J. Membrane Biol. 115, 109–121 (1990)

The Journal of
Membrane Biology

Topical Review

Subunit Assembly and Functional Maturation of Na,K-ATPase

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Introduction

All eukaryotic animal cells maintain high intracellular K and low intracellular Na concentrations, a situation which is reversed in the extracellular medium. It is the Na,K pump or its molecular equivalent the Na,K-ATPase located in the plasma membrane which is responsible for the creation and preservation of these ionic gradients (Fig. 1). This enzyme is able to transport Na and K against the electrochemical gradients existing across the plasma membrane by utilizing the energy of the hydrolysis of ATP to ADP [for review, *see* 55]. The potential energy of the Na gradient created by the Na,K pump is used by many transport systems to move, e.g., phosphate, amino acids, or glucose into the cell or to remove protons or Ca from the cells. Thus, ultimately, the Na,K pump is responsible for a cell assuming not only its basic, but also its specialized functions, namely the regulation of intracellular pH, regulation of cell volume, the propagation of nerve impulses, muscle contractions, uptake of nutrients, etc. [for review, *see* 75].

In view of the prominent physiological role played by the Na,K-ATPase, the understanding of how this pump works and how it is regulated in response to changing physiological demands is a major concern of cell biology [for review, *see* 118].

One particular aspect of this topic concerns the question of whether the protomeric organization of the Na,K-ATPase is of importance for the functional cellular expression of this enzyme.

We are indeed confronted with a particular situation. The Na,K-ATPase in its purified form is composed of two subunits, an α-subunit and a glycosylated β-subunit (Fig. 1). The α-subunit carries all presently known functional properties of the enzyme, such as the ability to hydrolyze ATP, to bind Na and K or else cardiac glycosides, specific inhibitors of Na,K-ATPase [for reviews, *see* 3, 57]. The β-subunit, on the other hand, has not yet a definite role assigned, though its presence has been identified in crystalline Na,K-ATPase [85, 90, 152] and in a minimal functional enzyme unit consisting of an α-β protomer [8, 17, 56, 57, 100]. In addition, the β-subunit is a glycoprotein, and the role of the sugar moiety remains obscure as for many other glycoproteins.

The elucidation of the functional interplay between the glycosylated β-subunit and the catalytic α-subunit has long been hampered by the only availability of purified enzyme preparation as experimental models. Indeed, due to the strong association of the two subunits with the lipid bilayer of the plasma membrane, which cannot be broken by detergents without destroying the functional integrity of the enzyme, and due to the higher proteolytic sensitivity of the α-subunit compared to the β-subunit, which does not permit a selective proteolytic manipulation of the β-subunit [61], it was so far impossible to assess the potential implication of the β-subunit in the catalytic cycle of the Na,K-ATPase. That the interaction between the α- and the β-subunit might be important in the hydrolyzing function of the Na,K-ATPase was nevertheless inferred from the observation that reduction of a disulphide bond existing between Cys 158 and Cys 175 of the β-subunit [97] results in loss of enzyme activity of the purified enzyme [66]. In addition, disulphide bond reduction of the β-subunit can be prevented by Na and K binding to the α-subunit in parallel with protection of the enzyme activity, lending further support for a functional α–β interaction [67].

The recent preparation of molecular probes in the form of α and β cDNA clones provide us now with new possibilities to study the importance of the

Key Words ($Na^+ + K^+$)-dependent adenosine triphosphate phosphorylase · protein biosynthesis · protein folding · protein oligomerization · posttranslational processing · glycoproteins, protein sorting · protein conformation · multi-subunit membrane proteins · protein degradation

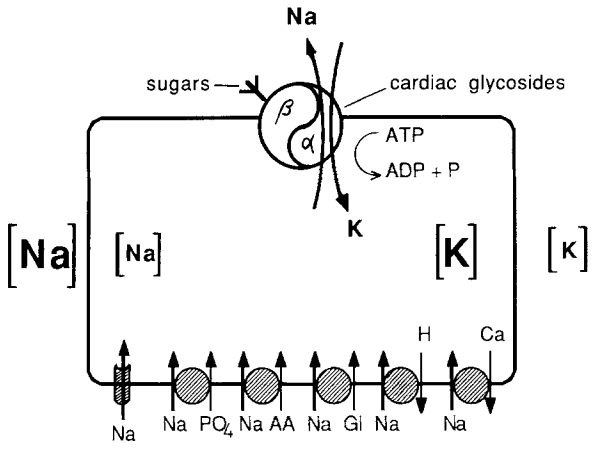

Fig. 1. Na,K-ATPase: structure and function. The Na,K-ATPase found in all eukaryotic cells of higher animals moves Na and K against their electrochemical gradient and provides the necessary energy for the proper functioning of many Na^+-dependent transport systems. The enzyme is composed of a catalytic α-subunit carrying the main functional properties and a glycosylated β-subunit

subunit assembly for the Na,K-ATPase function. At last we will be able to assess such essential aspects as the dynamic interplay between the subunits which is likely to occur during the early cellular life of the two subunits. In this review we would like to evaluate the functional consequences for the enzyme issuing of the assembly of the two subunits in the light of information on biosynthesis, membrane insertion, posttranslational maturation and cell surface expression of the Na,K-ATPase.

Structural Information Helps to Understand the Functional Properties of Na,K-ATPase Subunits

α-Subunit

Knowledge of the amino acid sequence of the cloned cDNAs has complemented our information on the membrane organization of the α- and β-subunits of Na,K-ATPase and ultimately will help us to understand the molecular mechanism of active cation transport [for reviews, *see* 57, 127].

Hydropathy analysis [126] confirms earlier data obtained by chemical labeling of proteolytic fragment [60] or by antibody reactivity of purified enzyme [31] that the α-subunit has several transmembrane segments and a large cytoplasmic loop containing the phosphorylation site and the ATP binding site. A number of conformation-dependent proteolytic sites which are exposed to the cytoplasmic side of the membrane have been determined and by inference locate the N-terminus of the α-subunit to the cytoplasmic domain [58]. Seven transmembraneous segments with the C-terminus of the α-subunit positioned to the extracytoplasmic domain have been inferred on the basis of immunoreactivity with intact cells of an antibody against a synthetic decapeptide of the C-terminus of the α-subunit [102].

