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Feature Article

Finding Na,K-ATPase

I - From Cell to Molecule

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Abstract. The oppositely oriented concentration gradients of Na^+ and K^+ ions across the cell membrane as found in animal cells led to the requirement of an active ion-transport mechanism that maintains this steady-state condition. As solution of this problem the Na,K-ATPase was identified, a member of the P-type ATPase family. Its stoichiometry has been defined as 3 Na^+/2 K^+/1 ATP, and a class of Na,K-ATPase-specific inhibitors, cardiac steroids, was established, which allow the identification of this ion pump. In an effort lasting for several decades structural details were uncovered down to almost atomic resolution. The quaternary structure of the functional unit, either αβ heterodimer or $(\alpha\beta)_n$ complexes with $n \geq 2$, is still under discussion.

Keywords. Sodium pump, active transport, discovery, physiological role, structure.

I. HISTORY OF THE NEED FOR A SODIUM PUMP

In the 1930s it was already well known that inside living cells the composition of the ionic contents was significantly different from that of the extracellular space. At that time the physiological investigations were focused mainly on muscle and red blood cells, and the evident asymmetry of high K^+ and low Na^+ concentrations inside and vice versa outside was well documented. During this period hardly any functional properties of the cell (or 'cytoplasmic') membrane were known, not to mention understood. It is not surprising that various concepts were developed to explain the asymmetry and how it was sustained. The initially preferred and widely supported idea was that the cell membrane is an almost perfectly impermeable barrier for ions. Some scientists were even willing to sacrifice the validity of the second law of thermodynamics in the field of biology in order to explain the experimental observations. A monograph on the historical development of our understanding of membrane transport, which is comprehensive and worth reading, was published about twenty years ago by Joseph D. Robinson.[1]

Major breakthroughs were achieved when new experimental techniques became available, such as the use of radioactive isotopes of K^+ and Na^+ that allowed the detection of unidirectional fluxes. Probably medical requirements of the Second World War also contributed, since physiologists were

forced to study every detail on the optimization of blood preservation.

When $^{24}Na^+$ was introduced into physiological experiments it was shown that this radioactive isotope readily and rapidly exchanged with the stable isotope on the other side of the cell membrane. The membranes of the muscle cells were evidently permeable for Na^+.[2,3] It was also demonstrated that the cytoplasmic K^+ concentration could be very well modulated by the extracellular K^+ concentration, a clear indication of a K^+ permeability of the cell membrane.[4] Nevertheless, the asymmetric distribution of both cation species remained preserved despite the fact that both ion species were able to permeate through the membrane. As an inevitable consequence, a counter-movement of Na^+ and K^+ had to be assumed that keeps up the concentration gradients. In 1940 H. Burr Steinbach mentioned in a contribution to a Cold Spring Harbor Symposium for the first time the request for a "pumping out the sodium" from the cytoplasm.[5] It became clear that ion translocation driven by their electrochemical potential gradients, the so-called passive ion transport, had to be counteracted by energy-consuming transport processes (or "active transport") that ensured the indispensable condition of stationary high K^+ and low Na^+ concentrations inside the cells and even the electric membrane potential. Figure 1 shows a schematic representation of transport pathways and the transporters that were identified as leading actors during decades of investigations of cell membrane properties.

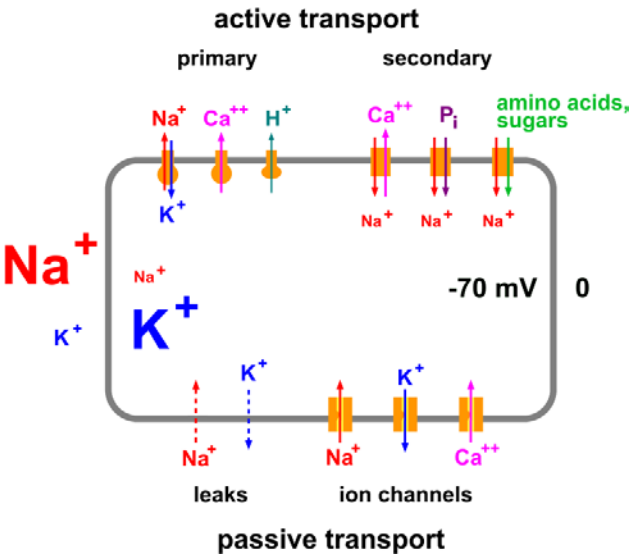

Figure 1. Ion transport pathways in animal cells. Common to all cells is the inside negative electric potential and the ion-concentration gradients oppositely oriented for Na^+ and K^+, with high Na^+ concentrations outside and low in cytoplasm and vice versa in the case of K^+. In principle, two different categories of transport mechanisms have to be discriminated, active and passive transport. The active transport is split into primary and secondary active transport. In primary active transport so-called ion pumps utilize ATP as energy source to translocate ions "uphill", i.e. against their electrochemical potential gradient. Secondary active transport is performed by antiporters (e.g. the Na,Ca-exchanger) or cotransporter (e.g. the Na,P_i- or Na,glucose-cotransporter) which translocate one of their substrates "uphill" while the other substrate, usually Na^+, provides the necessary free energy by its transport "downhill". A selection of examples is shown in this figure. Passive ion transport occurs either by leak conductance which is minimized by the nature and structure of the cell membrane (but unavoidable) or it is facilitated by channels or carriers. These transporters are regulated by different mechanisms to meet the metabolic needs of the cells. Key players for the passive cation transport are Na^+, K^+ and Ca^{2+} channels.

