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Topical Review

Topogenic Motifs in P-type ATPases

K. Geering
Institut de Pharmacologie et Toxicologie, Université de Lausanne, rue du Bugnon 27, CH-1005 Lausanne, Switzerland

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Introduction

P-type ATPases comprise a superfamily of ubiquitous pumps that are involved in the transport of charged substrates across biological membranes including K^+, Na^+, H^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and phospholipids. Most of these P-type ATPases function with a single subunit but some are two or multi-subunit complexes. Transport of substrates involves at least two conformational states E1 and E2 and is driven by the energy of the hydrolysis of ATP. The distinctive feature of P-type ATPases is the formation of a covalent phosphoryl-enzyme intermediate in the catalytic cycle. The phosphorylation site is an aspartic acid in the second cytoplasmic loop of catalytic α subunits located in the invariant sequence DKTG which together with 7 other conserved regions defines the P-type ATPases family (Axelson & Palmgren, 1998). These 8 domains of the polypeptide are believed to be involved in general functions common to all P-type ATPases such as ATP binding, phosphorylation, coupling of energy from ATP hydrolysis to conformational changes and ion translocation (for review *see* Moller et al., 1996).

Diversity of Membrane Topology Among P-type ATPases

For most P-type ATPases, the exact topology of the membrane domain that permits specific substrate transport, is not known. Comparison of hydropathy profiles suggests that the membrane topology of P-type ATPases correlates with the type of ion transported. This has led to the distinction of P1-ATPases (heavy metal pumps) and P2-ATPases (nonheavy metal pumps and phospholipid translocases) with very distinct hydropathy profiles and P3-ATPases (bacterial K-pumps) which share structural features with both P1- and P2-ATPases (Lutsenko & Kaplan, 1995). A more stringent analysis including sequence comparison and alignment of conserved core sequences of 159 catalytic subunits divides P-type ATPases into 5 major branches with several subfamilies including Type I (heavy metal pumps, K-pumps of bacteria), Type II (Ca-, Na,K-, H,K-pumps), Type III (H- and Mg-pumps), Type IV (phospholipid pumps) and Type V ATPases (pumps with no assigned function) (Axelson & Palmgren, 1998). According to this classification and the presence or absence of these ATPases in bacteria, archaea and/or eukarya, it appears that P-type ATPases did not evolve at a constant rate and that acquisition of new ion specificity may have involved abrupt changes in the rate of sequence adaptations (Axelsen & Palmgren, 1998).

Regardless of the classification adopted, the structure of all P-type ATPases can be dissected into a core or minimal domain which is common to all P-type ATPases and into additional variable regions (Fig. 1). The core unit in all P-type ATPases comprises 3 putative transmembrane pairs and several connecting loops which together encompass the 8 basic motifs of P-type ATPases (Axelsen & Palmgren, 1998). Apart from this core unit, P-type ATPases significantly diverge in their predicted structure, for instance in the most N-terminal, cytoplasmic region and in the putative membrane domain. According to Lutsenko and Kaplan (1996), hydropathy pro-

Correspondence to: K. Geering

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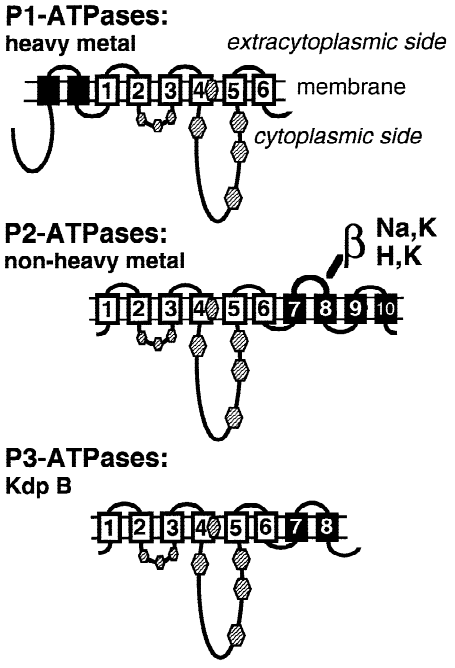

Fig. 1. Putative membrane topology of different P-type ATPase α subunits. The 6 membrane domains of the core structure are shown in white and the additional membrane segments in black. Diamonds indicate the approximate position of the 8 signature sequences of P-type ATPases which distribute in the cytoplasmic loop between transmembrane segment M2 and M3, in M4 and in the large, cytoplasmic loop connecting M4 and M5. In P2-ATPase α subunits, the interaction site of β subunits in the extracytoplasmic loop between M7 and M8 of Na,K- and H,K-ATPase α subunits is indicated. For more details *see* text.

files predict that heavy metal pumps (P1-ATPases) have one additional N-terminal membrane pair while non-heavy metal (P2-ATPases) and bacterial Kdp-ATPases (P3-ATPases) have 2 or 1 putative, additional C-terminal membrane pairs, respectively. A large body of biochemical and immunological evidence (for review *see* Moller et al., 1996) and recent analysis of two-dimensional crystals of H-ATPases and of Ca-ATPases (for review *see* Kühlbrandt et al., 1998) have led to the consensus that at least H-, Ca-, Na,K- and H,K-ATPases have 10 transmembrane segments most likely all α-helices. These data support the existence of 3 core membrane pairs in all P-type ATPases and the existence of additional C-terminal membrane segments in most non-heavy metal pumps. Finally, a particular feature which characterizes Na,K-, H,K- and bacterial K-ATPases is that these ATPases, in contrast to all other ATPases, are oligomeric proteins. Na,K- and H,K-ATPases contain a β subunit in the active enzyme and bacterial K-ATPases 3 subunits in addition to the catalytic α subunit.