Throughout the animal kingdom the amino acid sequence of the α-subunit is highly conserved. About 80% similarity is found between the α-subunit of invertebrates such as *Drosophila* [74], of fish [64], of amphibia [144], of birds [139], and of mammals [103, 104, 126].

In addition, amino acid sequence and membrane topology of the α-subunit of Na,K-ATPase are similar enough to transport ATPases of the aspartylphosphate type (P-type ATPases; for review *see* 106), the gastric H,K-ATPase [125], the sarcoplasmic reticulum Ca^{2+} ATPase [83], the plasma membrane Ca^{2+} ATPase [123, 143], the H-ATPases of yeast [122] or *Neurospora* [2] and the K-ATPase of *Escherichia coli* [48] or *Streptococcus faecalis* [132], to predict that the Na,K-ATPase belongs to a family of related genes, probably all deriving from a common ancestor [for review, *see* 57, 127].

Information on amino acid sequences have permitted mapping certain functional domains on the α-subunit. By comparing tryptic peptides labeled with ATP analogs to the deduced amino acid sequence of cDNAs, it could be shown that the various transport ATPases form a common structure at the catalytic site for ATP hydrolysis in the central cytoplasmic loop of the polypeptide [29, 96, 101].

Finally, with the help of chimeric or point-mutated cDNAs, the domains that determine the species differences in the sensitivity of Na,K-ATPase toward cardiac glycosides have been identified [95, 110]. Indeed, charged amino acid residues exposed in the first N-terminal extracytoplasmic loop are likely to be responsible for the fast dissociation of cardiac glycosides, characteristic for ouabain resistance [110].

At least five different sequences related to the Na,K-ATPase α-subunit have been detected and partially characterized in the human genome [105, 128, 135, 136, 147]. Actually, at the protein level three isoforms have been identified which differ, among other characteristics, in their tissue distribution, sensitivity to cardiac glycosides, affinity for Na and K, conformational equilibrium between the E_1 and E_2 state and in hormonal regulation [for review, *see* 137].

β-Subunit

Hydropathy analysis of the amino acid sequence deduced from cDNAs indicate that in contrast to the α-subunit, the β-subunit has a short cytoplasmic tail, one transmembrane segment and a large extracytoplasmic domain containing three to four glycosylation sites [9, 65, 94, 124]. This transmembrane topology is consistent with earlier findings from immunochemical [30, 44] and chemical labeling [21] as well as from in vitro translation studies [39].

Amino acid sequence similarity of the β-subunit is high (over 90%) within mammalian species [9, 65, 86, 103, 124, 140] but, in contrast to the α-subunit, decreases in amphibia (65% similarity with mammalian β-subunit [144]) or fish (61% similarity with mammalian β-subunit [94]).

The definition of the molecular organization of the β-subunit did not yet bring us any closer to an understanding of the β-subunit function. However, a newly emerging topic, namely the identification of β-subunit isoforms, provides us with a new though yet indirect argument that not only α-isoforms but also β-subunits are likely to determine the functional properties of the Na,K-ATPases.

It has long been known that the electrophoretic mobility of the β-subunit from different tissues differ on SDS-polyacrylamide gels [for review, *see* 36]. Multiple *m*RNA species of the β-subunit were indeed identified in kidney and brain, but restriction mapping and DNA sequencing suggested that the same β-subunit form is expressed in the two tissues [149]. The data indeed suggested that the apparent difference in the molecular mass of the glycosylated β-subunit in different tissue was mainly due to tissue-specific differences in oligosaccharide processing.

Several other data, however, still speak for the existence of multiple isoforms of the β-subunit. On the basis of peptide mapping, Hubert et al. [54] indeed provided evidence that the liver might contain β-subunit variants. These data are consistent with hybridization analysis, which failed to detect β-subunit mRNA in the liver [86] or which show a great discrepancy in the abundance of α- and β-*m*RNA in this tissue [109]. In addition, other hybridization studies revealed a complex pattern of tissue expression of β-*m*RNA which was distinct from any of the α-subunit gene products [26, 99]. Finally, monoclonal antibodies to the β-subunit revealed differences in the tissue distribution of β-subunit [28].

Most recently, more direct evidence for the existence of at least two β-subunit genes has been provided by the isolation of a putative β2 cDNA from brain and liver, which exhibits a high degree of similarity with the primary and secondary structure of the main β1 isoform but whose mRNA transcripts are distributed in a tissue-specific and developmentally regulated fashion distinct from the β1 *m*RNA [84].

In the context of our search for a functional role of the β-subunit, it is interesting to compare the structural organization of the Na,K-ATPase to other P-ATPases. Definitely, the presence of a glycoprotein component in the functionally active enzyme is best defined in Na,K-ATPase, but some evidence exists that other ATPases might be associated with a β-subunit equivalent. Thus, it has been suggested that a glycoprotein in the sarcoplasmic reticulum might be involved in regulating the coupling of ATP hydrolysis to Ca^{2+} transport by the Ca^{2+}-ATPase [77]. In addition, a 60–80 kDa glycoprotein has recently been identified and isolated from H,K-ATPase enriched membrane preparations [98]. Finally, the KdpC protein, a 190 amino acid subunit of the K-ATPase, shows a certain sequence homology with the β-subunit of Na,K-ATPase and a similar membrane topology [124]. Though these data point to a general significance of a glycoproteinic component associated to P-ATPases, it is actually too early to speculate on a common possible role of these structural elements.

A final aspect of the functional implication of the α-β organization of Na,K-ATPase concerns the question whether the enzyme in vivo exists as a single α/β or rather as multiple α/β protomer units. For the Na,K-ATPase, this question has not yet been definitely resolved [for review, *see* 85, 115]. Interestingly, studies on H,K-ATPase suggest that partial enzyme functions may reside in a single monomer but that transport activity might necessitate subunit interactions [111]. On the other hand, the monomeric Ca^{2+}-ATPase, as the protomeric αβ-unit of Na,K-ATPase constitutes the minimal unit required for all functional properties [for review, *see* 57]. However, recent data suggest that Ca^{2+}-ATPase activity might be stimulated by self-association of enzyme molecules at high concentrations and that monomer oligomerization might be the basis of enzyme regulation by calmodulin [70].

