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The Functional Role of β Subunits in Oligomeric P-Type ATPases

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Na,K-ATPase and gastric and nongastric H,K-ATPases are the only P-type ATPases of higher organisms that are oligomeric and are associated with a β subunit, which is obligatory for expression and function of enzymes. Topogenesis studies suggest that β subunits have a fundamental and unique role in K^+-transporting P-type ATPases in that they facilitate the correct membrane integration and packing of the catalytic α subunit of these P-type ATPases, which is necessary for their resistance to cellular degradation, their acquisition of functional properties, and their routing to the cell surface. In addition to this chaperone function, β subunits also participate in the determination of intrinsic transport properties of Na,K- and H,K-ATPases. Increasing experimental evidence suggests that β assembly is a highly ordered, β isoform-specific process, which is mediated by multiple interaction sites that contribute in a coordinate, multistep process to the structural and functional maturation of Na,K- and H,K-ATPases.

KEY WORDS: Oligomeric P-type ATPase; subunit assembly; ER quality control; protein degradation; topogenesis of polytopic and type II membrane proteins; protein maturation.

INTRODUCTION

The Na,K-ATPase is one of the few members of the P-type ATPase transporter family that are oligomeric. Indeed, of the over 200 identified members of the P-type ATPase family (Axelsen and Palmgren, 1998), only animal Na,K- and H,K-ATPase isozymes and the bacterial Kdp-ATPase contain, in addition to the catalytic α subunit, one or two subunits, respectively, which are obligatory for the enzymes function. Alpha subunits of Na, K-ATPase and H,K-ATPase share the highest sequence identity among the P-type ATPases and belong to the P2-ATPases (Lutsenko and Kaplan, 1995) which, according to the crystal structure of the sarcoplasmic Ca-ATPase (Toyoshima *et al*., 2000), have ten transmembrane (TM) segments. The β subunits associated with Na,K- and H,K-ATPase α subunits are type II membrane proteins with one transmembrane segment, a short N-terminal, cytoplasmic tail, and a large C-terminal ectodomain. Three Na,K-ATPase β isoforms (NKA β1, NKA β2, and NKA β3) and one gastric H,K-ATPase β subunit (HKA β) from different species have so far been identified, which exhibit ~20–30% overall sequence identity. NKA and HKA β subunits are glycoproteins containing 2–8 consensus glycosylation sites (Asn-X-Ser/Thr, where X can be any amino acid) in the ectodomain with NKA β2 and HKA β being most heavily glycosylated. The presence in the ectodomain of 6 cysteine residues forming three consecutive disulfide bonds (Kirley, 1989) and of a highly conserved Tyr-Tyr/Phe-Pro-Tyr-Tyr motif can be considered as signature features of the β subunit protein family.

To study the question why only NKA and HKA α subunits need association with a β subunit, while other P-type ATPases can function with an α subunit alone, we have studied several aspects of the functional role of the NKA and HKA β subunits. Our results establish that β subunits have two main functions. Of fundamental importance is the role of the β subunit as a specific chaperone, which assists in the correct membrane insertion of the newly synthesized NKA and HKA α subunits and, hence, in their structural and functional maturation. In addition, association of the β subunit determines some of the

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intrinsic transport properties of Na,K- and H,K-ATPases. Finally, our results provide evidence that functionally relevant α–β assembly is a multistep, coordinated process, which implicates interactions of various β domains and necessitates specific interactions of particular α–β isoforms. Although particular β subunits may have other specialized functions, e.g., during development (Magyar *et al*., 1994) or in cell sorting of NKA (Burrow *et al*., 1999) or HKA (Courtois-Coutry *et al*., 1997), we will concentrate in the following, on the discussion of the basic functional role of the α–β interaction in oligomeric P-type ATPases.

THE β SUBUNIT FUNCTIONS AS A SPECIFIC CHAPERONE IN THE STRUCTURAL AND FUNCTIONAL MATURATION OF Na,K- AND H,K-ATPase α SUBUNITS

Synthesis, ER Insertion, and Quality Control of Membrane Proteins

To understand the fundamental role of β subunits in the maturation of NKA and HKA α subunits, it is important to remind the general processes that are involved in the synthesis and membrane insertion of membrane proteins (for review and references see Dalbey *et al*., 2000). The synthesis of these proteins starts in the cytoplasm from mRNA translated on free ribosomes. A signal sequence emerging from the ribosome is recognized by a signal recognition particle (SRP), the complex is attached to the membrane of the endoplasmic reticulum (ER) via a SRP receptor, and the membrane insertion process is initiated. An aqueous, protein-conducting channel, the translocon, is responsible for the translocation of hydrophilic domains of membrane proteins across the ER membrane as well as for the partitioning of hydrophobic TM domains into the lipid bilayer. Several mechanisms have been proposed for the integration of membrane proteins into the lipid bilayer. According to the signal hypothesis (Blobel, 1980), topogenesis of polytopic membrane proteins is mediated by a sequential insertion mechanism of independent topogenic sequences. These involve signal anchor (SA) sequences, which initiates C-terminal translocation and have a N_{in}/C_{out} orientation and stop transfer (ST) sequences, which halt translocation and have a N_{out}/C_{in} orientation. Alternatively, topogenic sequences may form pairs in the cytoplasm, so-called helical hairpins, before inserting into the translocon and ultimately the lipid bilayer (Engelman and Steitz, 1981). Recent studies suggest that the time point during synthesis when a TM segment is fully inserted into the lipid bilayer depends on the hydrophobicity of the TM segment. Sufficiently hydrophobic TM domains may be released into the lipid bilayer as soon as they have access to it, while less hydrophobic TM domains may dynamically equilibrate between the lipid and the aqueous phase until intermolecular interactions shield hydrophilic or charged residues present in TM domains (Heinrich *et al*., 2000).

During ER translocation and elongation, membrane and secretory proteins undergo α-helical packing, acquire N-linked sugars, form cysteine bonds, and interact with molecular chaperones in cytoplasmic and ER lumenal domains (Fink, 1999). In addition to these processes, which assist in the correct folding of newly synthesized proteins, assembly of partner subunits in oligomeric proteins participates in the protein maturation process. Each step of protein maturation is essential to produce correctly folded proteins that can leave the ER and become functionally active. An ER quality control system prevents misfolded proteins from leaving the ER and mediates their degradation. Details of the degradation process are still poorly understood. According to the current view, misfolded proteins are recognized by molecular chaperones such as Bip or calnexin, a lectin-like chaperone which specifically associates with monoglycosylated trimming intermediates of N-linked glycoproteins (for review see Ellgaard *et al*., 1999). Misfolded membrane proteins are retranslocated from the lipid bilayer to the translocon and redirected to the cytosol where they are degraded by the proteasome (Hirsch and Ploegh, 2000).

The oligomeric nature of NKA and HKA, the distinct membrane topology of α and β subunits, and the extensive cotranslational processing of the β subunit, make these two P-type ATPase interesting model proteins for the study of (1) the mechanisms and the molecular determinants, which are involved in the degradation of misfolded, e.g., unassembled, polytopic membrane proteins or type II glycoproteins and (2) the role of cotranslational processing, interaction with molecular chaperones, and subunit assembly in the correct folding of oligomeric proteins. As an experimental system to study these questions, we preferentially use *Xenopus* oocytes, which exhibit a very low synthesis of endogenous NKA subunits, in particular, of β subunits (Jaunin *et al*., 1992) and therefore allow the overexpression of exogenous α and/or β subunits by cRNA injection and assessing of the properties of unassembled and assembled subunits.