A further fruitful approach to advance the understanding of transport processes across the cell membrane was contributed by investigations of red blood cells. Already in the late 1930s the blood banks tried to find optimized preservation conditions of red blood cell batches. It was found that the stored cells lost their internal K^+ in the course of time, and the concentration of free K^+ in the blood plasma reached toxic levels when blood was preserved in the cold. In addition, it was shown that the cytoplasmic Na^+ concentration increased and that these changes were not primarily caused by deteriorated blood cells.[6] The net outflow of K^+ in the cold could be reversed at a temperature of 37 °C and in the presence of glucose. This observation indicated that K^+ and Na^+ ions were transported across the membrane against their concentration gradient. In the end, the physiological asymmetry was restored and this action was dependent on glycolysis.[7-9] In addition, inhibition of glycolysis by incubation with fluoride caused a delayed loss of K^+ from the red blood cells which was no longer balanced by K^+ uptake, even at physiological temperature.[9] This observation pointed out that glycolysis may not be immediately responsible for K^+ inward transport. At that time the underlying metabolic functions were still subject to speculation.

Later in the 1940s sufficient experimental evidence had been collected to conclude convincingly that active transport of Na^+ maintains the Na^+/K^+ asymmetry across the cell membrane and that this condition is a steady state and not an equilibrium.[10] Another step forward was the realization that the Na^+ flux out of the cell is coupled to the presence of external K^+.[11]

In the early 1950s a focus was set on the energy sources that fuel the concentration asymmetry for Na^+ and K^+ across the cell membrane of the red blood cell. The fact that glucose was metabolized but was not the direct source of energy had been made evident already ten years earlier.[9] It was also discussed that glycolysis

plays a role as ATP-generating process.[12] In 1954, first experimental studies demonstrated that the K^+ accumulation in red blood cells was an ATP-requiring process which was activated by Mg^{2+}.[13] A few years later, the positive proof was provided that active K^+ transport occurred only when ATP was present,[14] and a corresponding finding was made also in squid giant axons.[15] In studies of frog skin significant experimental evidence was collected that active Na^+ transport was a forced exchange of Na^+ against K^+,[16] like in the case of red blood cells.[11]

Constructive findings concerning the sodium pump were made possible by another crucial discovery in the early 1950s which eventually turned out to be of eminent importance for all following investigations of the Na,K-ATPase. When studying the cation transport in red blood cells, Hans J. Schatzmann found that cardiac steroids blocked the K^+ uptake and the Na^+ outflux. He named the causative process "Na-K-Pumpe".[17] He was interested in identifying the mode of the blockers' action and with detailed experiments he proved in his study that these compounds did not affect glycolysis and oxygen consumption. He also concluded that there was a direct blockade of the transport mechanism.[17] This finding was supported and fortified by others.[18,19] Transport was blocked from the outside of the cell,[20] and external K^+ had an antagonistic effect on cardiac steroids.[19] Eventually, it became clear that Schatzmann had discovered a class of compounds, of which ouabain was the most well-known, that provides highly selective inhibitors of the Na,K-ATPase. Cardiac steroids have become the "custom-made" tool to discriminate the sodium pump from all other ATP-hydrolyzing enzymes in whatever biological tissues.[21]

Since at that time enough experimental evidence was collected that Na^+ and K^+ were coupled to maintain steady-state concentrations for both ion species inside the cells, the question was raised whether there exists a constant coupling ratio. To find the answer to that question was not so simple. It was necessary to discover a reliable experimental approach since the active ion transport balanced passive leak fluxes that in turn were dependent on the prevailing ion concentrations on the outside of the cells. From experiments with squid axons it was concluded that because of the passive ion permeabilities an active "secretory mechanism driven by metabolism" had to be present that moved Na^+ and K^+ against their electrochemical gradients.[22] The experiments also supported strongly the idea of a coupled system in which Na^+ was moved out of the cell "on one limb of the cycle" and K^+ taken up on the other.[22] In 1956 Ian M. Glynn was able to present experimental data from red blood cells using radioactive tracers, in which active and passive transport could be separated distinctly and Na^+ efflux and K^+ influx were tightly coupled in the active transport. He suggested a one-to-one exchange of Na^+ and K^+.[23] A year later Robert L. Post was able to measure net fluxes of Na^+ and K^+ with an unprecedented accuracy and determined a ratio of 3 Na^+ for 2 K^+, which was constant over the whole experimental concentration range of the transported ions.[24] This coupling ratio withstood all challenges and was found to be generally valid (except for a few extremely unphysiological electrolyte compositions).

II. TRACING THE PROTEIN

In the 1950s it was quite a challenge to propose the concept that a single protein molecule comprised both enzymatic function, in this case ATPase activity only known so far to soluble proteins, and transport function, i.e. vectorial ion movements across the cell membrane. Although during these years evidence was accumulated that there exists a tight coupling between both functions, the final proof, the identification of a protein (or protomer) that unites ATPase activity and ion transport was still pending. In 1957, the state of the art – with respect to red blood cells – was presented in a meticulously elaborated review by Glynn.[25] However, even on the basis of this amassed knowledge, no convincing experimental approach was developed to solve the puzzle of the Na,K-pump.