After this basic information on the structural and functional organization of the Na,K-ATPase, we would like to concentrate now on what we can and have learned so far on the structure-function relationship of the α-β protomer by (i) looking at the biosynthesis, the assembly and the posttranslational maturation of the two subunits in cellular and acellular systems and (ii) exploiting the cellular biosynthesis machinery of a given cell to follow the recon-

stitution of a functional enzyme from foreign α- and/or β-subunits.

Biosynthesis and Membrane Insertion of α- and β-Subunits of Na,K-ATPase

The general model of the biosynthesis and membrane insertion of membrane proteins destined for the plasma membrane or the endoplasmic reticulum (ER) of secretory proteins and of lysosomal proteins involves the following basic steps [for reviews, *see* 53, 113, 116, 129, 130]: (i) synthesis is initiated on free ribosomes in the cytoplasm; (ii) a signal sequence, which is located at or near the amino-terminus of the nascent chain, is recognized by a signal recognition particle (SRP) which might transiently inhibit translation, (iii) the ribosome-SRP complex then binds to the docking protein in the ER membrane whereby SRP is removed and the translational arrest is released; (iv) the signal sequence is handed over to a signal sequence receptor, an integral glycosylated membrane protein; (v) the nascent polypeptide chain is translocated across the ER membrane by a yet unknown mechanism; (vi) the signal sequence is or is not cleaved in the ER lumen, and nascent polypeptides might acquire N-linked core sugars.

Though not yet established in all details, the available information is consistent with the notion that both subunits of Na,K-ATPase are synthesized and anchored in the membrane as other membrane proteins [Fig. 2; for review, *see* 36]. Namely, the β-subunit of Na,K-ATPases requires the signal recognition particle for membrane insertion [36, 63] and it acquires its core sugars during translation [39]. According to its transmembrane topology and its mode of insertion, the β-subunit belongs to the class II integral membrane proteins, which comprises proteins such as the rat asialoglycoprotein receptor, the human IgE receptor, the human transferrin receptor, etc. [for review, *see* 46]. Such proteins are characterized by one membrane-spanning segment which functions both to anchor the mature protein in the membrane and in biosynthesis as an internal uncleaved signal sequence [133]. This latter characteristic yields a membrane topology with the N-terminus at the cytoplasmic side. Kawakami and Nagano [63] have recently shown that in the β-subunit of Na,K-ATPase, the hydrophilic N-terminus is indeed not required for membrane insertion while a 16 amino acid stretch in the membrane-anchor domain is a necessary and sufficient signal for this process.

Besides coreglycosylation, the β-subunit of Na,K-ATPase is most likely subjected to at least one other covalent modification, namely the formation of its disulphide bridges which are three in

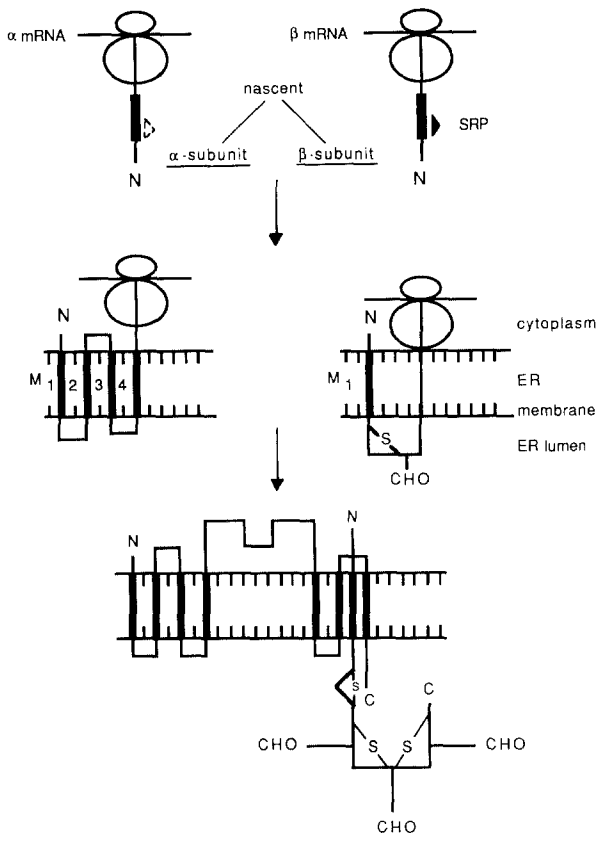

Fig. 2. Biosynthesis and membrane insertion of α- and β-subunits of Na,K-ATPase. Both α- and β-subunits are coordinately synthesized and inserted into the membrane of the endoplasmic reticulum (*ER*) during their translation. While the synthesis and membrane insertion of the β-subunit depends on the interaction with a signal recognition particle (*SRP*), this requirement has not yet been demonstrated for the α-subunit. During synthesis, the β-subunit acquires core sugars (*CHO*) and disulphide bonds. Soon after completion, the two subunits assemble into stoichiometric complexes. For further details, *see* text. *M* = putative transmembrane segments

number in the mature enzyme [68]. Disulphide bridge formation has been shown to occur in the lumen of the endoplasmic reticulum either during or soon after completion of secretory and cell-surface protein synthesis [for review, *see* 84] and is believed to be catalyzed by the enzyme protein disulphide-isomerase [10].

The α-subunit of Na,K-ATPase is a complex membrane protein of the class IV with more than one membrane-spanning segment in the final structure. All P-ATPases as well as proteins such as adrenergic receptors, subunits of the acetylcholine receptor or the human glucose transporter fall into this class [for review, *see* 46]. Their amino and carboxy termini can be on the same or on the opposite sides of the membrane and the signal sequence can

be cleaved or not. Membrane integration of such proteins most likely occurs by segmental units, namely by successive hydrophobic units which function as alternating insertion and stop-transfer sequences [for review, *see* 130]. Recently, evidence has been provided that only the first hydrophobic domain in multispanning membrane proteins needs to be a signal sequence interacting with SRP and that subsequent translocation events do not require SRP [145].