Membrane Topogenesis and Fate of Unassembled α and β Subunits

Since the original work of Noguchi *et al*. (1987) who expressed α subunits without β subunits in *Xenopus*

oocytes, it is well established that NKA α subunits alone are unable to direct the formation of functional NKA. More recent experiments permit drawing the same conclusion for gastric and nongastric HKA. The early observations that individual NKA α subunits can insert into ER membranes during synthesis (Geering *et al*., 1985; Caplan *et al*., 1986), but are highly trypsin sensitive and prone to degradation in intact cells (Geering *et al*., 1989), indicated that the β subunit is not required to initiate membrane insertion but rather to complete the correct membrane topogenesis and folding of the newly synthesized α subunit, which is necessary to render it trypsin-resistant and to impede its cellular degradation (Geering *et al*., 1989).

A recent analysis on the membrane topogenesis of truncated NKA and HKA α subunits of different lengths, expressed in *Xenopus* oocytes in the absence or presence of β subunits, confirmed this hypothesis and revealed details on the incomplete membrane insertion of individual NKA and HKA α subunits and on the helper function of the β subunit in the structural maturation of the α subunit. The use of a reporter glycosylation sequence (RGS), added to the C-terminal end of each truncated α subunit, permitted the determination of whether a translated sequence ends in the lumen of the ER (RGS glycosylated) or in the cytoplasm (RGS not glycosylated) and whether a C-terminal membrane segment acts as a SA or a ST sequence. Results of such studies show that the first 4 N-terminal membrane sequences of NKA and HKA α subunits act as efficient alternating SA/ST sequences (Bamberg and Sachs, 1994; Xie and Morimoto, 1995; Béguin *et al*., 1998; Beggah *et al*., 1999). They insert sequentially into the membrane and form a membrane anchor which is not susceptible to cellular degradation (Béguin *et al*., 1998; Beggah *et al*., 1999) and produces a 34-kDa fragment after controlled proteolysis of newly synthesized, individual α subunits (Geering *et al*., 1985, 1987, 1989). As indicated by results from RGS assays on Ca-ATPase (Bayle *et al*., 1995), H-ATPase (Lin and Addison, 1995a), or a heavy metal ATPase (Melchers *et al*., 1996; Bayle *et al*., 1998), formation during synthesis of two- or three-membrane inserted N-terminal pairs, is likely to be a universal feature of P-type ATPases.

Significantly, individual, truncated NKA α proteins which contain, in addition to the two N-terminal membrane pairs, the large M4/M5 cytoplasmic loop, comprising more than one-third of the amino acids of the α subunit, are also resistant to cellular degradation in contrast to full length, individual α subunits (Béguin *et al*., 2000). Probably, the cytoplasmic M4/M5 loop rapidly folds into a native structure during synthesis, which prevents exposure of degradation signals, which could be recognized from the cytoplasmic side and mediate significant degradation of α subunits before synthesis is completed. That the large cytoplasmic loop can adopt an ordered structure, independent of the presence of the membrane domain, is also observed after expression of the isolated central loop, which produces a protein with a E2-like conformation (Gatto *et al*., 1998; Tran and Farley, 1999).

RGS assays performed on α proteins, which contain the C-terminal TM domains after the large cytoplasmic loop, revealed that misfolding and ensuing sensitivity to trypsin or cellular degradation of unassembled α subunits is related to the poor efficiency of membrane insertion of C-terminal TM segments. In contrast to M1 and M3, which are efficient SA sequences, M5, M7, and M9 of NKA and HKA α subunits are poor SA sequences, as reflected by the only partial or the lack of glycosylation of the RGS-tagged M1-5, M1-7, and M1-9 α proteins (Bamberg and Sachs, 1994; Béguin *et al*., 1998; Beggah *et al*., 1999). The efficiency of membrane insertion of TM segments is a reflection of their hydrophobicity. Comparison of amino acid sequences reveals that the 6 C-terminal TM segments of NKA and HKA α subunits are less hydrophobic and contain more polar or charged residues and helix-breaking proline residues than the four N-terminal membrane domains, which may explain the less efficient membrane insertion of the C-terminal TM segments. Significantly, replacing charged or polar amino acids in M7 or M9 by leucine residues render these TM segments efficient SA sequences (Béguin *et al*., 1998). Based on our results, we propose a model for the membrane topology of unassembled NKA and HKA α subunits in which the two N-terminal TM pairs adopt a stable conformation due to integration into the lipid bilayer, whereas the C-terminal TM segments may be in a dynamic equilibrium between the lipid phase, the translocon, and the cytoplasm with a high preference for the aqueous phase (Fig. 1).

Significantly, available experimental data or sequence analysis do not provide a conclusive answer to the question of why topogenesis of C-terminal TM segments of individual NKA and HKA α subunits is incomplete, while it is fully effective in other P2-ATPases, such as Ca- or H-ATPases (Bayle *et al*., 1995; Lin and Addison, 1995b), despite a similar low hydrophobicity of the C-terminal TM segments (for review see Geering, 2000). It is possible that in monomeric P2-ATPases, efficient membrane integration can be achieved through interactions of TM segments, which annul the repulsive effect of hydrophilic or charged residues. For instance, it has been suggested that M5 and M6 of H-ATPase are linked by a salt bridge formed between a positively and a negatively charged residue (Sen Gupta *et al*., 1998), which are conserved in M5 and M6 of Ca- and H-ATPases and this salt bridge may favor the formation of a hairpin