These data raise several questions concerning the topogenesis of the different P-type ATPases. What are the mechanisms of membrane topogenesis that permit different P-type ATPases to adopt a similar topology of the core domain (M1–M6) despite low sequence identity? Since in P2-ATPases, no direct function in cation binding and transport of the additional C-terminal segments has so far been documented, is it possible that this membrane domain has evolved to assist the topogenesis process in these ATPases and in the same line, are the additional subunits, in particular the β subunit of Na,K- and H,K-ATPases that are not directly implicated in the transport function, helper proteins necessary for the structural maturation of these particular ATPases?

Recent studies on the biosynthesis and topogenesis of individual and combined membrane segments of various P-type ATPases have provided some, but still incomplete, answers to these questions. The results focus on the presence, in certain transmembrane segments, of specific topogenic information that is required for the transport of specific ions but which dictate the necessity in some P-type ATPases of additional transmembrane segments or even additional subunits to achieve a correct membrane packing of the basic core structure.

Mechanisms Involved in the Topogenesis of Polytopic Membrane Proteins

Integral membrane proteins destined for the plasma membrane or the membranes of other cellular compartments are inserted into the lipid bilayer at the level of the ER (Fig. 2). This cotranslational membrane insertion is initiated in the cytosol when a signal recognition particle (SRP) recognizes a signal sequence which emerges from the translating ribosome (for review *see* Bernstein, 1998). The complex is then targeted to the ER membrane by interaction of SRP with the SRP-receptor and of the ribosome with the Sec61/Tram complex that is part of the translocon. This polar transmembrane channel permits passage of hydrophilic sequences across the membrane but also allows transfer of hydrophobic sequences into the lipid bilayer by lateral translocation (for review *see* High et al., 1997).

A transmembrane α helix that is compatible with stable association with the lipid bilayer contains 15–30 residues and a threshold overall hydrophobicity. Typically, it has a central region rich in aliphatic amino acids which is bordered by aromatic residues and then charged residues and which ends with so-called cap residues such as Pro, Gly and Asn (Wallin et al., 1997). Nevertheless, transmembrane domains often contain helix-breaking residues such as glycine and proline as well as hydrophilic or even charged residues in the central region (for review *see* Deber and Goto, 1997). These residues are important determinants of the tertiary structure in polytopic membrane proteins and are likely to be involved in transport functions or signaling of transport proteins or receptors, respectively, but at the same time they may

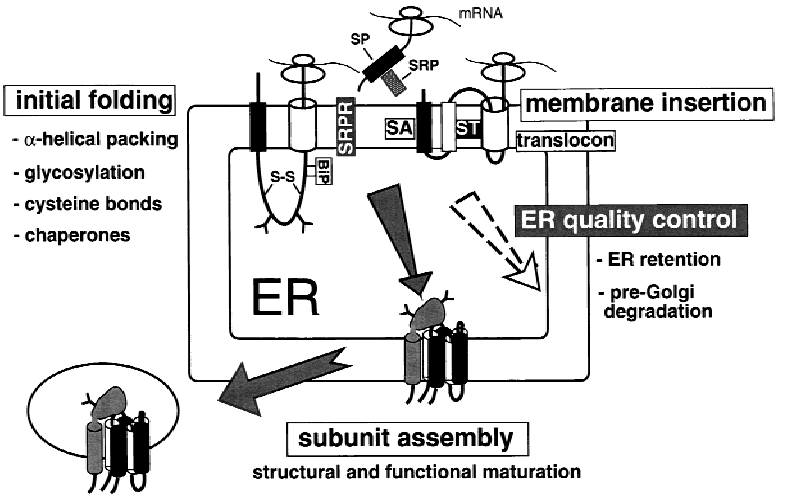

Fig. 2. Biosynthesis, membrane insertion and ER quality control of membrane proteins. The synthesis of membrane proteins starts in the cytosol from mRNA translated on free ribosomes. After interaction of a signal recognition particle (SRP) with a signal peptide (SP) emerging from the ribosome, the nascent polypeptide is targeted to the ER membrane by interaction with a signal recognition particle receptor (SRPR) on the ER membrane. A polar transmembrane channel, the translocon permits the passage of hydrophilic protein domains to the ER lumen as well as the membrane integration of hydrophobic segments by lateral translocation into the lipid bilayer. For polytopic membrane proteins, it has been suggested that they integrate into the lipid bilayer by a series of alternating signal anchor (SA) and stop transfer (ST) sequences. During membrane insertion, membrane proteins are subjected to an initial folding process that is mediated by α-helical packing, glycosylation, cysteine bond formation and interaction with molecular chaperones such as BiP. In addition, in oligomeric proteins, the maturation process is completed by assembly of partner subunits. Each step of this maturation process in the ER is necessary for intracellular routing e.g., for ER exit and for the acquisition of functional properties of the newly synthesized protein. The ER exerts an efficient quality control on misfolded forms of proteins which can be produced because of a mutation or because a partner subunit of an oligomeric protein is missing. Misfolded proteins are recognized, their exit from the ER is prevented and their final fate is degradation. For further details *see* text.

impede integration of membrane domains into the lipid bilayer making accessory, helper mechanisms necessary for this process.