The breakthrough was eventually provided by a scientist from a completely different field. Jens Christian Skou studied the effect of local anesthetics on nerve conduction with the view of finding a membrane preparation that could be used in monolayer experiments, in which a well-defined enzymatic activity should be detected as function of applied local anesthetics. He finally attained his goal in 1956 by a membrane-fragment preparation from crab nerves and showed Mg^{2+}, Na^+ and K^+ dependent ATPase activity.[26] The history of this development is elaborately described in a comment on his original paper, published in 1989[27], in Skou's Nobel Lecture in 1997[28] and in his autobiographical book, "Lucky Choices. The Story of my Life in Science", published recently.[29] Since active transport of ions was not his field of interest, initially he was not aware of the contribution he had made. Only after Post triggered the crucial test when both met at a conference in 1958, namely to confirm inhibition of the enzyme activity by ouabain, it was decided that he had identified the Na,K-ATPase.[30] In retrospect Skou described his posi-

tion in those early years: "I felt like an intruder in a field that was not mine."[28] The break-through was possible because he serendipitously worked with a membrane preparation from crab nerves that consisted of open membrane fragments in which both sides of the membranes were accessible simultaneously. In contrast, cell membranes of most other cells formed closed vesicular structures upon homogenization. Those preparations needed to be treated with detergents before the desired simultaneous access to both sides access of the membrane was obtained. With this information Post was able to identify briefly afterwards the Na,K-ATPase also in red blood cells.[31] In 1965 Skou had already published a review with reference to numerous tissues in which the presence of Na,K-ATPase had been verified too.[32] Today we know that the Na,K-ATPase is present in virtually all animal cells. A schematic biochemical characterization of the Na,K-ATPase is shown in Figure 2.

Because cell membranes contain scores of different proteins, at that time the question still remained open, whether the protein which performs ATPase activity was also responsible for ion pumping or whether more than one protein had to be coupled to a functional complex. It became a prominent task to isolate and purify the (minimal) enzyme complex that performed as Na,K-ATPase and analyze its components. In the early 1960s this project was, however, a major challenge because on one hand no standard methods were available to isolate and purify membrane proteins and keep them concurrently functional. On the other hand in isolated complexes no

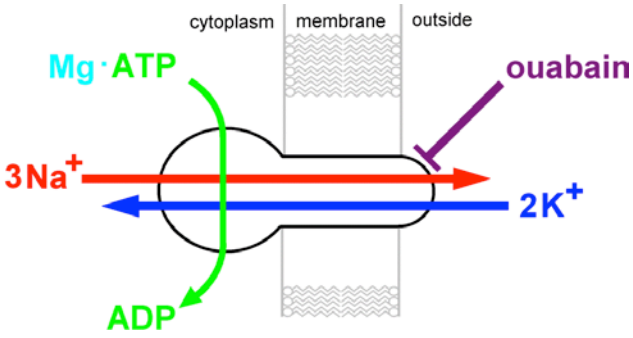

Figure 2. Biochemical characterization of the Na,K-ATPase. The ion pump is an integral membrane protein of animal cell membranes with its enzymatic machinery located on the cytoplasmic side. It hydrolyzes one MgATP complex into ADP and inorganic phosphate, P_i, and utilizes the released free energy to expel 3 Na^+ ions from the cytoplasm and to translocate 2 K^+ ions into the cytoplasm. Ouabain, a cardiac glycoside, is a specific inhibitor that completely blocks the Na,K-ATPase from the extracellular side of the membrane.

sidedness was given, and therefore, ion transport could not be proven. To overcome this problem, the solubilized complexes had to be reconstituted back into membranes that formed the interface of two compartments which were separate from each other and provided the required sidedness.

Tissues from which the Na,K-ATPase can be isolated in reasonable amounts are found either in mammalian brain and electroplax from fish that both contain excitable cells or in tissues specialized to transport sodium, such as the outer medulla of kidney, rectal glands of shark or salt glands of ducks.[33,34] Early findings showed that the outer medulla of mammalian kidneys is a fairly easily accessible and convenient source. The basolateral membranes of the cells forming the thick ascending limbs of the loops of Henle are specifically abundant in Na,K-ATPase.[35,36]

When cells rich in Na,K-ATPase are broken up by homogenization, a membrane preparation can be separated by centrifugation, the so-called crude microsomal fraction of which ATPase activities were measured in the order of 200 – 500 µmol inorganic phosphate (P_i) released per mg protein and hour. These membranes form vesicular structures with the cytoplasmic surface facing the outside.[37] To obtain a purified preparation from such a vesicle suspension that still contains all proteins of the plasma membrane, Peter L. Jørgensen elaborated in 1969 a specific treatment using a low concentration of the detergent sodium dodecyl sulfate (SDS). This method provides, after separation by differential centrifugation, open membrane fragments containing Na,K-ATPase in high density, the so-called purified microsomal preparation.[38,39] By this treatment most of the other proteins and a considerable fraction of the membrane lipids are removed. This approach became subsequently – with minor improvements – the standard routine to isolate and purify the Na,K-ATPase.[40] The protein density is so high that these fragments become stiff enough that they are no longer able to form vesicles. The hydrophobic membrane interior at the edge of the fragments is apparently covered by a layer of SDS molecules which prevent contact of the hydrophobic core to the aqueous phase. The resulting fragments contain ion pumps with densities of up to 10^4 per μm^2 as determined from electron micrographs.[41] When Jack Kyte applied in 1971 the shortly before introduced SDS polyacrylamide gel electrophoresis to a purified microsomal preparation, he proved a purity of better than 95% and that the Na,K-ATPase is a protomer of two distinct polypeptides with proposed molar masses of 84 kDa and 57 kDa.[42] The purified microsomal preparations from pig kidney attained ATPase activities of up to 2400 µmol P_i per mg

protein and hour.[43] When incubated in solutions with Mg^{2+} and sodium vanadate, an inhibitor of the Na,K-ATPase,[44] the proteins spontaneously form in the microsomal membranes large two-dimensional crystal lattices in the membrane fragments.[43]