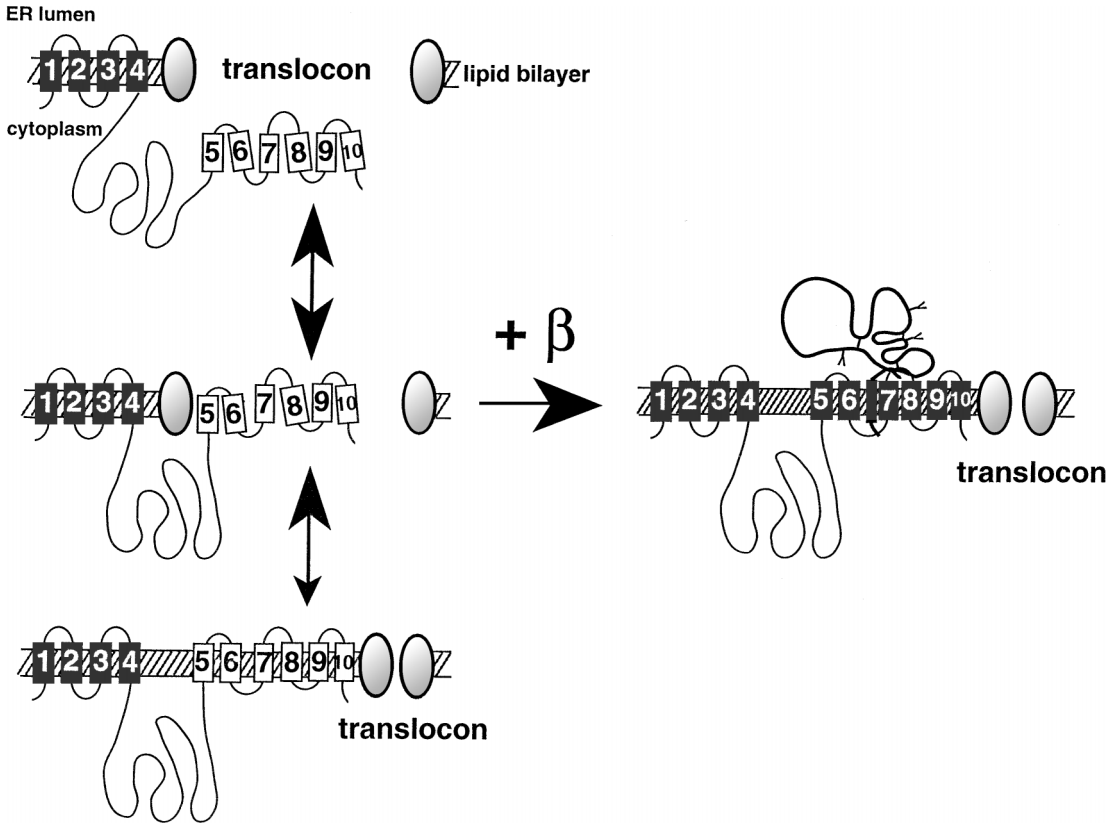

Fig. 1. Membrane topogenesis of the α subunit of Na,K-ATPase. During synthesis, the first four N-terminal hydrophobic segments of the α subunit are integrated from the translocon into the lipid bilayer by sequential insertion of signal anchor and stop-transfer sequences. The large, central cytoplasmic loop adopts a conformation that does not expose any primary degradation signals. On the other hand, due to specific sequence information, which impedes interaction with apolar surfaces, the efficiency of integration of C-terminal membrane segments into the lipid bilayer is very low. In the absence of coexpressed β subunits, C-terminal membrane segments are in a dynamic equilibrium between the lipid bilayer (left, lower panel), the translocon, and the cytoplasm, with a high preference for the aqueous phase (middle and upper panels). In this configuration, the α subunit is highly sensitive to cellular degradation and trypsinolysis. Only the extreme situations in which all C-terminal membrane segments are distributed to one particular compartment are shown. Coexpressed β subunits associate with the α-population, which exposes the M7/M8 extracytoplasmic loop to the ER lumenal side (middle panel). The assembly process will retain the M7/M8 pair in the membrane, permit intramolecular interactions and integration of C-terminal membrane segments into the lipid bilayer, and render the α subunit resistant to cellular degradation and trypsinolysis. The β subunit's ectodomain is shown with three loops formed by disulfide bridges and three sugar chains (V) as found in certain β1 isoforms. For further details, see text.

structure compatible with membrane integration. On the other hand, in NKA and HKA α subunits, efficient intramolecular interactions may not be possible due to the presence of specific sequence information in certain TM segments, at least until β subunits are associated.

The inefficient membrane insertion of the C-terminal TM segments of individual NKA and HKA α subunits correlates with their susceptibility to cellular degradation mediated, at least in part, by the proteasomal system. During synthesis of the α subunit, certain membrane domains, which in the mature protein are exposed to the extracytoplasmic side, are transiently exposed to the cytoplasm due to inefficient membrane insertion and potentially become targets for proteasomal attack (Béguin *et al*., 2000). A candidate for a degradation signal, that may be recognized by the proteasomal system from the cytosolic side, is located in the M5/M6 loop and consists of a Pro–Leu–Pro motif, highly conserved in NKA and HKA α subunits. Other putative degradation signals that may be involved in proteasome-dependent degradation of NKA α subunits exist in M7 and M9. In addition to degradation signals that are directly exposed to the cytosolic side due to inefficient membrane insertion of C-terminal TM segments, other degradation signals, e.g., in the M7/M8 extracellular loop, have been identified which may be targets for ER lumenal proteases (Béguin *et al*., 2000). Although mechanistic

details of degradation may differ among different misfolded proteins, it is likely that the results obtained with the NKA α subunit represent a paradigm for the degradation of polytopic membrane proteins. The poor efficiency of membrane insertion due to specific natural sequence information or of mutations in certain TM segments may be the main determinant for degradation of such proteins since it permits exposure of degradation signals during synthesis. Furthermore, our results provide evidence that degradation of polytopic membrane proteins involves proteasome-dependent mechanisms acting from the cytosol as well as proteasome-independent mechanisms acting from the ER lumenal side. Finally, molecular chaperones such as BiP probably play an important role in the ER quality control of misfolded, polytopic membrane proteins since they interact with unassembled, full length or truncated NKA α-proteins, but not with β-assembled α subunits, and the interaction time correlates with the proteins stability and ER retention (Beggah *et al*., 1996).

In contrast to α subunits, the overall membrane topogenesis of the β subunit is not dependent on partner subunit interaction. However, as suggested by the sensitivity to ER degradation or the ability of unassembled β subunits to leave the ER, stable membrane insertion and correct folding of the β subunit may or may not rely on α subunit interaction depending on the β subunit species. Insertion of the β subunit into the ER membrane requires interaction with SRP (Geering, 1988; Kawakami and Nagano, 1988) and results in a protein with a type II orientation, exposing its short N-terminus to the cytoplasmic side (Geering *et al*., 1985; Kawakami and Nagano, 1988). The three disulfide bridges are formed during elongation of the β subunit, while core sugars may be added to consensus glycosylation sites during or after completion of synthesis, depending on the time β subunits spend in the ER (Hasler *et al*., 2000). Cysteine bond formation (Noguchi *et al*., 1994; Beggah *et al*., 1997) and glycosylation (Zamofing *et al*., 1988, 1989; Beggah *et al*., 1997; Asano *et al*., 2000) play a distinct role in the acquisition of a correctly folded structure of newly synthesized NKA as well as HKA β subunits, which is compatible with efficient α subunit interaction. For instance, lack of glycosylation of NKA β1 indeed reduces NKA α-assembly competence (Zamofing *et al*., 1988, 1989; Beggah *et al*., 1997) but still permits for the formation of functionally active, stable Na,K-pumps at the cell surface (Tamkun and Fambrough, 1986; Takeda *et al*., 1988; Beggah *et al*., 1997). On the other hand, although nonglycosylated β2 isoforms can associate with α2 isoforms, nonglycosylated NKA β2 subunits cannot interact with α1 isoforms (Schmalzing *et al*., 1997) similar to nonglycosylated HKA β subunits, which cannot assemble with HKA α subunits (Asano *et al*., 2000). In addition, partial glycosylation of HKA β subunits impedes cell surface expression of H,K-pumps (Asano *et al*., 2000). In this context, it is interesting that nonglycosylated NKA β1 subunits expressed in *Xenopus* oocytes, remain stably expressed in the ER (Beggah *et al*., 1997), while nonglycosylated HKA β subunits are degraded (unpublished observations). Possibly, the partial misfolding of nonglycosylated β1 subunits and, in consequence, their reduced competence to assemble with α subunits, can at least partially be overcome by a prolonged half-life of the protein, which increases the time allowed for interaction with the α subunit. This may also explain why nonglycosylated HKA β subunits, which are not degraded in transfected, insect Sf9 cells, associate with HKA α subunits (Klaassen *et al*., 1997). Significantly, the HKA α–β complexes formed with nonglycosylated HKA β are not functional and their delivery to the plasma membrane is impeded (Klaassen *et al*., 1997).