Membrane domains can insert into the membrane in two orientations either bearing the N-terminus in the cytoplasm and the C-terminus in the extracytoplasmic side (Ncyt/Cout) or in the inversed configuration (Nout/Ccyt). The mechanisms by which the topology of a protein, especially of polytopic membrane proteins, is achieved are complex and only partially elucidated. According to a simple model, after insertion of a first hydrophobic sequence into the membrane, following hydrophobic sequences are inserted sequentially while alternating their orientation (Blobel, 1980). However, intrinsic features of topogenic sequences have been shown to affect the orientation of a membrane domain. An important determinant of the direction of the insertion is the distribution of charged residues flanking the hydrophobic sequences ('positive-inside rule,' Wallin & von Heijne, 1998 or 'charge difference hypothesis,' Hartmann et al., 1989). According to the 'positive-inside rule,' signal anchor (SA) sequence topology with a Ncyt/Cout orientation is favored by N-terminal positive charges while stop transfer (ST) sequence topology with a Nout/Ccyt orientation is favored by C-terminal positive charges. Other features including the length of the hydrophobic segment and the folding state of adjacent domains may also contribute to the topology (for review *see* Hegde and Lingappa, 1999). Furthermore, in the case of polytopic proteins, interactions between closely spaced hydrophobic segments that would lead to the formation of a helical hairpin prior to membrane insertion should also be considered as a topogenic determinant.

Based on the observation that several proteins do not follow these general rules and some proteins even can adopt multiple topologies (Zhang & Ling, 1995), it was recently suggested that the topogenic process may not only be determined by sequence determinants in the nascent polypeptide but may also be regulated by components of the translocation machinery (Hegde & Lingappa, 1999). Such regulatory mechanisms, mediated by selectively activated or inactivated, accessory factors including components of the translocon, cytosolic and lumenal molecular chaperones or processing enzymes, could reduce or favor the insertion efficiency of strong or weak hydrophobic sequences, respectively, and could, in principle, also dictate their membrane orientation. Finally, acquisition of the ultimate three-dimensional topology that is compatible with function of transport proteins or hormonal receptors needs additional folding mechanisms which consist in intramolecular and often intermolecular packing of transmembrane helices (Lemmon et al., 1997). These helix-helix interactions can be relatively nonspecific but in some cases where complementary helix surfaces are involved (e.g., formation of salt bridges between charged residues) may become highly specific.

Membrane Topogenesis of the Core Structure of P-type ATPases

Membrane topogenesis has been studied for P1-ATPases such as bacterial Cu-ATPases and for several P2-ATPases such as yeast H-ATPase and mammalian, SERCA Ca-, gastric H,K- and Na,K-ATPases. So far no

such analysis has been performed for P3 bacterial Kdp-ATPases. In most studies, the glycosylation reporter scanning (RGS) assay has been used to define the topogenic properties of transmembrane segments of P-type ATPases. This assay exploits the fact that N-glycosylation of newly synthesized proteins only occurs in the ER lumen. A protein moiety containing consensus glycosylation sites is added to the C-terminal end of individual transmembrane segments or truncated α-proteins of different length and the glycosylation state of the various constructs is followed. The presence or absence of glycosylation indicates whether the C-terminal of a translated sequence ends on the ER lumenal or the cytoplasmic side and thus whether a transmembrane segment acts as a signal anchor (SA) or a stop transfer (ST) sequence. Glycosylated proteins can be distinguished from nonglycosylated proteins by their differences in the migration on SDS-polyacrylamide gels and by their sensitivity to endoglycosidase H which specifically cleaves N-linked high mannose core sugars. RGS assays have been performed in in vitro translation systems supplemented with ER microsomes as well as in intact cells, in the *Xenopus* oocyte expression system. Comparative studies have shown that membrane insertion efficiency and orientation properties of certain membrane domains can slightly differ in intact cells compared to in vitro translation systems. This is likely due to the longer synthesis time allowed in intact cells compared to that used in in vitro translations which may be necessary for the steady-state level of folding and membrane insertion of certain membrane segments. Thus, only in vivo studies permit to distinguish between efficient, complete and inefficient, partial membrane insertion of a particular membrane segment.

Topogenesis of the 2 N-Terminal Membrane Pairs of the Core Structure

Early studies on the biosynthesis of Na,K-ATPase α subunits performed in in vitro translation systems supplemented with ER microsomes provided evidence that initial membrane insertion occurs cotranslationally (Geering et al., 1985). Furthermore, since translation products of M1–M2 and M3–M4 constructs were alkali-resistant, it was concluded that at least two SA sequences must be contained within the four N-terminal transmembrane segments of the Na,K-ATPase α subunit (Homareda et al., 1989). The insertion properties of M1–M4 of several P2-ATPase α subunits were later analyzed by using the RGS assay. In vitro translation assays as well as in vivo biosynthesis studies provided compelling evidence that in SERCA Ca-ATPase (Bayle et al., 1995), Na,K-ATPase (Xie & Morimoto, 1995; Béguin et al., 1998) and gastric H,K-ATPase (Bamberg & Sachs, 1994; Beggah et al., 1999) α subunits, the first 2 N-terminal membrane pairs are formed by sequential insertion of M1 up to M4 acting as alternating SA and ST sequences. In vitro as well as in vivo, all 4 N-terminal membrane segments of H,K-ATPase α subunits can insert individually into the membrane and can adopt both Ncyt/Cout or Ccyt/Nout orientation under appropriate conditions (Bamberg & Sachs, 1994; Beggah et al., 1999). This implies that in the nascent polypeptide, the ultimate orientation of M2, M3 and M4 in the membrane is dictated by the orientation of the preceding membrane segment. This does not exclude that the ST activity of certain membrane segments, such as M4, may be favored by the presence of positively charged residues located downstream of its C-terminal end (Beggah et al., 1999). The determinants that permit the first transmembrane segment in these ATPases to adopt the correct membrane orientation with its N-terminus anchored on the cytoplasmic side remain to be explored. Indeed, only Na,K- and H,K-ATPases α subunits have a net positivity in the sequence preceding the first transmembrane segment which according to the 'inside positive rule' (Wallin & von Heijne, 1998) may favor an Ncyt/Cout orientation of membrane segments. In the SERCA Ca-ATPase α subunit sequence, for instance, negatively charged residues predominate in the cytoplasmic tail. The topogenic signal for Ncyt orientation of M1 may be the 'negative outside' distribution of Asp and/or Glu residues in all these P-type ATPases which is characteristic of the most N-terminal transmembrane segment in several eukaryotic, polytopic membrane proteins (Hartmann et al., 1989).