III. DEFINING PROPERTIES

In the 1970s advancing biochemical techniques promoted the study of membrane proteins. These were applied successfully to characterize the Na,K-ATPase with increasing precision in the following decades. A detailed review of the progress yielded during this period has been compiled in a 1979 review by Robinson and Flashner.[45]

At first, the most fundamental open question was probably about the subunit composition of the Na,K-ATPase: Does the functional ion pump consist of a single polypeptide chain or of a complex of two or more subunits? Although purified preparations displayed two different proteins in SDS gel electrophoresis,[42] it was not clear whether the lighter glycoprotein "is a true component of the NaK ATPase" or a tenaciously bound or coincidentally co-purified unrelated component.[46] From the gel-analysis method, applied to numerous purified protein preparations from different sources, the determined ratios of both subunits varied between 2:1 and 1:2 when the heavier subunit was compared with the lighter.[42,47,48] These diverse stoichiometries were obtained from experiments in which Na,K-ATPase preparations from different tissues were used, different amounts of proteins were applied on the gels, and at that time the behavior of glycoproteins on gels was not well understood. It lasted another few years until common agreement was reached that the stoichiometry is 1:1 and that only both subunits together form the active Na,K-ATPase.[33,49,50] In 1980 the final notation was introduced, in which the large polypeptide was named α subunit and the smaller glycoprotein β subunit.[49] It was established that the α subunit is phosphorylated by ATP[51] and that cardiac glycosides bind to it.[52] The location of the ion-binding sites was assumed to be also in the α subunit, but this problem was still under discussion in 1988.[53] The role of the β subunit was largely unknown at that time. Although it does not carry out enzymatic functions, it is crucial for the activity of the Na,K-ATPase.[54] Reduction of a single disulfide bridge that the β subunit possesses in its extracellular C-terminal part leads to a complete loss of the pump's activities.[55] How far various functions of the Na,K-ATPase are modulated by this subunit was under scrutiny for a long time. Convincing evidence, however, was compiled throughout the years that the β subunit is crucial for structural and functional maturation, trypsin resistance in ER preparations, appropriate trafficking in the cells and cell-cell adhesiveness.[56,57] Only after the Na,K-ATPase could be expressed from cloned cDNA it has been shown directly that the α subunit alone is incapable to perform Na,K-ATPase specific functions.[58]

The question whether an αβ complex is sufficient to perform Na^+ and K^+ transport fueled by ATP hydrolysis could be answered only after the Na,K-ATPase has been purified functionally and reconstituted as single protein species in a lipid membrane. This experimental approach was successfully introduced in 1974, when the purified Na,K-ATPase was incorporated in lipid vesicles and inside-out oriented reconstituted pumps transported ^{22}Na ions into the vesicles.[59] The transport was inhibited when ouabain was present inside the vesicles. As will be shown later, the application of Na,K-ATPase containing vesicles (or 'liposomes') turned out to become an extremely useful tool to investigate functional properties of the ion pump.[60]

In 1978 for the first time reliable evidence was presented that Na,K-ATPase isolated from pig kidneys contained a third subunit, a small polypeptide with a molar mass in the order of 12 kDa.[61] It took until the 1990s before more systematic investigations of this third subunit started, and during the following years a tissue specific distribution was found. Some tissues lacked of a third subunit and in others, where it was present, it was a member of the so-called FXYD protein family.[62-64] This family consists of seven members. These proteins possess less than 165 amino acids, have a single membrane-spanning segment, and they share the (eponymous) extracellular motive FXYD (with X as place holder for either T, E, Y, F). They interact with the α subunit and their function is a modulation of the ion-transport kinetics that allows short-term adaptation to specific metabolic needs of the cells.[64]

Skou reported already rather early experimental results that the Na,K-ATPases of rabbit kidney and brain exhibited different sensitivity to g-strophantin (ouabain). It was higher by a factor of 5 in the enzyme from the brain than from the kidney.[37] In 1976 experimental results were published from ouabain-binding studies using enzyme isolated from ox brain that are explained by the existence of two (or more) enzyme populations.[65] In 1979 Kathleen Sweadner advanced the field considerably when she proved by SDS-gel electrophoresis that two forms of the Na,K-ATPase existed in the brain that differ by 2 kDa in molar mass[66] which differed in their sensitivity to strophanthidin by almost a factor of 1000.

By 1989 three isoforms of the α subunit were identified and a common nomenclature fixed (α1 – α3).[67,68] In 1994 a fourth isoform, α4, was identified that is specific to testis.[69,70] α1 is the dominant isoform in kidney and heart, the tissue-specific distribution is catalogued extensively.[68] For the β subunit three isoforms (β1 – β3) were found.[71,72]