As the sarcoplasmic Ca^{2+}-ATPase [114] or the H^+-ATPase of *Neurospora* [1], the α-subunit of Na,K-ATPase lacks a cleavable N-terminal signal sequence but yet appears to be cotranslationally inserted into membranes [13, 33, 39]. The only detectable posttranslational modification of unknown function is the removal of the first five amino acids of the α-subunit of various species [103, 126]. A SRP-dependent membrane integration, similar to the β-subunit, has not yet been demonstrated for the α-subunit, but internal membrane insertion signals are likely to be included in the first four transmembrane segments [51].

The interesting hypothesis has been put forward that the β-subunit might function as a receptor for the α-subunit to be anchored in the membrane [49]. Even though α-subunits as well as β-subunits can be inserted into microsomal membranes in vitro independent of each other (39, 43] in the intact cell, several observations indeed point to a rapid interplay between the two subunits. Thus, synthesis of α- and β-subunits are highly coordinate, both in basal conditions [37, 141] or during upregulated synthesis induced by hormones [37] or low K [7], and stoichiometric α-β complexes are rapidly formed during or soon after synthesis [141].

In view of these data, it is tempting to speculate that association of the two subunits might be an important event in some early maturation process of the Na,K-ATPase. In the following chapter we will discuss what we have learned so far on the modifications the α- and the β-subunits are subjected to after their synthesis in the endoplasmic reticulum and during their intracellular transport to the plasma membrane.

Both α- and β-Subunits of Na,K-ATPase are Subjected to Posttranslational Processing

Structural Maturation of the α-Subunit is Required for the Expression of its Functional Properties

By using a controlled trypsinolysis assay, it could be shown that the α-subunit synthesized in vitro in a reticulocyte lysate supplemented with rough microsomes was inserted into membranes since it was partially protected against proteolysis [39]. However, only one small trypsin-resistant fragment could be recovered, indicating that the α-subunit was in a very loose configuration permitting extensive attack by trypsin. Since in intact cells, pulse-labeled for very short time periods, the α-subunit exhibited the same characteristics (Fig. 3), this result was not an artifact of the in vitro translation but indicated that the newly synthesized α-subunit is indeed highly trypsin sensitive [39].

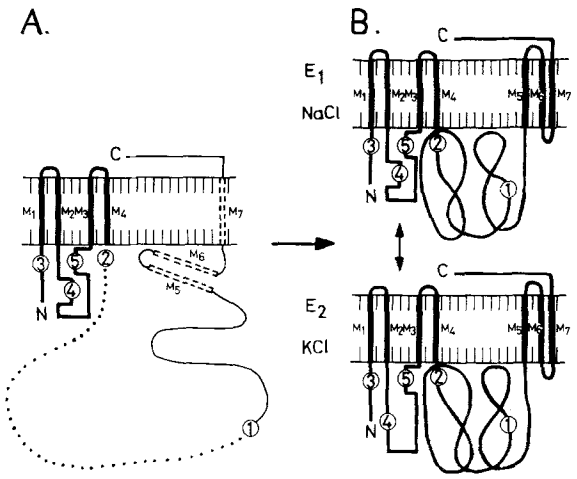

Fig. 3. Model for the structural and functional maturation of α-subunit of Na,K-ATPase. (*A*) Immediately after synthesis, the α-subunit is structurally in a relaxed form, as suggested by its high trypsin sensitivity. Trypsinolysis, in the presence of both Na and K, gives rise to two small fragments: a 27.5-kDa soluble fragment through the attack of tryptic bonds *1* and *2* and a membrane-bound fragment through the attack of tryptic bonds *1* and *3*. The structural organization of the C-terminus is not determined, as indicated by the thin line. (*B*) Within 20 min after synthesis, the α-subunit is structurally modified as suggested by an increased trypsin resistance. Tryptic bond *1* and *2* are occluded most likely by a rearrangement of the large cytoplasmic loop. In parallel, the α-subunit acquires the ability to perform cation-dependent conformational changes, which can be visualized by a different tryptic pattern in the presence of Na and K. Tryptic site *3* is exposed in the presence of Na or K. Tryptic bond *4*, in the first cytoplasmic loop is K^+ specific, and its exposure gives rise to an 85-kDa tryptic fragment. Tryptic site *5* is revealed with high trypsin concentrations. In the Na condition, it is produced after cleavage of site *3*, in the K condition it is produced after cleavage of site *4*. M_1–M_7 = putative transmembrane segments

Controlled trypsinolysis has frequently been used to study the structural rearrangements occurring during conformational transitions of purified active Na,K-ATPase [for review, *see* 59] and Ca^{2+}-ATPase [4, 153]. By combining a pulse-labeling protocol with a controlled trypsinolysis assay and immunoprecipitation, it could be shown that in the intact cell, the α-subunit is subjected within 20 min-