Correct folding of NKA β subunits is also strongly dependent on the presence of the cytoplasmic N-terminal tail and of a positively charged residue at the N-terminal start of the TM domain (Hasler *et al*., 2000). N-terminal truncations of β1 or β3 isoforms affect the positioning of the transmembrane domain of β isoforms, lead to the exposure of cryptic, signal peptidase cleavage sites in β3 isoforms and produce a population of β3 proteins unable to insert into membrane. This latter effect can also be produced in β1 isoforms when a lysine residue flanking the β1 TM domain is replaced with a leucine residue in truncated β1 isoforms, and the effect can be reduced in β3 isoforms when the leucine residue flanking the β3 TM domain is replaced by a lysine residue in the truncated β3 isoform (Hasler *et al*., 2000).

In addition to the cytoplasmic N-terminal tail, regions in the ectodomain, e.g., the highly conserved YYPYY motif play an important role in the structural integrity of the β subunit as suggested by the higher protease sensitivity of a Pro to Gly mutant of NKA β3 subunits, which is no longer able to associate with NKA α subunits (Geering *et al*., 1993).

In the *Xenopus* oocyte system, certain NKA β subunits, such as *Xenopus* β1 and β3 subunits expressed without α subunits are retained in the ER in association with BiP and are degraded with specific rates (Ackermann and Geering, 1990; Jaunin *et al*., 1992; Beggah *et al*., 1996), while HKA β subunits can escape the ER quality control and be routed to the plasma membrane in various cell types (Horisberger *et al*., 1991; Jaunin *et al*., 1993; Gottardi and Caplan, 1993). The reason why HKA β subunits can adopt a more correct conformation than NKA β1 and β3 subunits is not known, but since results, obtained with chimeric β subunits, indicate that the ectodomain

determines the fate of the β subunit (Jaunin *et al*., 1993), it is possible that the more extensive glycosylation plays a role in favoring a rapid and correct folding of the HKA β subunit.

α-β Assembly Process and Its Consequences on Na,K-ATPase Maturation

Assembly of α and β subunits of oligomeric P-type ATPases occurs at the level of the ER and mediates the structural and functional maturation of the enzymes. A first demonstration of the fundamental role of β-assembly with NKA α subunits was provided by experiments which showed that an endogenous, immature, and trypsin-sensitive NKA α subunit pool, synthesized in *Xenopus* oocytes in excess over β subunits and residing in the ER, can be rendered trypsin-resistant and can be mobilized to the plasma membrane as functional Na,K-pumps by the sole injection of β cRNA (Geering *et al*., 1989). Later experiments performed in β and/or α cRNA-injected *Xenopus* oocytes or DNA-transfected cells confirmed and refined the functional role of α–β assembly both in NKA and HKA. Assembly of β subunits is indeed a multistep process, which depends on several discrete steps of intersubunit interactions necessary for the complete maturation of the α subunit. The most obvious consequence of β subunit assembly is a structural change in the newly synthesized NKA and HKA α subunit, which leads to its resistance against cellular degradation and trypsinolysis (Geering *et al*., 1989; Ackermann and Geering, 1990; Geering *et al*., 1996). Additional, more subtle, folding events may be mediated by β interaction, which are necessary for the observed acquisition of functional properties of NKA α subunits such as the ability to adopt different ligand-dependent conformations and to hydrolze ATP in a Na^+-and K^+-dependent, ouabain-inhibitable fashion (Geering *et al*., 1996), and/or for the ability of the α subunit to leave the ER as α–β complexes (Jaunin *et al*., 1992). Not only α subunits but also β subunits undergo a structural change after assembly, which results in a global change in its protease resistance (Geering *et al*., 1996).

Subunit assembly probably does not occur during, but soon after completion of α subunit synthesis. This is suggested by the observation that endogenous NKA of various cell types undergoes a maturation process, which is comparable to that observed after coexpression of α subunits with β subunits in *Xenopus* oocytes, namely, it is processed from a trypsin-sensitive, functionally immature form to a trypsin-resistant form, which can undergo ligand-dependent conformational changes within 7–15 min after synthesis (Geering *et al*., 1987, Caplan *et al*., 1990). Experimental evidence suggests that post-translational assembly of NKA α and β subunits is indeed possible. Not only a presynthesized β subunit (Noguchi *et al*., 1990; Ackermann and Geering, 1992), but also a presynthesized α subunit (Ackermann and Geering, 1992) pool, build up in *Xenopus* oocytes during a pulse period and maintained constant by β or α cRNA-specific antisense oligonucleotides, retain an assembly-competent state, which permits assembly with the corresponding partner subunit synthesized at later time points. The molecular chaperone BiP remains associated with individual subunits until oligomerization occurs (Beggah *et al*., 1996) and thus probably plays an important role in the maintenance of an assembly-competent state of the two subunits. The potential physiological relevance of posttranslational assembly of α and β subunits is illustrated during the early development of *Xenopus laevis*. *Xenopus* oocytes possess a stable α subunit pool in the ER which is inactive for lack of association with β subunits (Geering *et al*., 1989; Jaunin *et al*., 1992). There is experimental evidence that, during the transcriptionally silent phase of early development, this presynthesized α subunit pool may be recruited by $\beta 1$ subunits newly synthesized from polyadenylated mRNA, to form active Na,K pumps needed for blastocoel formation (for review and references see Geering, 1998). It is also likely that the selective hormonal regulation of the transcription and/or translation of one or the other of the NKA subunits, which is observed under certain physiological conditions (for review see Geering, 1998), reflects the necessity as well as the possibility of post-translational subunit assembly.

α-β Interaction Domains and Their Role in Na,K-ATPase Maturation

By studying chimera-containing Ca-ATPase and regions of the NKA $\alpha 1$ subunit, Lemas *et al.* (1994b) revealed that 26 amino acids comprising part of the extracytoplasmic M7/M8 loop and of M8 of NKA α subunits are involved in assembly with NKA β subunits. Studies with HKA α and β subunits led to similar conclusions (Melle-Milovanovic *et al*., 1998). Alanine scanning mutagenesis combined with the yeast two hybrid assay permitted to identify a SYGQ motif, conserved in the M7/M8 loop of all α subunits of oligomeric P-type ATPases, which is crucial to α-β interactions (Colonna *et al*., 1997). Recent experimental data demonstrates that interaction of the β subunit with a ten amino acid region encompassing the SYGQ motif is indeed the key event for the correct packing of newly synthesized NKA α subunits (Béguin *et al*., 2000). Alanine mutations in this region of the $\alpha 1$ subunit