More precise access to the molar masses became available when amino-acid sequences were obtained from exploiting the analysis of complementary DNA that was introduced in the late 1970s[73] and the molar mass of proteins could be calculated precisely. In 1985, accurate numbers were published: 1016 amino acids (AAs) were determined for the (mature) α subunit of sheep kidney,[74] and 1022 AAs for the α subunit of *Torpedo californica*.[75] That led to a calculated molar mass of 112,177 Da for the sheep subunit. For the β subunit the first sequences were published in 1986: 302 AAs (sheep kidney)[76] with a calculated molar mass of 34.937 Da, and 305 AAs (*Torpedo californica*).[77] Due to the fact that glycosylation varies between animals and tissues, a detectable variation in total molar mass has to be expected.[78] With this technique and its success, further sequences of α and β subunits from several other tissues were published in 1986: α,β pig kidney,[79] β rat brain,[80] β human tumor cells.[81] Thereafter, numerous additional sequences followed in quick succession.[82,83] Whole families of Na,K-ATPase genes were identified and their transcriptional competence confirmed.[84,85] In 1987 the cloned cDNAs of both subunits from *Torpedo californica* were used to produce mRNAs by transcription in vitro. These were transferred by microinjection into *Xenopus* oocytes, expressed and trafficked functionally into the cell membrane.[58] It was shown that both subunits were necessary for correct folding and transfer to the cell membrane. With an increasing variety of molecular-biological tools that became available, the vast field of amino-acid mutations and protein expression in "foster cells", such as oocytes,[86] yeast,[87] or various cell lines,[88,89] became accessible. This leap in development opened a completely new dimension of experimental investigations, and they enabled us to gain a major part of our contemporary understanding of function and structure function relationship (see subsequent part II).

With the knowledge of the gene sequence not only of the Na,K-ATPase but also of other ion motive ATPases a comparison of their sequences revealed that there is a whole family of ATPases which were named P-type ATPases because of their covalently phosphorylated intermediate.[90] Throughout the years increasingly complex phylogenetic trees of the P-type (super) family have been compiled[91,92], in which the Na,K-ATPase belongs to the type-II ATPases and its closest family members are the H,K-ATPase and the SR Ca-ATPase.

IV. INSIGHTS INTO THE STRUCTURE

Before the amino-acid sequence (or primary structure) became available, information on the structure of the Na,K-ATPase was mostly restricted to gross spatial features from electron-microscopical images[41,93] or from spectroscopic studies that allowed an estimation of the percentage of α helices and β sheets present in the protein at various substrate compositions.[94] More detailed concepts on the secondary structure were proposed after the tool of hydropathy analysis of the amino-acid sequence of proteins was introduced by Jack Kyte in 1982[95] and applied to the α subunit of the Na,K-ATPase. Between 1985 and 1994 many groups tried to derive from the primary structure a spatial organization of the protein in the membrane and especially the number of transmembrane segments. The count varied between 6 and 10 membrane-embedded α helices.[53,74,75,79,82,83,96] The proposal of an odd number of transmembrane segments, 7 or 9,[79,97] was quickly ruled out, because experimental evidence was presented that both N and C terminus of the α subunit were located on the cytoplasmic side of the membrane.[96,98] Eventually, consensus was obtained at a count of 10 helices,[99] which was confirmed in the end, when detailed tertiary structures became available by X-ray structure analysis from crystals of the complete Na,K-ATPase.[100,101] As shown in Figure 3, two major cytoplasmic loops were identified between the second and third transmembrane segment with about 140 amino acids, and between the fourth and fifth transmembrane segment with about 440 amino acids. In the latter loop the phosphorylation site as well as the FITC and IAF binding sites were located, which play important roles for function and analysis of the enzymatic activity of the pump.[102-105]

In the case of the β subunit it was accepted from the beginning that this small protein has no more than one transmembrane segment. The larger extracellular part with the C terminus[76] carries the essential disulfide bridges and is glycosylated at three asparagines.[53,78]

For a long time the insight to gain understanding of the tertiary structure was confined to low-resolution data provided by electron microscopy, starting with the identification of knob-like structures with a diameter of about 45 Å.[107,108] A few years later a 'stalked knob' was resolved on the cytoplasmic side of the catalytic subunit of the Na,K-ATPase.[93] The next step was the investigation of vanadate-induced two-dimensional Na,K-ATPase

crystals[109] and a three-dimensional model reconstructed thereof,[110,111] as shown in Figure 4.

No further real improvement in the revelation of structural details was achieved until almost a decade later, the first structure of the SR Ca-ATPase was solved with a resolution of 2.6 Å.[112] The structure of this closely related enzyme was used for homology modeling of the α subunit of the Na,K-ATPase. The resulting structure proposals gained a lot of popularity and were used quite successfully to identify crucial amino acids as targets for mutation studies. Yet, another seven years had to pass until in 2007 the first original crystal structure of the Na,K-ATPase became available at a resolution of 3.5 Å in an E_2P-analogous conformation with two K^+ ions bound.[100] Two years later, another structure of the Na,K-ATPase in the same conformation became available at a resolution of 2.4 Å,[113] as well as another four years later a complex with a Mg^{2+} and a ouabain bound.[114] Since then, structures in the E_1 conformation with 3 Na^+ ions in their binding sites were resolved and published.[115,116] As already introduced in the analysis of the first Ca-ATPase structure, the cytoplasmic portion of the α subunit is subdivided into three domains named *N*, *P*, and *A*.[112] This organization was found similarly for all P-type ATPases studied so far. The *N* domain is the largest of the three domains and contains the nucleotide-binding site to which the Mg-ATP complex binds in a specific orientation that subsequently enables phosphorylation of the enzyme. The *P* domain includes the conserved aspartate that is phosphorylated by ATP. This domain is formed from two segments of the large cytoplasmic loop (between M4 and M5). The *A* domain is formed by the loop between transmembrane helices M2 and M3 and part of the sequence before M1. It is assumed to be an actuator that moves the phosphate hydrolysis machinery in and out of the active site by large rotational motions. A comparison of the crystal structures in the E2P and E1 conformation is shown in Figure 5. These represen-