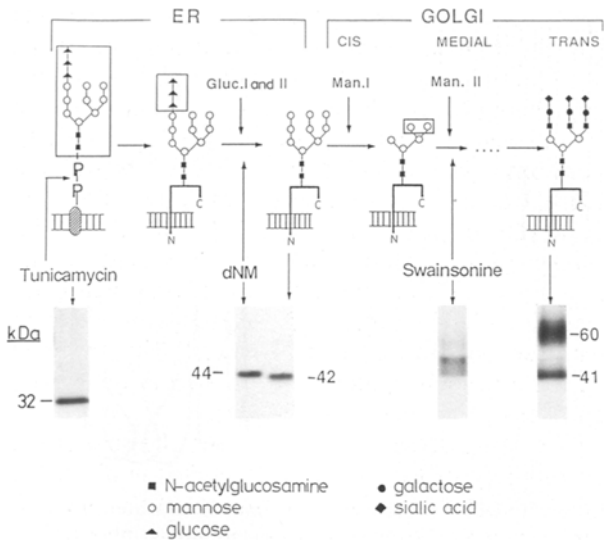

Fig. 4. Posttranslational processing of the β-subunit in the presence and absence of glycosylation inhibitors. During synthesis, the β-subunit acquires core sugars from a high mannose dolichol precursor as other N-linked glycoproteins. The sugar moiety is trimmed through the action of glucosidases (*gluc I and II*) and mannosidases (*man I* and *man II*) during the transport of the β-subunit from the ER to the Golgi compartment. In a *trans*-Golgi compartment, the β-subunit acquires complex-type sugars, a process that yields the mature glycoprotein expressed at the plasma membrane. For further details, *see* text. Glycosylation inhibitors such as tunicamycin, interfere with the synthesis of the dolichol precursor, leading to the formation of the nonglycosylated β-subunit (32 kDa). Deoxynojirimycin (*dNM*) inhibits glucosidases I and II, which prevents the removal of the three distal glucose residues. The β-subunit accumulated in the presence of dNM thus shows a higher molecular mass (44 kDa) than the coreglycosylated β-subunit (42 kDa) produced in the absence of the drug. Swainsonine inhibits mannosidase II, leading to incomplete trimming of mannose residues. Complex type glycosylation only occurs on the fully trimmed branch of the glycomoiety and, in consequence, a hybrid glycosylated β-subunit with a molecular mass between the trimmed (41 kDa) and the fully glycosylated (60 kDa) β-subunit accumulates in the cell. The β-subunit forms shown are immunoprecipitates from TBM cells (derived from the toad urinary bladder)

utes after synthesis to a structural maturation, as reflected by an increased trypsin resistance. In parallel, the α-subunit acquires the ability to change its conformation in response to Na and K as demonstrated by the production of cation-specific tryptic fragments [38] (Fig. 3).

Functional maturation of the α-subunit during intracellular transport is supported by experiments by Caplan et al. [12] who showed that about 10 minutes are required for the newly synthesized enzyme to be able to bind ouabain in an ATP-dependent fashion. Since intracellular routing of Na,K-ATPase from its site of synthesis to a distal Golgi compartment takes about 45 minutes [38, 141], these results clearly indicate that the α-subunit acquires at least some of its functional properties at the level of the endoplasmic reticulum or proximal Golgi, but definitely before it reaches the plasma membrane.

The Enigma of the Functional Role of β-Subunit Glycosylation

The β-subunit is a N-linked glycoprotein and as such subjected to extensive co- and posttranslational processing during its synthesis and intracellular routing to the plasma membrane. As outlined in Fig. 4, for all N-linked glycoproteins, processing in eurkaryotic cells starts with the transfer of a core sugar ($Glc_3\ Man_9\ GlcNA_2$) from a dolichol precursor to susceptible asparagine residues on the nascent polypeptide in the ER lumen. This high-mannose form, which is susceptible to endoglycosidase H (Endo H) digestion, rapidly loses the three terminal glucose residues by the action of glucosidases I and II residing at the level of the ER. During the transport through the ER and the Golgi, the glycoprotein is further trimmed by ER and Golgi mannosidases until it reaches medial or distal Golgi compartments where complex sugars are added to the glycoprotein. The mature form is insensitive to Endo H digestion [for reviews, *see* 50, 69, 119].

Due to differences in electrophoretic mobility and in Endo H sensitivity of the various glycoforms, this general scheme of glycosylation processing can easily be illustrated for the β-subunit of Na,K-ATPase in intact cells metabolically labeled for different times [39, 141, 150] (Fig. 4). On the other hand, very little is known on the actual content and specific sugar composition of the different glycoforms. The composition of the core sugars of the β-subunit is likely to be of the high-mannose type, which is identical in all glycoproteins. Species differences in sugar content in the coreglycosylated forms might, however, arise from differences in the number or in the glycosylation frequencies of the necessary N-glycosylation sequence Asn-X-Ser/Thr on the polypeptide bone [for review, *see* 148]. Mammalian species such as sheep [124], dog [9], pig [103], human [65] and amphibia such as *Xenopus* [144] have three, while fish [94] and probably amphibia such as *Bufo marinus* [150] have four of these N-glycosylation consensus sequences in the β-subunit of the β_1 type. In mammalian β_1-subunit [88, 141] as well as in *Xenopus laevis* β_1-subunit (unpublished observation) all of the three potential N-glycosylation sites appear to be glycosylated. Interestingly, in the recently described rat and human β_2-subunit cDNAs, seven and eight potential glyco-

sylation sites, respectively, were identified [84]. However, since the corresponding proteins have not yet been identified, nothing is known of their actual glycosylation pattern.

While the composition and content of the β_1-subunit core sugars appear to be quite homogeneous and predictable, the structure of mature glycomoieties is highly variable as judged by total sugar analysis [91, 107, 108], or even in different organs of the same species as judged by their differences in electrophoretic mobility [36]. This phenomenon is not unique to the β-subunit of Na,K-ATPase, but true for all glycoproteins. Indeed, production of the final glycan unit of a given protein depends not only on the particular set, the specificity and relative activity of processing enzymes in different cells, but also on the protein matrix itself, which determines the microenvironment of each glycan and thus its processing [for reviews, *see* 121, 148]. In consequence, a single glycoprotein with identical core-sugar structures might contain different glycan structures after completion of processing.

The potential of these complex and highly diversified carbohydrate structures to encode specific information is evident. Research on the role of sugar moieties on glycoproteins has indeed provided us with a large body of information but the picture remains highly complicated. The spectrum of proposed glycan functions ranges from stabilization of protein structure to specific signaling for intracellular transport [for review, *see* 69]. Perhaps the most promising field of research concerns the role of glycomoieties as recognition signals, meaning that glycans can function as messages which are recognized by a counterpart and thereby modulate molecule-molecule, cell-molecule and cell-cell interactions. The importance of such processes in development and morphogenesis [for review, *see* 22] as well as in tumorogenesis and in the pathogenesis of other diseases is well documented [for reviews, *see* 32, 112, 134].

The role of the sugar moiety in the β-subunit of Na,K-ATPase is, as in many other glycoproteins, poorly understood. Complex sugars such as sialic acid or galactoses are probably not involved in the catalytic cycle of the enzyme since treatment with neuraminidase or galactose oxydase of the purified enzyme does not affect Na,K-ATPase activity [76, 107].