Though all P2-ATPases have 2 N-terminal membrane pairs as part of the core structure, their formation may not always be mediated by sequential membrane insertion of M1–M4 as in Na,K-, H,K- and SERCA Ca-ATPase α subunits. For instance, neither M1, M2 or M4 membrane domains of yeast plasma membrane H-ATPases can insert independently into membranes and only co-expression of M1 and M2 or M3 and M4 permits membrane pair formation and insertion (Lin & Addison, 1995*a*). It is likely that specific sequence information within these membrane domains of H-ATPases is responsible for their inefficient membrane insertion. Compared to Ca-, Na,K- or gastric H,K-ATPases, the M1 domain of H-ATPases contains, for instance, a proline residue in addition to a negatively charged residue, the M2 domain is richer in glycine residues and H-ATPase M4 has the lowest overall hydrophobicity among all P2-ATPases. The proposed mechanisms to facilitate membrane insertion of the 2 N-terminal membrane pairs in H-ATPases consists in the antiparallel arrangement of M1/M2 and M3/M4 prior to membrane insertion to form hairpin structures (Lin & Addison, 1995*a*) which may mask hydrophilic residues and annul the helix-breaking effects of proline and glycine residues. At least for M1/

M2, hairpin arrangement may be favored by their close positioning due to a very short connecting loop which only contains 4 amino acids including a flexible helix-braking glycine residue.

Despite differences in the mechanisms of membrane insertion, topogenesis studies on P2-ATPases indicate that the first 2 N-terminal membrane pairs of the core structure of P-type ATPases are stably inserted into the lipid bilayer during the synthesis of these proteins. This concept may indeed also apply to the topogenesis of bacterial P1-ATPases. Results from in vitro RGS assays suggest that the 6 N-terminal membrane segments of Cu-ATPases including the 2 N-terminally located, additional membrane segments characteristic of P1-ATPases and the 4 membrane domains of the core structure probably insert into the membrane by an alternating SA/ST mechanism (Melchers et al., 1996; Bayle et al., 1998).

Topogenesis of the Third C-Terminal Membrane Pair of the Core Structure

The question of the topogenesis of the last membrane pair of the core structure is particularly interesting. This domain is separated from the N-terminal, core membrane domain by the large cytoplasmic loop and together with M4 has been implicated in the transport function of P-type ATPases. The involvement in ion transport suggests that this domain may undergo conformational changes and thus may have an intrinsic 'flexibility.' Experimentally, this flexibility has been documented in Na,K- (Lutsenko et al., 1995) and H,K-ATPase (Gatto et al., 1999) α subunits by showing that the M5/M6 membrane domain is selectively released to the extracellular side upon extensive trypsinolysis but only in the absence and not in the presence of potassium.

Flexibility of a membrane domain is a reflection of a particular amino acid composition rich in helix-breaking and/or charged residues that are likely to impose severe constraints on the membrane insertion during synthesis. Sequence analysis shows that the last 2 membrane segments of P1-ATPases contain one highly conserved proline residue in the C-terminal and the N-terminal end, respectively, but no charged residues. Furthermore, overall hydrophobicity of the last membrane segment of P1-ATPases is higher (about 85% hydrophobic amino acids) than that of M6 of P2-ATPases (62–74% of hydrophobic residues). The predicted loop linking the 2 last membrane segments in P1-ATPases contains 1 proline residue and is twice as long as the M5/M6 loop of P2-ATPases. Though still insufficient, data from recent topogenesis studies on *H. pylori* CopA ATPase (Bayle et al., 1998) are consistent with the idea that the amino acid composition of the last 2 membrane segments of P1-ATPases permits membrane integration by an alternating SA/ST mechanism or after hairpin formation.