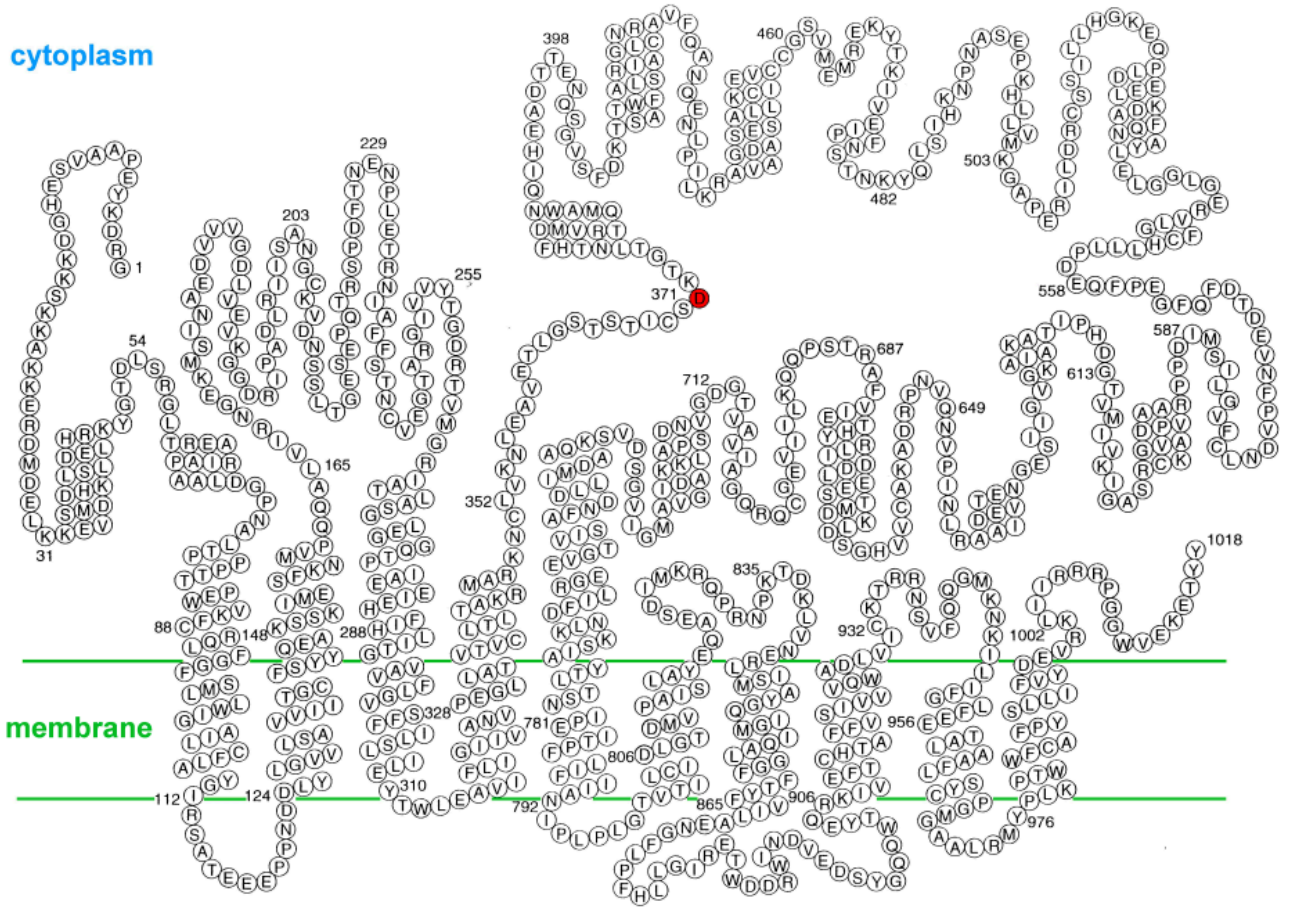

Figure 3. Secondary structure of the rat Na,K-ATPase α1 subunit with ten transmembrane helices. The high-lighted aspartate 371 is the amino acid phosphorylated by ATP. The drawing is adapted from Vilsen et al.[106] with permission.