Several drugs have been described that interfere at different steps in N-linked oligosaccharide formation [for reviews, *see* 24, 25]. These inhibitors lead to the cellular accumulation of abnormal glycoproteins and thus have become valuable tools for studies on the role of oligosaccharide in glycoprotein function. In Fig. 4, three of these compounds have been listed, namely (i) tunicamycin (TM), which inhibits the synthesis of the dolichol precursor, leading to the synthesis and accumulation of a nonglycosylated corepeptide [for review, *see* 24]; (ii) deoxynojirimycin (dNM), which inhibits glucosidase I and II, preventing trimming and complex glycosylation [for review, *see* 25]; and (iii) swainsonine (SW), which inhibits mannosidase II, thereby preventing the trimming of one high-mannose branch. Complex type glycosylation can in this case only occur on the second fully trimmed mannose branch, which leads to hybrid glycosylated forms [for review, *see* 24, 25].

By using dNM and SW to study the role of the glycomoiety of the Na,K-ATPase β-subunit in the intact cells, it could be confirmed that the acquisition of complex sugars is not needed for the enzyme to express Na,K-ATPase activity [150].

The role of the complex-type sugars in the β-subunit of Na,K-ATPase thus remains a challenging problem for future research. In view of the increasing experimental evidence that complex carbohydrates are directly implicated in cell recognition processes [22] and the identification of molecules such as lectins [20] or carbohydrates themselves [23] that recognize carbohydrates on the counterpart cell, it is tempting to speculate that the complex-type sugars on the β-subunit might be specific recognition signals implicated in cell coupling. Ultimately, such a cell coupling through the β-subunit could lead to a fine regulation of the ionic milieu between cells.

After these speculations on a possible role of the complex carbohydrates on the β-subunit, we are finally left with the question whether coreglycosylation of the β-subunit has any impact on the catalytic function of the α-β complex. The answer is "no," since even complete inhibition of glycosylation with TM permits the expression of functional Na,K-pumps in the plasma membrane. Several groups have indeed observed that the sugar moiety of the β-subunit is not needed for the intracellular transport of the Na,K-ATPase to the plasma membrane [138, 141, 151]. In addition, the nonglycosylated enzyme is able to participate in transepithelial Na^+ transport in cultured cells [151] and exhibits very similar ouabain-binding capacity and ^{86}Rb transport activity as the fully glycosylated enzyme in *Xenopus* oocytes, microinjected with α and β *m*RNA [138]. These data are consistent with the observation that assembly of α-β complexes are not impeded by inhibition of glycosylation of the β-subunit [141].

Despite the apparent lack of a role in the functional maturation of the Na,K-ATPase, it is likely

that the sugar moiety of the β-subunit has an influence on the structural organization of the α-β complex. Though the half-life of the nonglycosylated Na,K-ATPase does not appear to be different from the glycosylated enzyme [141], the trypsin sensitivity of both the β- and α-subunits is higher in nonglycosylated enzyme complexes than in glycosylated complexes [150]. These results might indicate that core sugars shield the enzyme from proteolytic attack. However, this interpretation could only explain the higher trypsin sensitivity of the β-subunit but not of the α-subunit, which exposes all tryptic sites to the cytoplasmic and thus sugar-free side of the membrane. Since trypsin sensitivity of the two subunits is particularly pronounced in the newly synthesized enzyme, we might rather postulate that, as suggested for certain other glycoproteins (for reviews, *see* 42, 117], acquisition of core sugars during synthesis might be important to achieve a correct initial configuration of the β-subunit. It is possible that an improperly folded unglycosylated β-subunit might not be able to impose a stable structural organization to the α-subunit after its association.

Interestingly, trypsin resistance of both β- and α-subunits increases in the nonglycosylated enzyme en route to the plasma membrane. These data suggest that Na,K-ATPase is subjected not only to glycosylation-dependent but also to glycosylation-independent structural rearrangements during intracellular transport. Since the α- and β-subunits remain, however, always more trypsin sensitive in the nonglycosylated enzyme complex than in the core of fully glycosylated enzyme complex, we have to conclude that the nonglycosylated enzyme complex, which retains functional properties, is in a more relaxed form than the glycosylated enzyme [150]. So far, all studies concerned with the functional integrity of the nonglycosylated Na,K-ATPase have been performed under V_{max} conditions for substrates and ligands. Indeed, we need more detailed kinetic data on the transport properties of the nonglycosylated enzyme in order to definitely determine the importance of the core sugars in the enzyme properly functioning under physiological conditions.

Even in the absence of this information, however, the actually available data point to a role of the glycosylated β-subunit in a specific conformation conferred to the α-subunit rather than to a direct involvement of the carbohydrate moiety in the catalytic cycle of the enzyme. This hypothesis is supported by the intriguing observation that in TM-treated epithelial cells the accumulation not only of the newly synthesized nonglycosylated β-subunit but also of the α-subunit, itself not a glycoprotein, is significantly decreased in the absence of any notable effect on total protein, or on specific nonglycosylated protein synthesis [150, 151]. In view of the high trypsin sensitivity of the newly synthesized nonglycosylated β-subunit of Na,K-ATPase, the decrease in the cellular amount of the β-subunit in TM-treated cells might be explained by a rapid degradation of the polypeptide after its synthesis. Alternatively, efficient protein synthesis might be coupled to core glycosylation as has been suggested for certain other glycoproteins [45]. In any case, the reduction of cellular β-subunit in TM-treated cells further supports the hypothesis that core glycosylation essentially participates in the efficient cellular accumulation of the β-subunit.