Compared to P1-ATPases, the amino acid composition of M5 and M6 of P2-ATPases appears less compatible with membrane insertion. In most P2-ATPases, M5 is particularly rich in proline and/or charged residues and M6 in charged residues. M5 of Na,K- and H,K-ATPase α subunits, for instance, contains 2 proline and 1 and 2 charged residues, respectively, in the central region. In contrast to M1 and M3 which have a highly efficient SA function and are completely membrane-inserted, the SA activity of M5 determined in vivo in truncated M1–5 α-proteins of Na,K- (Béguin et al., 1998) and gastric H,K-ATPases (Beggah et al., 1999) is only partial. Only 5 and 50% of the total M5 population of Na,K- and H,K-ATPase, respectively, are found inserted in the membrane. In in vitro translations, M5 of H,K-ATPase have no SA activity at all (Bamberg & Sachs, 1994). Mutations of the 2 proline residues in M5 of Na,K-ATPase M1–5 α-proteins improve considerably M5 membrane insertion in vivo (Béguin et al., 1998) indicating that these two proline residues are at least partially responsible for the poor membrane insertion efficiency of M5. Another important topogenesis signal in P2-ATPases is provided by the very short extracytoplasmic M5/M6 loop which contains negatively charged aspartic acid and/or proline residues. Na,K- and H,K-ATPase α subunits have 2 proline residues in the 4 amino acid long, conserved M5/M6 loop. Mutations of these proline residues in M5 of Na,K-ATPase α subunits permits full SA function of M5 but, at the same time, impedes ST activity of M6 and the proper membrane insertion of all following membrane segments which, with the exception of M10, become exposed to the ER lumenal side (Béguin et al., 1998).

Altogether, these results indicate that, due to a particular amino acid composition of the M5/M6 domain which is likely to be related to the ion transport function, the membrane insertion of the last membrane pair of the core structure of Na,K- and H,K-ATPase α subunits must proceed by a different mechanism than the classic alternating SA/ST mechanism determined for the N-terminal membrane domains. Proper membrane insertion of the M5/M6 membrane pair of Na,K- and H,K-ATPase α subunits probably requires prior masking of the helix-breaking internal proline and charged residues. This could be achieved by hairpin formation of M5 and M6 which should be strongly favored by the short M5/M6 loop and the presence in there of kink-forming proline residues. RGS assays in vivo show that addition of M6 to M1–5 α-proteins of Na,K- (Béguin et al., 1998) or H,K-ATPases (Beggah et al., 1999) abolishes their partial glycosylation which is consistent with M5/M6 hairpin formation. It remains to be shown whether M5/M6 hairpin formation of H,K-ATPases is favored by salt bridge formation between the Lys and the Glu residues present in M5 and/or the Lys residue in M5 and the Asp

residue in M6. Such a mechanism could explain the more efficient membrane insertion of M5/M6 of gastric H,K-ATPase compared to that of M5/M6 of Na,K-ATPase which have no positively charged residue in M5.

Hairpin formation of M5/M6 in Na,K- and H,K-ATPase α subunits may be necessary but probably is not sufficient for correct membrane insertion of the M5/M6 pair. Indeed, recent studies show that M1–6 α-proteins of Na,K-ATPase expressed in intact cells are rapidly degraded which contrast with M1–4 α-proteins including the large cytoplasmic loop which are stable (P. Béguin, U. Hasler, O. Staub and K. Geering, *submitted*). This result indicates that M1–6 α-proteins are recognized by the ER quality control system which is responsible for the elimination of proteins that are misfolded (for review *see* Kopito, 1997). A degradation signal was mapped to the 2 proline studies in the M5/M6 loop which is recognized from the cytoplasmic side. The degradation of the M1–6 α-protein implies that the M5/M6 pair in this truncated α-protein is not membrane-inserted and suggests that its stable membrane insertion needs, in addition to the specific M5/M6 interaction, intramolecular interactions with downstream membrane segments.

The experimental data are still too few to confirm a general role of downstream membrane segments for the proper membrane insertion of the M5/M6 pair of P2-ATPases. In vitro RGS assays suggest that M5/M6 membrane insertion of SERCA Ca-ATPase, similar to M5/M6 of Na,K- and H,K-ATPase α subunits, does not proceed according to an alternating SA/ST mechanism since M5 has no SA activity. On the other hand, under similar experimental conditions, M5 of H-ATPase show SA function suggesting that M5/M6 membrane insertion may occur cotranslationally (Lin & Addison, 1995*b*). Since topogenesis of these ATPases has only been studied in in vitro translation systems, the real efficiency of membrane insertion of M5 remains, however, unknown. Sequence analysis and recent mutational analysis of M5 and M6 of H-ATPase would predict that M5 of these two ATPases may use a similar mechanism of insertion. Both these ATPases indeed contain a positively and a negatively charged residue in M5 and a negatively charged residue in M6 which is conserved in P2-ATPases. Mutational analysis has shown that single neutral substitution of Arg-695 in M5 or of Asp-730 in M6 leads to defects in protein folding of H-ATPases (Dutra et al., 1998). Since these defects could be overcome when the two amino acids were exchanged for one another or when both were replaced by alanine residues, it was concluded that Arg-695 and Asp-730 form a salt bridge which links M5 and M6 (Gupta et al., 1998). These results provide evidence that the negative charge in M5 of H-ATPase and probably also of SERCA Ca-ATPase impedes membrane insertion and must be masked prior to membrane insertion by helix packing involving salt bridge formation between M5 and M6. From the available data, it cannot be decided whether formation of a salt bridge-stabilized hairpin structure is sufficient as a topogenesis signal for M5/M6 of H- and Ca-ATPases or whether stable membrane insertion of this domain depends on additional interactions with distal membrane segments as it is the case in Na,K and H,K-ATPases. Perhaps, the constraints for membrane insertion of M5/M6 of Ca- and H-ATPases are indeed less stringent than for M5/M6 of Na,K- and H,K-ATPases since the former ATPases have no helix-breaking proline residues in M5.

