determined that the voltage dependence of displaced charge for this construct follows a Boltzmann distribution which reflects the distribution of the enzyme between $E_1P$ and $E_2P$ states (Fig. 2B). As the change in fluorescence intensity