The effect of TM treatment on the cellular expression of the α-subunit is more difficult to explain. Interestingly, a similar marked decrease was observed in the amount of the delta or Δ subunit (~80% of the wild type) of the acetylcholine receptor when expressed in the *Xenopus* oocyte concomitantly with the ACHR α-subunit mutated at the single N-glycosylation site [89]. It was suggested that the lack of N-glycosylation of the α-subunit may affect the assembly of the delta or Δ subunit. With respect to the phenomenon observed with Na,K-ATPase, the best guess is that, due to the diminished synthesis or degradation of the nonglycosylated β-subunit, the concomitantly synthesized α-subunit cannot adopt a stable membrane organization through its assembly to the β-subunit and is itself degraded. We can, however, not yet exclude the possibility that the synthesis of the two subunits is coordinated by a yet undefined linking factor which might be the β-subunit itself, or else, another glycoprotein, the accumulation of which could be decreased in TM-treated cells.

These latter speculations lead us now to the final topic of this review, namely the discussion of subunit assembly and its potential importance in the functional expression of Na,K-ATPase in the plasma membrane.

Assembly of α- and β-Subunits is Essential for the Expression of Functional Na,K-ATPase

Molecular biological strategies such as cell transfections with cloned cDNAs are limited in their usefulness to study the functional aspects of Na,K-ATPase assembly. One major obstacle is that all eukaryotic cells of higher animals synthesize and express Na,K-ATPase themselves. These endogenous Na,K pumps often impede the analysis of certain functional aspects of exogenous α- and β-sub-

units expressed in these cells. Yeast cells, which do not synthesize Na,K-ATPase but which show a great similarity in the biosynthesis and processing of membrane and secretory proteins, might become an attractive alternative to animal expression systems [52].

Despite the obvious disadvantages, studies using transfection of animal cells with cloned cDNAs or microinjection of *m*RNA into *Xenopus* oocytes have provided some interesting data. Thus, it turns out that functional hybrid α-β complexes are formed between exogenous α or β subunits and their cellular counterpart [139, 140]. Animal cells containing ouabain-resistant Na,K-ATPase express ouabain-sensitive α-β complexes when transfected with α cDNA [140], but not when transfected with β cDNA [140] derived from an ouabain-sensitive species. Similarly, ouabain-resistant Na,K-ATPase is expressed in a ouabain-sensitive cell after transfection with a rat α cDNA [27]. These data confirm the view that ouabain sensitivity of Na,K-ATPase is mainly determined by the α-subunit.

Interestingly, in *Xenopus* oocytes, efficient expression of functional pumps at the plasma membrane could only be observed after injection of both α and β *m*RNAs [93]. The fact that in *Xenopus* oocytes, exogenous α- and β-subunits cannot form functional hybrid pumps with endogenous subunits, in contrast to the situation in somatic cells, can partly be explained by the observation that *Xenopus* oocytes synthesize little β-subunit [40, and *see below*]. Alternatively, the assembly between *Torpedo* and *Xenopus* Na,K-ATPase subunits might be inefficient. In any case, the results presented by Noguchi et al. [93] clearly demonstrate that only an enzyme complex composed of α- and β-subunits becomes functionally active. Either subunit alone has no catalytic or transport activity.

Why is the coexistence of the α- and β-subunit essential to form an active enzyme? Is it needed to impose a specific configuration to the α-subunit necessary for the functional maturation and/or for the correct intracellular transport of the enzyme?

Though the number of well-characterized multisubunit membrane proteins is small and the structural diversity among them is great, a comparison between their subunit assembly process and its structural and functional consequences might be helpful to approach these questions. The best documented examples of multisubunit membrane proteins comprise homo-multimeric proteins composed of homologous subunits such as influenza hemagglutinin (HA; with a subunit combination of A_3), vesicular stomatitis virus G protein (VSV G, A_3) or hetero-oligomeric proteins composed of heterologous subunits such as membrane bound IgM and IgG ($[AB]_2$), class I histocompatbility antigens (AB), class II HLA-DL histocompatibility antigens (AB) and the nicotinic acetylcholine receptor (AChR, A_2BCD) [for reviews, *see* 14, 117].

Subunit assembly of most oligomeric proteins occurs at the level of the ER. Na,K-ATPase [141] shares this characteristic with, e.g., AChR [131], influenza HA [15, 41] VSV G protein [18, 71], immunoglobulins [14] and HLD-DR histocompatibility antigens [73]. For Na,K-ATPase it is not yet clear whether subunit assembly occurs during or soon after synthesis [141]. In other multisubunit proteins, assembly is most often a posttranslational but rapid event occurring within 7–10 min after synthesis [for review, *see* 117].

The interesting question then arises how the strictly defined subunit composition of a given multisubunit protein is established. No definite evidence has been provided for a strict coordination of subunit synthesis perhaps with the exception of type I procollagen chains, the mRNA of which form specific supramolecular assemblies with ribosomes [142]. In addition, no active process of assembly has yet been demonstrated. It indeed appears from studies with influenza HA, that newly synthesized monomers freely diffuse in the ER and that the assembly process depends mainly on random subunit collisions [117].

Since individual subunits often represent only a small fraction of total ER proteins [15, 18, 41], it is striking that assembly is indeed an efficient process. Experimental evidence suggests that in many multimeric proteins individual subunits are subjected to a conformational change either during or soon after synthesis which might allow subunits to recognize each other. By using conformation-specific antibodies, it could be shown that in VSV G protein [19] or in HA [16] locally folded structures form in the nascent monomeric subunits before trimerization. Similarly, IgG heavy chains [5] or the α-subunit of the AChR [for review, *see* 87] undergo significant folding prior to assembly into heterooligomers. Early co- or posttranslational events, e.g., glycosylation in VSV G-proteins [81, 82], formation of intrachain disulphide bonds in the α-subunit of the AChR [14] or in immunoglobulin light chains [5] or *cis-trans* isomerization of prolyl peptide bonds [34, 35] are potentially associated with initial correct folding of subunits.

By monitoring the changes in trypsin sensitivity of the newly synthesized polypeptide, a folding process occurring soon after synthesis could also be demonstrated for the α-subunit of Na,K-ATPase [*see above* and 38]. In parallel, the α-polypeptide acquires certain of its functional properties, namely the ability to perform cation-dependent configura-

tion changes [38] and to bind ouabain in an ATP-dependent fashion [12]. The question arises whether this rapid structural change of the α-subunit occurs before or after the assembly to the β-subunit. For the newly synthesized α-subunit of the AChR, it has indeed been shown that it undergoes a conformational change before subunit assembly, which permits the binding of the antagonist α-bungarotoxin [87]. On the other hand, however, association of the α-subunit with either γ- or Δ-subunit and with any two of the β-, γ- or Δ-subunits is necessary for the binding of acetylcholine and for the expression of channel activity, respectively [72].