Topogenesis of the C-terminal Membrane Pairs in P2-ATPases and the Role of the β Subunit

The dependence of the membrane insertion of the M5/M6 membrane pair of Na,K-ATPase and possibly of other P2-ATPases on interactions with distal membrane segments raises the question of the topogenesis of the 2 additional C-terminal membrane pairs and of the particular role in this process of the β subunit which is associated with Na,K- and H,K-ATPase α subunits in this C-terminal domain.

Topogenesis of M7 up to M10 of H-ATPase and SERCA Ca-ATPase that do not contain a β subunit have only been studied in in vitro translations. In vitro, individual M7 of both ATPases act as SA sequences (Lin & Addison, 1995*b*; Bayle et al., 1995) which is compatible with the absence of proline and charged residues in the central part of these domains. M8 of Ca-ATPase shows ST activity when expressed individually but in combined M7/M8 α-proteins, M8 of Ca- (Bayle et al., 1995) and H-ATPase (Lin & Addison, 1995*b*) cannot act as efficient ST sequences in in vitro translations. As to the mechanism of membrane insertion of the last membrane pair, it was shown that M9 of SERCA Ca-ATPase has (Bayle et al., 1995) but that of H-ATPase has no SA activity (Lin & Addison, 1995*b*). Compared to M9 of SERCA Ca-ATPase, M9 of H-ATPase has lower hydrophobicity and contains a charged residue which could explain its lack of SA function in in vitro translations. Finally, individual M10 can act as a ST signal in SERCA Ca-ATPase but only as a SA signal in H-ATPase. M10 of both ATPases are, however, able to form a membrane-inserted pair if combined with M9. Though definitive conclusions cannot yet be drawn, the in vitro RGS assays suggest that (i) in SERCA Ca-ATPase, all membrane segments are cotranslationally inserted into the membrane with the exception of M5, M6 and M8 which may necessitate hairpin formation and/or interactions with succeeding membrane domains for proper membrane insertion and (ii) in H-ATPase, all membrane segments are post-translationally inserted after hairpin formation dependent or not on other intramolecular interactions with

the exception of M7 which may be cotranslationally inserted.

Based on the observations that only β subunit-assembled α-subunits of Na,K- and H,K-ATPases become trypsin resistant, are protected from cellular degradation and become functionally active (Geering et al., 1996; Chen et al., 1998), it was anticipated that the β subunit plays a role as a specific molecular chaperone necessary for the correct packing of Na,K- and H,K-ATPase α subunits. β subunits are type II membrane proteins which have one transmembrane domain, a short cytoplasmic N-terminal tail and a large ectodomain containing 3 disulfide bridges and several sugar chains. So far, 3 β isoforms of Na,K-ATPase and 1 β subunit of gastric H,K-ATPase have been identified while the authentic β subunit of nongastric H,K-ATPases is not known. Results obtained with the two-hybrid system have provided evidence that a conserved SYGQ motif in the extracellular loop linking M7 and M8 in Na,K-ATPase (Colonna et al., 1997) and H,K-ATPases (Melle-Miovanovic et al., 1998) α subunits is sufficient for the interaction with the β subunit.

Why, in contrast to other P2-ATPases, β assembly may be necessary for the correct packing of Na,K- and H,K-ATPase α subunits and how does it influence the topogenesis of the C-terminal membrane segments?

RGS assays on truncated M1–7 α-proteins of Na,K-, and gastric H,K-ATPases expressed in *Xenopus* oocytes without β subunits show that M7 of the Na,K-ATPase α subunit has only partial (Béguin et al., 1998) and that of the gastric H,K-ATPase α subunit has no detectable SA activity (Beggah et al., 1999). These results indicate that membrane insertion of M7 of Na,K- and H,K-ATPase α subunits must proceed by a different mechanism than the predicted cotranslational membrane insertion of M7 of H- and Ca-ATPase α subunits. Sequence analysis does not reveal any obvious reason for the observed differences in membrane insertion of M7 of different P2-ATPases except for M7 of gastric H,K-ATPase α subunits which contains exclusively a charged residue in the central region that could explain the lack of its membrane insertion compared to the partial insertion of M7 of Na,K-ATPase α subunits. The poor membrane insertion efficiency of M7 of both Na,K- and H,K-ATPase α subunits may, at least in part, be determined by the presence of conserved helix-breaking glycines or polar amino acids because when these residues are replaced with hydrophobic amino acids, M7 of Na,K-ATPase α subunits behaves as a fully effective SA sequence. Similar to M5 and M7, M9 of both Na,K- and H,K-ATPase α subunits have only partial SA properties. In contrast to M7, M9 of Na,K-ATPase α subunits does not retain its partial SA function when added to a M1–2 α-protein indicating that M7 of Na,K-ATPase α subunits has intrinsic, though partial SA function while M9 depends for its correct membrane insertion on the flexibility of the preceding membrane segment and on interactions with M10. M9 of Na,K-ATPase α subunits contains two negatively charged and a polar residue in the N-terminal region which may in part be responsible for its limited insertion efficiency since when these residues are changed to leucine, M9 has full SA activity.