abolishes assembly with coexpressed β1 subunits and maintains the α subunit in a misfolded state, which is retained in the ER, is trypsin-sensitive, and rapidly degraded (Béguin *et al*., 2000). Mutagenesis combined with topology and degradation studies has provided some clues to the question how β-assembly with the M7/M8 extracellular loop assists in the correct membrane insertion of the C-terminal TM segments of NKA α subunits. According to our model of the membrane topology of unassembled α subunits in which C-terminal TM segments are dynamically equilibrated between the translocon, the cytoplasm, and the lipid bilayer, the ectodomain of the β subunit will assemble with the population of the newly synthesized α subunit, which exposes the M7/M8 connecting loop of α subunits to the ER lumenal side (Fig.1). This process will retain M7 in the membrane but will also induce a conformational change in the M7/M8 extracytoplasmic loop, which renders M8 an efficient ST sequence and helps to form a M7/M8 pair (Béguin *et al*., 1998). As a result, intramolecular interactions with other TM segments may become possible permitting their membrane integration and ultimately an overall stabilization of the α subunit. Experimental evidence also suggests that β-assembly with the M7/M8 extracellular loop masks a degradation signal present in this region, which may be important for the recognition of the misfolded state of unassembled α subunits by a component acting from the ER lumenal side (Béguin *et al*., 2000). Finally, it is likely that secondary interaction events with other regions of the β subunit may be necessary to complete the maturation of the α subunit (see below).

Although fundamentally similar, some subtle differences exist in the β-mediated stabilization process of HKA α subunits. In contrast to M7 of NKA α subunits, which has partial SA properties (Béguin *et al*., 1998), M7 of gastric HKA α subunits is devoid of any SA activity (Bamberg and Sachs, 1994; Beggah *et al*., 1999) and, therefore, the probability of β-interaction with the M7/M8 loop of HKA α subunits at the ER lumenal side is lower than with that of NKA α subunits. This is reflected by a poor stabilization of a truncated HKA M1-8 α-protein compared with a complete stabilization of a truncated NKA M1-8 α-protein in the presence of β subunits (Béguin *et al*., 1998; Beggah *et al*., 1999). According to our results, formation of a M9/M10 pair in HKA α subunits and its interaction with the M7/M8 pair is essential for membrane integration of the M7/M8 pair and in consequence for the exposure of the β-interaction domain to the ER lumenal side. The particular role of the M9/M10 pair for efficient assembly of the β subunit with the M7/M8 loop in HKA α subunits is also demonstrated by the observation that chimeras containing M1–M8 of human, nongastric HKA and M9 and M10 of NKA α subunits completely lose the ability to associate with β subunits (Geering *et al*., 2000).

The assembly site in the ectodomain of the β subunit, which interacts with the M7/M8 loop of the α subunit, has been mapped to 63 amino acids adjacent to the C-terminal end of the putative TM domain of NKA β1 subunits by using the two-hybrid assay (Colonna *et al*., 1997). Similar experiments for HKA β subunits (Melle-Milovanovic *et al*., 1998) and specific Cu^{2+}-catalyzed oxidative cleavage of NKA β subunits (Shimon *et al*., 1998) point to two interaction regions, one which is located before the first S–S bridge and another between the second and the third S–S bridge. Although interactions of these β regions with the M7/M8 loop of the α subunit may be the first step necessary for the maturation process of the α subunit, it is likely that they are not sufficient to promote full maturation of NKA or HKA α subunits. Indeed, experimental evidence suggests that other putative assembly sites exist both in the ectodomain and the TM domain of the NKA β subunit, which participate in the correct folding of the NKA α subunit. First, deletion of only ten amino acids from the C-terminal end of NKA β subunits, or alanine replacements of the four aligned hydrophobic residues, but not of the charged residues, in the β strand-like structure of this region, completely prevent stabilization of NKA α subunits (Beggah *et al*., 1993). Although it has been suggested that deletion of as little as four C-terminal amino acids of the NKA β subunit may prevent its proper folding (Hamrick *et al*., 1993), it is not likely that a moderate change in the hydrophobicity of four C-terminal amino acids may lead to misfolding of the cysteine-bonded and glycosylated β subunit's structure. Our results rather indicate that the aligned, hydrophobic C-terminal amino acids cooperate, in a so far unknown way, with amino acids adjacent to the TM domain in the correct assembly with the α subunit. Finally, by using a monoclonal antibody, which only recognizes unassembled β subunits, Okamoto *et al*. (2000) have also provided evidence that a four amino acid sequence in the HKA β subunit's ectodomain, which overlaps with the YYPYY motif, may be implicated in HKA α–β interactions.

Cross-linking studies show that the NKA β TM domain is in close contact with M8 of the NKA α subunit suggesting that α and β subunits not only interact in the ectodomain but also in the transmembrane domain (Or *et al*., 1999; Ivanov *et al*., 2000). At least in NKA, interactions in the transmembrane domain may play a role in certain steps of the α maturation process. Indeed, β chimeras containing the TM domain of HKA β subunits and the ectodomain of the NKA β subunit produce less functional Na,K-pumps than β chimeras with the inverse topology (Jaunin *et al*., 1993; Eakle *et al*., 1994; Hasler

et al., 1998). Significantly, the TM domain of the NKA β subunit cannot be replaced by the corresponding domain of the HKA β subunit without important consequences on the intracellular routing of the NKA α-β complexes formed. Most α–β complexes containing β subunits with the TM domain of the HKA β subunit are indeed retained in the ER (Hasler *et al*., 1998), which reflects an improper folding of the enzyme that appears also to be incompatible with efficient function.

Controlled proteolysis assays of NKA β subunits suggest that α and β subunits also interact in the cytoplasmic domain (Geering *et al*., 1996). However, no default in any step of the maturation process leading to stabilization, ER exit, or activity of the α subunit, could be revealed by mutational analysis of the cytosolic N-terminus of the NKA β1 subunit (Hasler *et al*., 1998) confirming previous observations that cytoplasmic interactions are not implicated in the proper folding of the α subunit (Renaud *et al*., 1991; Geering *et al*., 1996).

β SUBUNIT CONTRIBUTES TO INTRINSIC TRANSPORT PROPERTIES OF NKA AND HKA α SUBUNITS

In addition to its chaperone function in the maturation of newly synthesized α subunits, the β subunit also plays a role in the determination of the transport properties of mature NKA and HKA pumps. First evidence for a role of NKA and HKA β subunits in cation binding was provided by experiments which showed that the function of purified NKA and HKA is impeded by reduction of disulfide bonds of the β subunit, but that it can be restored in the presence of cations (Kirley, 1989; Chow *et al*., 1992; Lutsenko and Kaplan, 1993; Kawamura and Nagano, 1984; Tyagarajan *et al*., 1995). Transport or kinetic studies on NKA or HKA isozymes, expressed in cells and composed of the same α isoform but different NKA or HKA β isoforms, confirmed that the β subunit indeed influences the apparent K^+-affinity of oligomeric P-type ATPases (Eakle *et al*., 1992; Schmalzing *et al*., 1992; Jaunin *et al*., 1993; Jaisser *et al*., 1994; Blanco and Mercer, 1998; Hasler *et al*., 1998; Koenderink *et al*., 1999; Crambert *et al*., 2000). According to transport studies on Na,K-pumps, different NKA β subunits result in differences in the K^+ affinity, which are not directly related to the K^+ binding site whereas HKA β subunits associated with NKA α subunits may directly alter the K^+ binding or translocation mechanism of Na,K pumps (Jaisser *et al*., 1994). In view of the tight coupling between α and β subunits, it is likely that the K^+ transport function is mediated by a concerted conformational change of the two subunits rather than by an α-independent effect of the β subunit on a particular reaction step of the enzyme (Chow and Forte, 1995). In addition to the apparent K^+ affinity, the β structure was also shown to influence the apparent Na^+ affinity (Hasler *et al*., 1998) and the Na^+ dependence of phosphoenzyme formation (Eakle *et al*., 1995).