That the conformational change observed in the α-subunit of Na,K-ATPase is rather a consequence of, than a prerequisite for subunit assembly, is supported by experiments performed in the *Xenopus* oocyte. In these cells, a large excess of α-subunit is synthesized over β-subunit [40]. The overexpressed α-subunits in oocytes remain highly trypsin sensitive, even after prolonged periods of metabolic labeling and thus differ from α-subunits in somatic cells, which are coexpressed with β-subunits and which rapidly acquire trypsin resistance [38]. In addition, trypsin resistance of the α-subunit in *Xenopus* oocytes can be induced by microinjection of β cRNA, indicating that association of exogenous β-subunit to the endogenous occyte α-subunit leads to a conformational change of the α-subunit [40].

While the mechanisms underlying the establishment of the final stoichiometric subunit relation in multimeric proteins are still poorly understood, it becomes increasingly clear that initial correct folding of monomers and subsequent assembly into oligomers are often a prerequisite for a protein to be transported from the ER [for review, *see* 79, 117]. The requirement for proper folding and assembly is at least partly due to the fact that misfolded proteins tend to accumulate in the ER and/or degrade rapidly. Indeed, in certain cases, resident ER proteins appear to recognize and to retain unfolded, but not folded, proteins in the ER. The best-documented case is the association of unassembled Ig heavy chains to BiP (for immunoglobulin heavy chain binding protein) which is only released when assembly with light chain is complete [6, 92]. If association to BiP is prevented by a mutation of heavy chains, free heavy chains are secreted [47]. In addition, BiP appears to retain mutated unfolded [41] or unglycosylated [16] influenza HA which cannot form trimers in the ER. These observations have led to the conclusion that the function(s) of BiP is (are) to facilitate subunit assembly and/or to retain abnormal proteins in the ER awaiting degradation [for reviews, *see* 79, 120]. On the basis of the observation that BiP recognizes and binds avidly in vitro to unfolded or incorrectly glycosylated polypeptides, but not to all nascent proteins in the process of folding, the idea has also been put forward that BiP might not solely bind aberrant proteins, but rather be involved in an attempt to correct their anomaly [62].

The presence of BiP might not be the sole means by which a cell monitors the proper folding of proteins. Indeed, mutants of VSV G protein that prevent folding and trimerization are retained in the ER in large aggregates [19] but are not associated to BiP [117]. Similarly, mutant molecules of class I histocompatibility molecules unable to leave the ER appear not to be bound to BiP [146]. Finally, in some cases it might not be necessary to postulate a mechanism for ER retention since many misfolded or unassembled proteins exhibit a significantly higher degradation rate than the proteins integrated in functioning multimeric complexes [for review, *see* 80]. Recently, an ER proteolytic degradation pathway has been described that might play a role in the removal of unassembled membrane proteins [78].

Whatever the mechanism controlling the accumulation of misfolded or unassembled proteins may be, the present knowledge indicates that a correctly folded structure acquired by subunit oligomerization is a necessary and sufficient signal for the exit of most multimeric proteins from the ER.

Though not yet experimentally confirmed, circumstantial evidence suggests that a similar requirement holds for the Na,K-ATPase. Two observations indeed support the hypothesis that the β-subunit might be the limiting factor for the α-subunit to be transported to the plasma membrane in a functional form. First, Takeyasu et al. [139] have observed that in mouse cells transfected with an avian α cDNA, only a limited amount of hybrid α-β complexes reach the plasma membrane and that much of the avian α-subunit accumulated in an internal membrane pool likely to be the ER. This result indicated to them that the overexpressed avian α-subunit and the mouse α-subunit compete for a limited amount of mouse β-subunit and that only α-β complexes reach the plasma membrane. Further support for a role of the β-subunit in the intracellular transport of Na,K-ATPase can be derived from the observation that injection of β mRNA into *Xenopus* oocytes leads to an increased number of ouabain binding sites at the plasma membrane [40]. Since *Xenopus* oocytes produce more α-subunits than β-subunits, this result most likely indicates that exogenous β-subunits are able to provoke the cell surface expression of oocyte α-subunits. Finally, Caplan et al. [11] have presented

preliminary results suggesting that α-subunit overexpressed in *Xenopus* oocytes from injected α mRNA is associated to BiP. Since BiP is lost from immunoprecipitates after coninjection of β *m*RNA, the authors postulate that assembly of α and β subunits triggers the release of α-BiP complexes and thereby the transport constraint from the ER. While all these data are consistent with the idea that the α-subunit needs the β-subunit for efficient cell surface expression, nothing is known as to whether the reverse is also true or, in other words, whether the β-subunit alone is able to move out of the ER.

Concluding Remarks

In this review, we have tried to better understand the role of the β-subunit of Na,K-ATPase by looking at the importance of subunit assembly and post-translational processing for the correct structural and functional expression of Na,K-ATPase. Though little substantial data are yet available on this subject for Na,K-ATPase, comparison to other multimeric proteins has permitted us to formulate perspectives for which future research might provide the most relevant information. It becomes indeed increasingly clear that subunit assembly is likely to play a crucial role in the regulation of export of multimeric proteins from the endoplasmic reticulum and their functional maturation. The recently developed techniques that permit us to reconstitute functionally active Na,K-ATPase in vivo from individual subunits will, hopefully, soon give us more insight into the mechanisms of subunit assembly and its functional implication. In addition, by using this dynamic experimental approach, we can expect to learn more about the potential diversity of the functional properties of the enzyme subunits likely to exist in view of the presence of various α- and β-subunit isoforms.

I would like to thank Mrs. Nicole Skarda-Coderey for her excellent secretarial work and Mrs. Nancy Narbel and Mrs. Estelle Ryser for keeping up with the bibliography.

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Received 25 July 1989