In RGS assays performed in intact cells, M6, M8 of Na,K-ATPase and M10 of Na,K- and H,K-ATPases abolish glycosylation of M1–5, M1–7 and M1–9 α-proteins, respectively, indicating that M6, M8 and M10 can form a pair with the preceding hydrophobic sequence. However, since the SA function of M5, M7 and M9 is not complete, the RGS assay does not permit the decision of whether the helical pairs are membrane-inserted or released to the cytoplasmic side. Taking into consideration that individual α subunits of Na,K- and H,K-ATPase are misfolded proteins which are highly susceptible to degradation, a likely model for their membrane topography is that the C-terminal membrane pairs are in a dynamic equilibrium between the cytoplasm and the membrane or more precisely the translocon (Fig. 3).

For Na,K-ATPase, there is evidence that interaction of the β subunit with the M7/M8 extracytoplasmic loop is necessary for the efficient membrane insertion and retention of the M7/M8 pair and in consequence the correct packing of the entire α subunit. M1–8 α-proteins of Na,K-ATPase which are unstable in the absence of β subunits, become stable when expressed with β subunits. This result suggests that β-assembly favors membrane insertion of the M7/M8 pair which in consequence promotes correct packing and insertion of the M5/M6 pair and masks degradation signals (P. Béguin, U. Hasler, O. Staub, K. Geering, *submitted*).

β-assembly not only enables M7/M8 membrane retention but may also be implicated in its correct packing. M8 *per se* is indeed an inefficient ST sequence. A small reduction of its hydrophobicity, by changing existing Ile and Val residues to Ala, significantly decreases the ST activity of Na,K-ATPase M8 (Béguin et al., 1998). Furthermore, addition of M8 together with its preceding β-assembly domain to a membrane-fixed segment induces release of M8 to the ER lumenal side. In all these α-proteins, association of the β subunit renders M8 an efficient ST sequence. According to these results, it can be postulated that the extracytoplasmic loop between M7/M8 is a flexible structure which after interaction with the β subunit responds with a conformational change that favors retention of M8 in the membrane and in consequence M7/M8 membrane pair formation.

β-association may play a similar role in the formation of the M7/M8 pair of H,K-ATPase but compared to Na,K-ATPase, membrane insertion of the H,K-ATPase M7/M8 pair is subjected to more stringent constraints (Fig. 3). In contrast to Na,K-ATPase α-proteins, only

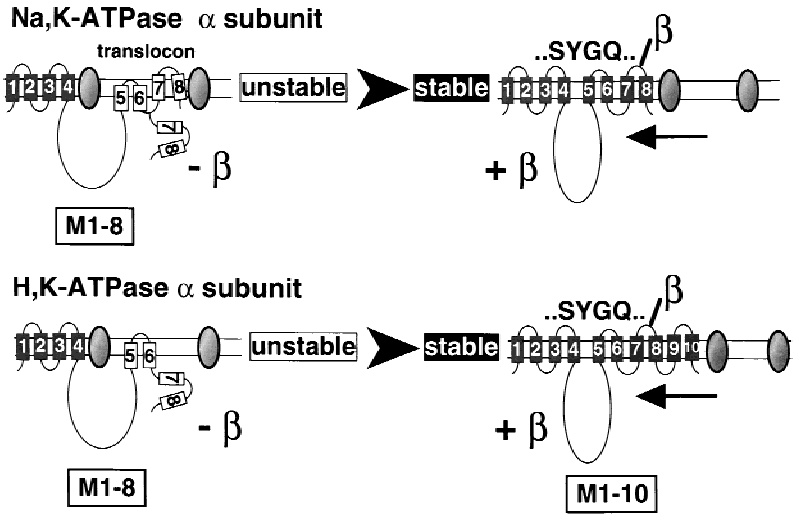

Fig. 3. Models for membrane insertion of unassembled Na,K- and H,K-ATPase α subunits and effects of β subunit assembly. In Na,K- and H,K-ATPase α subunits, the 4 N-terminal hydrophobic segments behave as efficient, alternating SA/ST sequences and permit for the formation of two membrane pairs that are likely to be transferred from the translocon into the lipid bilayer during or soon after their synthesis and which function as a stable N-terminal anchor of the nascent α-proteins. In contrast to the N-terminal membrane segments, the membrane insertion of the C-terminal membrane domains is very inefficient, mostly due to specific sequence information within or near the hydrophobic sequences that reduces interaction with nonpolar surfaces. Inefficient membrane insertion of the C-terminal membrane segments is responsible for misfolding and degradation of α-proteins which are synthesized without a β subunit. Association of the β subunit with a short stretch of amino acids in the M7/M8 extracellular loop of the α subunit containing a conserved SYGQ motif is the key event for the correct packing of the C-terminal membrane domain and in consequence for an overall stabilization of the α subunits of Na,K- as well as H,K-ATPase. However, the prerequisites for β assembly with the M7/M8 loop differ among Na,K- and gastric H,K-ATPase α subunits. M7 in Na,K-ATPase α subunits has poor membrane insertion properties but nevertheless it is partially inserted into the membrane during synthesis which permits exposure of the M7/M8 assembly loop to the extracytoplasmic side for β interaction. Thus, in Na,K-ATPase, β interaction and stabilization of the α-protein can already occur in a nascent M1–8 Na-K-ATPase α-protein by initially favoring the formation of the M7/M8 membrane pair and in consequence the correct packing of the entire α subunit and its integration into the lipid bilayer. On the other hand, in H,K-ATPase, M7 has no signal anchor function at all. The exposure of the M7/M8 β assembly domain to the extracytoplasmic side is only possible after synthesis of the M9/M10 membrane pair that supports the formation of the M7/M8 membrane pair. This explains why H,K-ATPase α subunits are only stabilized after complete synthesis of the α protein. For further details *see* text.