No consistent conclusions can be drawn so far as to the distinct effect and the physiological relevance of interactions of α subunits with different β isoforms. Indeed, whereas transport studies on human NKA isozymes, expressed in *Xenopus* oocytes, revealed highest, apparent K^+-affinities for $\alpha1$–$\beta1$, $\alpha1$–$\beta2$, $\alpha1$–$\beta3$ or $\alpha3$–$\beta1$ isozymes, intermediate K^+ affinities for $\alpha2$–$\beta1$, $\alpha2$–$\beta3$, $\alpha3$–$\beta2$ or $\alpha3$–$\beta3$ isozymes and lowest K^+ affinities for $\alpha2$–$\beta2$ isozymes (Crambert *et al*., 2000), kinetic analysis of the NKA activity of rat NKA isozymes, expressed in Sf9 insect cells, showed apparent K^+ affinities in the order of $\alpha1-\beta1 > \alpha2-\beta1 = \alpha2-\beta2 > \alpha3\beta1 = \alpha3-\beta2$ isozymes (Blanco and Mercer, 1998). The discrepancies in the results are likely to be due to the various experimental conditions and assays used and/or to a cooperative mechanism between α and β isoforms, which determines the apparent K^+ affinity (Eakle *et al*., 1994; Crambert *et al*., 2000) and which may differ among NKA isozymes of different species.

The role of the β subunit in determining intrinsic functional properties of NKA and HKA has been further documented by mutational, biochemical, and immunological analysis, which provided, in addition, some information on the intersubunit interaction domains that may be implicated in the β subunit's effects. Functional analysis of chimeras between NKA and HKA β subunits revealed that mainly ectodomain interactions between α and β subunits are responsible for the β subunit's effect on the apparent K^+ affinity as measured by the K^+ activation of Na,K-pump currents (Jaunin *et al*., 1993; Hasler *et al*., 1998) or by the K^+ competition of ouabain binding to NKA (Eakle *et al*., 1994). Similar conclusions were drawn from experiments on purified NKA that showed a loss of Rb occlusion by the reduction of S–S bridges in the ectodomain of the β subunit and a protective effect of RbCl, but not of Mg^{2+} and P_i, against reduction and loss of enzyme activity (Lutsenko and Kaplan, 1993, 1994). Based on proteolysis analysis, it was suggested that upon Rb binding, the NKA β subunit changes its conformation and interacts more tightly with the NKA α subunit, involving the loop between the first S–S bond, and thereby impedes access of extracellular Rb to its binding site (Lutsenko and Kaplan, 1994). Results from Cu^{2+}-catalyzed oxidative cleavage studies (Shimon *et al*., 1998), which provide information on interacting regions of α and β subunits, suggest that loss of Rb occlusion in NKA by reduction of S–S bridges in

the β subunit or the differential effects of different β subunits on the apparent K^+ affinity of NKA may, indeed, be mediated indirectly by the α–β interaction in the M7/M8 extracytoplasmic loop. Perturbations of α–β interactions or conformational diversity of the interaction region may influence interactions of this loop with M4, M5, and M6 and affect the inherent occlusion and transport function of these TM domains. Intramolecular interactions of the M7/M8 loop could occur at the extracytoplasmic surface of the protein or eventually in the membrane domain if, as suggested by Schneider and Scheiner-Bobis (1997), the M7/M8 loop acts as a P-loop invaginated within the plane of the membrane.

Studies on the putative implication of cytoplasmic α-β interactions in the control of the cation binding function of NKA and HKA have led to some conflicting results. Controlled proteolysis assays performed on NKA β subunits expressed in *Xenopus* oocytes revealed that coexpression of NKA α subunits leads to protection of the N-terminus of the β subunit against proteolysis in the presence of KCl but not of NaCl (Geering *et al*., 1996). Furthermore, truncation of the β N-terminus affects the apparent K^+ (Geering *et al*., 1996; Hasler *et al*., 1998) and Na^+ (Hasler *et al*., 1998) affinity of Na,K pumps as well as their E1–E2 conformation equilibrium (Abriel *et al*., 1999), but not their maximal transport capacity. These results suggested that the N-terminus of the β subunit interacts with the α subunit in a cation-dependent fashion possibly reflecting a modulatory role of the β N-terminus in the transport properties of the α subunit. Similar conclusions were drawn from experiments performed on "19-kDa membranes" (Shainskaya and Karlish, 1996; Shainskaya *et al*., 2000). These enzyme preparations which are produced by extensive tryptic digestion of purified NKA, contain fragments of transmembrane α-domains connected by extracytoplasmic loops and an intact or partially cut β subunit (16-k and 50-kDa fragments), and are fully competent to occlude Na^+ and K^+ and to bind ouabain (Karlish *et al*., 1990). Based on the proteolytic sensitivity and ion-binding capacity of "19-kDa membranes" under defined conditions, it was suggested that the cytoplasmic loop between M6 and M7 of the α subunit acts as an entrance port for Na^+ and K^+ and that the β N-terminus may interact with this region and contribute to the selectivity for Na^+ over K^+ (Shainskaya *et al*., 2000). This latter conclusion was supported by the observations that (1) a chymotrypsin split produced at 20°C in the NKA β N-terminus does not affect Rb occlusion but markedly reduces the Na^+ affinity of "19-kDa membranes" as measured by Na^+ occlusion assays or electrogenic Na^+ binding and (2) the selective chymotrypsin cleavage at the Phe14–Ile15 bond, producing a stable 15-kDa fragment at 20°C, is impeded in the presence of Na^+. Finally, a modulatory role of the HKA β N-terminus in the K^+ activation of H,K-pumps was suggested by experiments using a monoclonal antibody, which recognizes a cytoplasmic epitope of the HKA β subunit and which decreses the K^+ activation of *p*-nitrophenyl phosphatase activity of H,K pumps (Chow and Forte, 1993).