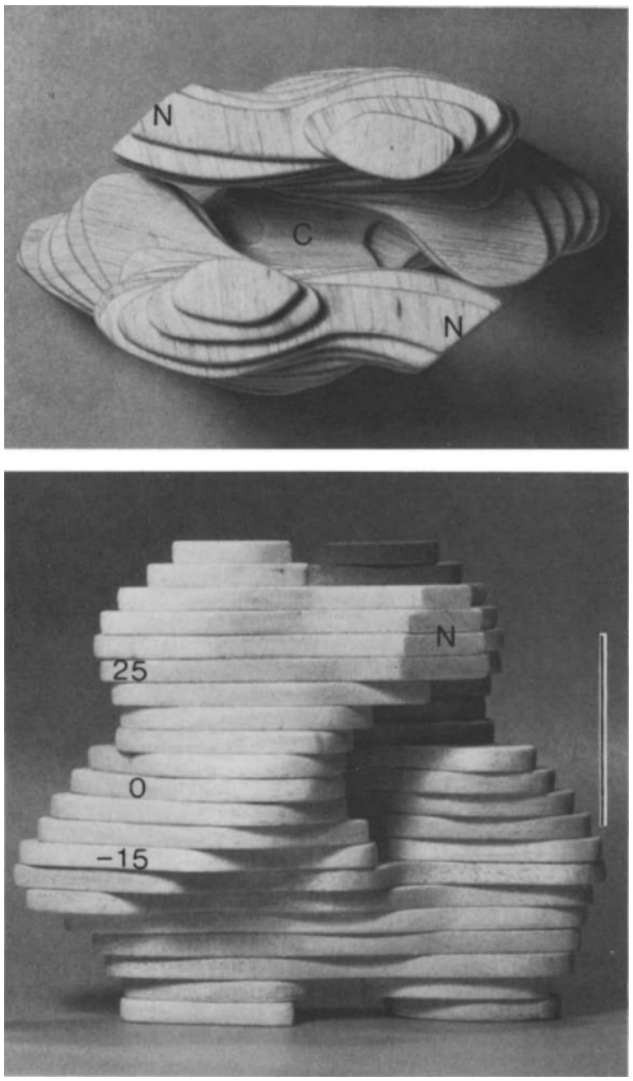

Figure 4. Reconstruction of a three-dimensional model of a Na,K-ATPase dimer from a tilt series of electron-microscopical images taken from a two-dimensional crystal of Na,K-ATPase in membrane fragments. Upper panel: top view, lower panel side view. The vertical bar indicates the assumed position of the lipid membrane, the cytoplasmic protrusion of the protein is on the bottom. (Figure taken from Ref. 109, with permission)

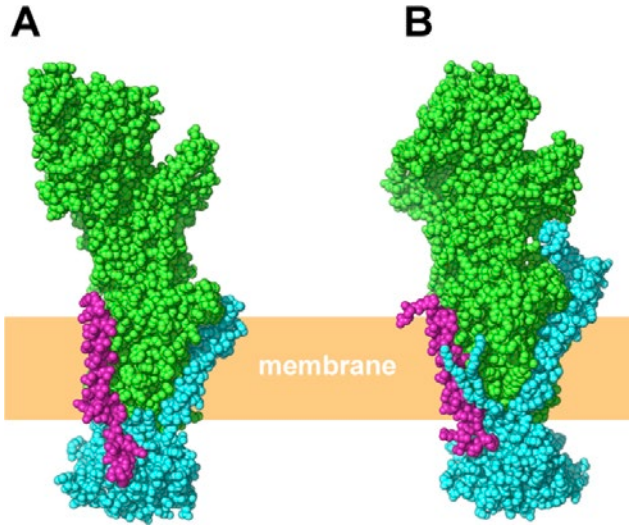

Figure 5. Crystal structure of the Na,K-ATPase in two conformations. The ion pump consists of the α (green), β (cyan) and an regulatory FXYD subunit (magenta). **A:** E_2 conformational state with 2 K^+ bound in a E_2P-like state in which phosphate is replaced by MgF_4^{2-} (pdb ID 2ZXE).[101] The analyzed crystal had a resolution of 2.4 Å. The enzyme was isolated and purified from shark rectal glands. It contains FXYD10 as regulatory subunit. **B:** Transition state of the Na,K-ATPase preceding the E_1P conformation with 3 Na^+ ions occluded after binding from the cytoplasmic side (pdb ID 3WGU).[115] The analyzed crystal had a resolution of 2.8 Å. The enzyme was isolated and purified from pig kidney. It contains FXYD2 as regulatory subunit.

tations of the Na,K-ATPase confirmed also that the structures derived by homology studies of the α subunit based on the SR Ca-ATPase structure have been rather well-suited. Furthermore, the original structures of the Na,K-ATPase revealed how the β and the additional regulatory FXYD subunit are connected to the α subunit. In combination with the biochemical and biophysical studies on the kinetics of the sodium pump, these structures with almost atomic resolution (and those in further different conformations that hopefully will come) are extremely useful to advance the comprehension of the molecular mechanism of enzyme and transport activity of the sodium pump.

For a long time a passionate discussion was carried out on the composition of the functional Na,K-ATPase, the protein's quaternary structure. The main opposing proposals were that under physiological conditions the ion pump consists either of a single αβ heterodimer or of an oligomer $(\alpha\beta)_n$ with n = 2 or even larger. This controversial issue was triggered by the rather early experimental finding that the Na,K-ATPase exhibits during the course of its catalytic activities two distinguishable affinities for ATP binding which were assigned to a high and low affinity site, accordingly.[33] This observation may be explained by three different concepts: First, a single αβ heterodimer has one ATP-binding site that changes its properties when the enzyme switches its conformation during the pump cycle.[34,117] The second proposal was that a single αβ heterodimer has two spatially different ATP-binding sites, one to perform the energizing enzyme phosphorylation while the other acts as a regulatory site, used to modulate the Na,K-ATPase activity,[118] a function found in numerous other ATP-controlled

enzymes. The third concept was that of an Na,K-ATPase oligomer, $(\alpha\beta)_2$, in which both heterodimers are (tightly) coupled to provide synergistic effects.[119] The experimental evidence collected over many years was multifarious and seemed often to be in favor of one proposal but rarely refuted the other(s).[120]