M1–10 but not M1–M8 H,K-ATPase α-proteins are completely stabilized after co-expression with β subunits (Beggah et al., 1999). This difference in the stabilizing effect of β subunits in H,K-ATPase α subunits is likely to be a reflection of the very poor SA function of M7 which lowers the probability of β-assembly with the extracellular loop in H,K-ATPase compared to that in Na,K-ATPase. The results also suggest that M9/M10 pair formation plays a different role in the correct packing of Na,K- and H,K-ATPase α subunits. In H,K-ATPase α subunits, M9 membrane insertion is favored after co-expression with β subunits but only M1–10 are completely stabilized. This infers that M9/M10 pair formation and interaction with M7/M8 is necessary for efficient M7/M8 membrane insertion, β subunit interaction and stabilization of H,K-ATPase α subunits. On the other hand, in Na,K-ATPase α subunits, β interaction with the M7/M8 loop is sufficient for α subunit stabilization but formation of membrane-inserted M7/M8 pairs impedes M9 insertion (Béguin et al., 1998). M9/M10 pair formation in Na,K-ATPase α subunits may thus necessitate hairpin formation and may only be possible due to the efficient ST function of M10.

Conclusions

Topogenesis studies of various P-type ATPases reveal that membrane insertion, orientation and ultimate packing of their membrane segments is governed by a multitude of different mechanisms. This result may be representative of the topogenesis of all polytopic membrane proteins. The data on P-type ATPases suggest that each membrane segment of polytopic membrane proteins contains its own, particular topogenesis signal that requires individual decoding according to the specific sequence information contained within or adjacent to the membrane segment. The presence of polar, charged or helix-breaking residues in the putative membrane segments often impedes a simple, alternating SA/ST mechanism for membrane insertion. A mechanism that is likely to be of general importance for the stable membrane insertion of such segments consists in the formation of hairpins between closely positioned membrane helices prior to membrane insertion which masks the deleterious effects of certain amino acids. Hairpin formation and membrane insertion may need assistance by salt bridge formation, the presence of kink-forming amino acid residues in the connecting loops and/or intramolecular interactions with distal membrane segments and, in particular cases, intermolecular interactions with a specific chaperone subunit.

Though still incomplete, the available data permit the formulation of the following hypothesis for the topogenesis of P-type ATPases. In P2-ATPases such as Na,K-, H,K- and SERCA Ca-ATPase α subunits or in

P1-ATPases, the amino acid composition of the N-terminal membrane segments allows for their cotranslational membrane insertion according to an alternating SA/ST insertion mechanism while in H-ATPase, the amino acid composition of these same domains necessitates hairpin formation of closely positioned membrane segments prior to membrane insertion. Though different, both these mechanisms are likely to permit insertion of the N-terminal membrane domain into the lipid bilayer during synthesis to provide a stable anchor for the nascent polypeptide.

The two fundamental topogenesis mechanisms observed for the N-terminal membrane segments may not be sufficient to assure membrane integration of the last membrane pair of the core structure of certain P-type ATPases. This membrane domain is separated from the N-terminal membrane domain by the large cytoplasmic loop and, in particular, it often has an amino acid composition which is indeed consistent with a certain flexibility necessary for transport function but which imposes severe constraints on the membrane insertion of this domain. As suggested for Na,K-ATPase α subunits, the amino acid composition of M5/M6 may be so stringent that hairpin formation must be assisted by additional intramolecular interactions with distal membrane segments to permit membrane insertion.

On the basis of the few data available, it cannot be decided whether in contrast to P1-ATPases, in P2-ATPases, distal membrane segments have evolved for the sole purpose to aid membrane insertion of M5/M6 pairs which may need a particularly membrane-repulsive amino acid composition to permit specific ion transport. Since as suggested by topogenesis (Béguin et al., 1998) and biochemical studies (Goldshleger et al., 1995; Arystarkhova et al., 1995), the 2 additional C-terminal membrane pairs of Na,K-ATPase α subunits exhibit themselves as having a certain 'flexibility' and since mutations that abolish this flexibility produce pumps with severe functional defects (Béguin et al., 1998), it is likely that this membrane domain not only has a helper function for membrane insertion of the M5/M6 pair but is also implicated in a fine coordination of ion transport. This hypothesis is also supported by recent experimental evidence which suggests that negatively charged amino acids in the N-terminal part of the M7/M8 extracellular loop may be involved in the release of Na to the extracytoplasmic side (Schneider & Scheiner-Bobis, 1997).

A particularly intriguing question related to the topogenesis of P-type ATPases remains the exclusive necessity of a β subunit for the structural maturation of Na,K- and H,K-ATPase α subunits. Though a similar role of the Kdp A subunit of bacterial K-ATPases remains to be confirmed (Altendorf et al., 1998), it is tempting to speculate that the K-transport function, common to all these oligomeric ATPases, necessitates a particular amino acid composition of the core structure and/or the additional C-terminal membrane pairs which is not compatible with membrane insertion mediated only by intramolecular interactions and has required during evolution the association of a helper protein that assists the correct packing of K-transporting P-type ATPases. It is likely that β subunits have additional functions as it was shown that they influence the transport properties of Na,K-ATPase (Hasler et al., 1999). So far, it is not known whether β subunits produce these latter effects by local conformational changes, by participating in the ion translocation pore and/or by a fundamental rearrangement of the helix-bundle packing compared to β subunit-deficient P1-ATPases.

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