In an attempt to define the molecular determinants, which are functionally relevant in cytoplasmic α-β interactions, we recently performed a detailed mutational analysis of the NKA β N-terminus. The results of these studies show that, in contrast to the complete truncation of the β N-terminus (Geering *et al*., 1996), internal deletions or multiple point mutations in the β N-terminus do not affect the K^+ activation of Na,K-pumps expressed in *Xenopus* oocytes (Hasler *et al*., 1998). Furthermore, addition of only four unrelated amino acids to the N-terminally truncated β subunit restored the wild-type phenotype of Na,K-pumps with respect to their apparent Na^+ and K^+ affinity (Hasler *et al*., 1998). These results suggested that the effects on the cation affinities of Na,K pumps containing the N-terminally truncated β subunit are not due to abolition of specific cytoplasmic α–β interactions, but rather to structural changes in other domains of the β subunit due to N-terminal β truncation. To probe potential, positional changes of the TM α-helix of the β subunit after N-terminal truncation, we applied a glycosylation mapping assay (Hasler *et al*., 2000). Glycosylation sites were engineered at different positions from the putative C-terminal end of the TM domain of full length and truncated β subunits and the glycosylation pattern of the various β mutants was compared. Since the active center of oligosaccharyltransferase is at a fixed position from the lipid bilayer and thus only permits glycosylation of engineered glycosylation sites at a minimal glycosylation distance, the glycosylation mapping assay allows the determination of the C-terminal end of TM helixes (Nilsson and von Heijne, 1993) and probing of its potential repositioning. By using this assay, the C-terminal end of the *Xenopus* wild-type β1 TM domain was mapped to Leu58 and it was found that N-terminal truncation significantly decreases the minimal glycosylation distance compared with wild-type β1 subunits indicating that removal of the cytoplasmic N-terminus leads to a repositioning of the TM domain, which may affect the conformation of the ectodomain (Hasler *et al*., 2000). Additional evidence for positional changes in the β TM domain after N-terminal truncations were obtained with β3 isoforms in which N-terminal truncation exposes a cryptic signal peptidase cleavage site (Hasler *et al*., 2000). Altogether, these results indicate that cytoplasmic interactions of α–β subunits may not be directly responsible for the observed

functional effects of β subunits on cation binding of Na,K pumps. On the other hand, it cannot be excluded that cytoplasmic α–β interactions contribute to some discrete steps in the catalytic cycle as suggested by Na^+ occlusion and electrogenic binding assays (Shainskaya *et al*., 2000), although selective truncation of the β subunit's first 14 N-terminal amino acids, which are assumed to interact with the cytoplasmic entrance to the alkali-metal cation sites (Shainskaya *et al*., 2000), does not affect the apparent affinity for internal Na^+ of Na,K pumps expressed in *Xenopus* oocytes (unpublished observations).

Although results from β chimeras and N-terminal truncations indicate that α–β interactions in the ectodomain are mainly responsible for the modulatory role of the β subunit, recent experimental evidence suggests that subunit interactions in the TM domain as well contribute to the determination of intrinsic NKA transport properties. Tryptophan substitution of two highly conserved tyrosine residues, which are aligned in the β1 subunit's TM α-helix, perturb the transport kinetics of Na,K-pumps in an additive way (Hasler *et al*., 2001). At present, it is not known which TM segment of the α subunit provides the putative interacting surface for the two tyrosine residues and may translate the β subunit interaction into a functional effect. The two tyrosine residues are on another face of the TM α-helix than the cysteine residue, which was shown to be closely located to M8 of the α subunit by using oxidative cross-linking (Or *et al*., 1999; Ivanov *et al*., 2000). Assuming that the membrane organization of NKA is similar to that reported from the crystal structure of the Ca-ATPase (Toyoshima *et al*., 2000) and that the cysteine residue of the β subunit's TM domain is positioned toward M8, then the two tyrosine residues of the β's TM domain would be directed toward M10 or alternatively M7 of the α subunit. Based on the observation that during heat denaturation, M8–M10 are released from the membrane to the extracytoplasmic side but M7 (which is close to the extracytoplasmic β interaction site in the α subunits M7 and M8 loop) and the β subunit's TM domain remain anchored in the membrane, the hypothesis was favored that the β subunit associates with M7 of the α subunit (Donnet *et al*., 2001).

Interestingly, the NKA β1 subunit's TM helix presents a third face, which is characterized by the presence of three aligned glycine residues, which are part of a well-characterized TM helix interaction motif, GxxxG, originally identified in glycophorin A (Brosig and Langosch, 1998). The interesting possibility arises that the GxxxG motif in β1 subunits may be implicated in NKA (α/β)2 dimer formation. However, if this is the case, monomeric and dimeric α-β complexes must exhibit only discrete functional differences, since mutational disruption of the putative β–β interaction motif produces Na,K pumps with similar transport properties than wild-type Na,K pumps (Hasler *et al*., 2001).

SPECIFICITY OF β INTERACTIONS

Despite differential, developmental, and tissue-specific regulation of the expression of different α and β isoforms (for review see Geering, 1998), several NKA or HKA α and β isoforms are coexpressed in certain cells providing the possibility for the formation of multiple α–β complexes. Promiscuous α–β interactions are, however, difficult to reconcile with the distinct physiological and pharmacological significance proposed for different isozymes (Blanco and Mercer, 1998; James *et al*., 1999; Crambert *et al*., 2000). Thus far, three NKA β isoforms and 1 gastric HKA β subunit, but no authentic β subunit for nongastric HKA have been identified. Efficient association, leading to functional Na,K pumps, is possible between the three NKA α and the three NKA β isoforms in artificial expression systems (Crambert *et al*., 2000; Blanco and Mercer, 1998; Schmalzing *et al*., 1997). Nevertheless, experimental data, using resistance to detergent dissociation of α–β complexes as criteria, indicate that some isozymes may have higher forces of interaction than others do and, therefore, may be formed preferentially *in situ*. For instance, α1–β1 and α2–β2 complexes are more resistant to Triton X-100 dissociation than α1–β2 complexes (Schmalzing *et al*., 1997; Geering *et al*., 2000) suggesting that these later complexes may be formed only when no α2 subunits are competing for assembly with β2 isoforms. It is likely that contact strength in NKA α-β complexes are determined directly by specific amino acids in α and β isoforms as well as indirectly by conformational diversity. Although little is known on such determinants, results obtained with truncated β isoforms containing only the ectodomain indicate that β ectodomain interactions with the M7 and M8 α loop may play a major role to direct NKA isoform-specific assembly (Schmalzing *et al*., 1997).

The efficient association of NKA α and gastric HKA α subunits with heterologous β subunits *in situ* appears to be subjected to particularly stringent constraints since no hybrid ATPases have been found in the stomach. Expression in heterologous systems suggests that formation of functional NKA α–HKA β or HKA α–NKA β complexes are prevented by different mechanisms. Also, it cannot entirely be excluded that thus far unknown stomach-specific factors are responsible for the lack of formation of heterologous ATPase complexes.

NKA α subunits can assemble (Lemas *et al*., 1994a; Hasler *et al*., 1998; Koenderink *et al*., 1999) and form

partially stable (Hasler *et al*., 1998, Eakle *et al*., 1992) detergent- (Geering *et al*., 2000) and trypsin-resistant (Horisberger *et al*., 1991; Noguchi *et al*., 1992) complexes with HKA β subunits that can leave the ER (Hasler *et al*., 1998) after expression in *Xenopus* oocytes, yeast or Sf9 cells. Several studies have now revealed that, despite assembly and partial folding, the NKA α–HKA β complexes are functionally deficient in terms of ouabain binding in the presence of ATP and Na^+ (Hasler *et al*., 1998), Na,K-ATPase activity (Ueno *et al*., 1997; Koenderink *et al*., 1999) and Na, K transport (Hasler *et al*., 1998). Analysis of NKA/HKA β chimeras suggests that α interactions with the loop between the first cysteine bond in the ectodomain of the NKA β subunit are important for folding events that lead to functionally active Na,K pumps (Ueno *et al*., 1995). Interestingly, however, Na,K pumps also become functionally active when expressed with HKA β subunits in which the TM domain was replaced with that of NKA β (Hasler *et al*., 1998). These apparently contradictory results again highlight the complexity and our poor understanding of the structural determinants and functional consequences of the α–β interaction event.