On one hand, it has been shown that an isolated, monomeric αβ was able to run the enzymatic reaction cycle and to occlude Na and Rb ions.[121-123] In this condition, however, there was no membrane present, hence no sidedness was given and ion transport through the Na,K-ATPase could not be proven. On the other hand, in membranes Na,K-ATPase molecules were frequently clustered, and $(\alpha\beta)_n$ complexes were found when isolated with mild detergents. It was easily possible to crosslink the subunits of two different pumps,[124] and in the presence of vanadate, and when the pumps were in a E_2 conformation, the emergence of long rows of dimeric two-dimensional crystals was detected.[125] Such a crystal formation was observed also in purified membrane preparations of Na,K-ATPase after a treatment with phospholipase A_2 by which the lipid content of the membrane fragments was reduced.[126] Extensive crosslinking studies were performed in the group of Amir Askari and, modulated by the chosen substrate conditions, they substantiated various links between α-α, α-β, and β-β subunits. Their interpretation of the results from their crosslinking studies led to the conclusion that the "minimum association state within the membrane must indeed be $(\alpha,\beta)_4$".[127] But no direct evidence was presented that furnished proof of a functional cooperation between αβ heterodimers. Robinson wrote in his book (p.160): "$(\alpha\beta)_n$ formulations were popular in the 1970s, but αβ was favored in the 1980s when *almost* all the observations were revised and reinterpreted and when new data favoring the αβ interpretation was reported".[1]

Conceptual discussions about the requirements of monomeric or oligomeric structures of the Na,K-ATPase are multifaceted. The finding that all reaction steps needed for enzyme and transport functions have been documented in the case of monomers confronts the frequently observed spatial arrangement of the Na,K-ATPase as patches of two or more αβ protomers in close neighborhood or even in a tight contact that would allow functional interaction. Why should such contacts be stabilized or maintained if they are not advantageous? And indeed, the list of published experimental findings is notably that resorts to functional interaction between two αβ protomers. For example, it was used to describe complex kinetic behavior detected in measured substrate dependencies. A first option would be that tight coupling of two protomers could promote substrate binding to one αβ and then modulate the interaction of the other αβ with a second substrate. Since in both αβ protomers the pump cycles can (or will) be out of phase, one substrate may affect the interaction with a different one, even on the other side of the membrane. With such additional degrees of freedom and additional selectable parameters that are provided by modeling functional coupling, a complex kinetic behavior may be represented much easier by mathematical reaction schemes.[128] Furthermore, instead of a permanent tight coupling between two αβ protomers, it may be proposed that an ATP-dependent aggregation and separation of the $(\alpha\beta)_2$ oligomer exists which leads to different turnover rates when acting as $(\alpha\beta)_2$ or (temporarily) separated αβ under control of the ATP concentration in the cell.[129] It is tempting to see how more complex reaction schemes result in equation systems that allow almost perfect fits of experimental results by mathematical modeling. However, such a success does not justify the reverse conclusion that the underlying model is the right one.

Another reason to ask for dimers of Na,K-ATPases is to consider synergistic effects. When the energetics of the ion transport in the sodium pump was analyzed in terms of basic free energies,[130] i.e. the amount of free energy needed or being released at each single (experimentally accessible) reaction step of the pump cycle, no 'power stroke' was found.[131] This fact implies that at no single reaction step of the pump cycle, a driving thrust of energy was released to the "out-side world". The steps of the pump cycle consuming most energy were found to be the dislocation steps for both K^+ ions from their binding sites to the cytoplasm in the E_1 conformation. The absence of a power stroke could have its origin, on the one hand, in the mismatch of the application of our comprehension of macroscopic motors to molecular machines. But on the other hand, it could be also the consequence of an energetic coupling between two ion pumps running around their cycles out of phase in a way that energy production and consumption in coupled protomers compensate each other largely like in coupled chemical reactions.

For a comprehensive view of quaternary-structure formation it is, however, necessary to consider also the fact that the ion pumps are embedded in a lipid bilayer which is in a liquid-crystalline phase. This two-dimensional liquid has a life of its own that is under control of entropy. The various species of lipid molecules forming the cell membrane differ in the nature of their polar head groups as well as in the lengths of their hydrophobic fatty-acid chains and their number of double bonds. Especially the latter properties affect the degree of membrane fluidity. The presence of large rigid particles

such as integral membrane proteins is able to promote separation into liquid-disordered and liquid-ordered phases. Studies of the molecular interaction mechanisms between proteins and lipids have shown that the match between the thickness of the hydrophobic domain of the integral protein and the bilayer core leads to an accumulation of specific lipids around the protein molecules.[132] This observation led to the conclusion that "the proteins end up in the membrane that provides for the best hydrophobic matching".[132] In addition, Na,K-ATPase has been found clustered in stable and rigid lipid rafts 'floating' in the membrane. Those rafts form spontaneously in membranes of (at least) ternary lipid mixtures.[133] Based on those findings an alternative line of arguments to explain clustering the Na,K-ATPase in cell membranes can be based on purely entropic effects that lead to an aggregation of Na,K-ATPase molecules without any functional coupling. But again, counter-arguments may be provided by the observation that in gastric acid-secreting cells an association between the K^+-Cl^- cotransporter-3a and the α1 subunit of the Na,K-ATPase was formed spontaneously in lipid rafts when cholesterol was present, and upregulated ATPase activity could be detected in a strictly cholesterol-dependent manner.[134] So far nothing is known about the molecular mechanism of the reported effects and about the role of cholesterol in the interactions between both ion transporters. Obviously, we still do not have all the pieces of a puzzle to recognize the complete raison d'être of Na,K-ATPase aggregation. "The paper is (still) open for discussion."

So far we have followed the trace of the discovery of the Na,K-ATPase, what it is good for and what it looks like, but the crucial question, how it works remains still open. Countless scientists contributed during decades to find answers to this question since this ion pump was identified and since protein preparations became available to work hands-on with physiological, biochemical and biophysical methods. An overview on this research will be presented in a subsequent article, "Finding Na,K-ATPase – From fluxes to ion movements."

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