In contrast to NKA α and HKA β subunits, which can assemble and form stable, although inactive complexes that are expressed at the cell surface, gastric HKA α subunits imperatively need HKA β subunits to be efficiently expressed at the cell surface (Gottardi and Caplan, 1993). When HKA α subunits are coexpressed with NKA β1 subunits, only low H,K-pump activity is observed (Koenderink *et al*., 1999) probably because of inefficient subunit association, as reflected by the degradation of the HKA α subunit (Geering *et al*., 1994). Analysis of chimeras between NKA and HKA α subunits indicates that a 26 amino acid sequence, comprising amino acids from the M7/M8 extracytoplasmic loop and part of M8 of the HKA α subunit, and containing the highly conserved SYGQ motif, may be involved in the discrimination between HKA or NKA β interaction (Wang *et al*., 1997). In particular, a glutamine residue at position 904 in gastric HKA appears to be sufficient to prevent assembly of HKA α subunits with NKA β1 subunits (Wang and Farley, 1998). The prominent role of the ectodomain interactions in the specificity of β subunit assembly is also reflected by NKA/HKA β chimeras, in which the presence of the ectodomain of HKA β subunit is necessary and sufficient for the stabilization of the HKA α subunit (Geering *et al*., 1994) and the production of functional H,K pumps (Asano *et al*., 1999). While a nonconserved four amino acid stretch adjacent to the β subunits TM domain and the loop between the second cysteine bond are not discriminatory for the interaction of HKA β or NKA β subunits with the HKA α subunit, all other parts of the HKA β ectodomain appear to be important for specific HKA β–HKA α interaction (Asano *et al*., 1999). It is not known, however, to which extent each part contributes directly, or indirectly through conformational effects, to the α–β assembly site(s).

Another enigma concerning the specificity of β subunit interaction in oligomeric P-type ATPases is related to the intrinsic subunit composition of the nongastric H,K-ATPases. So far no authentic β subunit for nongastric H,K-ATPases has been identified. On the other hand, both NKA β1 (Kraut *et al*., 1998; Codina *et al*., 1998) and NKA β3 isoforms (Sangan *et al*., 1999) have been proposed to be the real partner subunit for nongastric H,K-ATPases, based on the recognition by β1 and β3 antibodies, respectively, of proteins which coimmunoprecipitated with nongastric HKA α subunits from colon or kidney microsomes. The identity of the β-proteins was, however, not verified by amino acid sequencing. In addition, several β subunits, e.g., gastric HKA β (Modyanov *et al*., 1995; Codina *et al*., 1996; Grishin *et al*., 1996; Grishin and Caplan, 1998; Cougnon *et al*., 1998), NKA β1 (Codina *et al*., 1996; Cougnon *et al*., 1999), the NKA β2-like *Bufo* bladder β (Cougnon *et al*., 1996), and the NKA β3 subunit (Sangan *et al*., 2000), coexpressed with nongastric HKA α subunits in *Xenopus* oocytes or mammalian cells, were shown to produce functional H,K-ATPases. Despite these data, evidence exists that none of the known β subunits is the authentic β subunit of nongastric HKA. Indeed, human, nongastric HKA α subunits, expressed alone in various mammalian cells, accumulate intracellularly, indicating that they cannot efficiently associate with the endogenous NKA β1 subunit (Grishin *et al*., 1996; Reinhardt *et al*., 2000). This observation probably reflects similar constraints evoked above for NKA α and β isoform interactions. A comparative analysis of the efficiency of assembly of different β subunits revealed that multiple subtle interaction events are necessary to ultimately lead to a correctly folded HKA or NKA and that nongastric HKA α subunits exhibit more stringent requirements for β assembly than NKA α subunits. Testing fundamental criteria for the correct folding of newly synthesized α subunits, e.g., whether newly formed α–β complexes are resistant to detergent treatment, to proteolysis and cellular degradation, and whether they can leave the ER, indicates that probably none of the known β subunits fulfills all requirements to act as a natural counterpart of nongastric HKA α subunits. However, an as yet unidentified β subunit of nongastric HKA should have structural features resembling those of HKA β, NKA β2, and/or β2-like *Bufo* bladder β subunits (Geering *et al*., 2000). A further conclusion, which can be drawn from our studies, is that in cells, which coexpress nongastric HKA α and NKA α subunits with NKA β1,

NKA β3 and/or HKA β subunits, these β subunits would preferentially associate with NKA α subunits. Formation of functional nongastric HKA, observed with certain β subunits in various expression systems, may be mainly due to significant overexpression of HKA α subunits.

Finally, the high complexity of the β assembly event is illustrated by the paradoxical observation that expression of particular β subunits in certain expression systems leads to the degradation of some coexpressed α subunits despite or possibly because of transient subunit assembly. For instance, NKA and HKA α subunits expressed in *Xenopus* oocytes associate with *Xenopus* HKA β subunits similar to rabbit HKA β subunits, but the *Xenopus* HKA β - NKA/HKA α complexes are rapidly degraded (Chen *et al*., 1998). Similarly, human and *Xenopus* β3 isoforms associate with nongastric HKA α subunits after coexpression in *Xenopus* oocytes, but only *Xenopus* β3 isoforms can form stable α–β complexes whereas human β3 subunits lead to the specific degradation of nongastric HKA α subunits (Geering *et al*., 2000). It is possible that these results reflect the need of tissue-specific factors to facilitate efficient interactions of certain α–β combinations.

CONCLUSIONS

As outlined in this review, much progress has been made in the understanding of the role of the β subunit in oligomeric P-type ATPases, such as Na,K- and H,K-ATPases and in the elucidation of the mechanistic details of the β assembly process and its structural and functional consequences on the catalytic α subunit. Nevertheless, the question remains why among P-type ATPases only Na,K- and H,K-ATPases and bacterial Kdp-ATPases are associated with additional subunits. An interesting hypothesis is that their indispensable presence in these P-type ATPases may be related to the K^+-transport function, which is a common and unique feature of oligomeric P-type ATPases. The primary role of the β subunit is to act as a specific chaperone which favors the correct membrane insertion and folding of Na,K- and H,K-ATPase α subunits. Since KdpC subunits of the bacterial KdpFABC transporter may have a similar function (Altendorf and Epstein, 1994), it has been speculated that the β subunits of Na,K-and H,K-ATPases may be remnants of the bacterial KdpC subunit that have been eliminated in other P2-ATPases (Axelsen and Palmgren, 1998). The K^+-transport function, common to oligomeric P-type ATPases, may require particular sequence information in certain TM segments, which is incompatible with their proper membrane integration. β subunits may have evolved as helper proteins to overcome the sequence constraints of K^+-transporting P-type ATPases and permitting their membrane integration.

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