REVIEW

FUNCTIONAL SIGNIFICANCE OF THE β-SUBUNIT FOR HETERODIMERIC P-TYPE ATPases

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Summary

We have reviewed the structural and functional role of the β-subunit in a subfamily of the P-ATPases known as the α/β-heterodimeric, cation-exchange ATPases. The subfamily consists of the various isoforms of Na^+/K^+-ATPase and H^+/K^+-ATPase, both of which pump a cation out of the cell (Na^+ or H^+, respectively) in recycle exchange for K^+. Much of the earlier work has emphasized the functional activities of the α-subunit, which shares many characteristics with the broader P-ATPase family. It is now clear that the glycosylated β-subunit is an essential component of the cation-exchange ATPase subfamily. All β-subunit isoforms have three highly conserved disulfide bonds within the extracellular domain that serve to stabilize the α-subunit, α/β interaction and functional activity of the holoenzyme. Evidence strongly suggests that the β-subunit is involved in the K^+-dependent reactions of the enzymes, such as the E_1–E_2 transition and K^+ occlusion, and that the extracellular domain of the β-subunit plays an important role in determining the kinetics of K^+ interaction. In most vertebrate cells, the unassociated α-subunit is restricted to the endoplasmic reticulum (ER), and assembly of the α/β complex occurs within the ER. Signals for exiting the ER and directing the correct intracellular trafficking are primarily determined by the β-subunit; Na^+/K^+-ATPase typically terminates in the plasma membrane facing the basolateral membrane, whereas all isoforms of H^+/K^+-ATPase terminate in the apical membrane. The C-terminal extracellular domain of the β-subunit is important for proper interaction with the α-subunit and for correct intracellular trafficking. Oligosaccharides on the β-subunit are not essential for enzyme function, but do serve to enhance the efficiency of α/β association by increasing the lifetime of the unassociated β-subunit and the stability of the α/β complex to tryptic attack. We propose that highly specialized glycosylation on the β-subunit of the gastric H^+/K^+-ATPase may help to protect that enzyme from the harsh extracellular environment of the stomach.

Key words: H^+ pump, Na^+ pump, glycosylation, subunit assembly, trafficking, acid secretion.

Introduction

All cells possess an array of sophisticated membrane-bound enzymatic systems that perform various processes essential for life. These processes range from regulation of the intracellular milieu to the genesis of information transfer and communication between cells. A major functional class of these membrane-bound enzymes includes those categorized as primary active transporters and called ATPases because they catalyze the transport of molecules against an electrochemical potential by reactions directly linked to the hydrolysis of ATP. The ATPases that actively transport cations have been extensively studied and have been categorized by Pedersen and Carafoli (1987) into three classes: F-type ATPases (F-ATPases), V-type ATPases (V-ATPases) and P-type ATPases (P-ATPases). The F-ATPases are located in bacterial plasma membranes, inner mitochondrial membranes and thylakoid membranes of chloroplasts, and they actually operate *in vivo* as reverse ATPases, or ATP synthases, synthesizing ATP from ADP and inorganic phosphate using energy derived from electrochemical gradients of protons (Amzel and Pedersen, 1983). Using reaction mechanisms analogous to F-ATPases, the V-ATPases utilize ATP to create proton electrochemical gradients; they are ubiquitously distributed in eukaryotic vacuo-lysosomal organelles and archaebacteria and are also present in plasma membranes of various animal tissues (Harvey, 1992).

The P-ATPases are broadly distributed, active cation translocators having the distinctive feature of forming a covalent acylphosphate–enzyme intermediate (hence the P-

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designation) during the cycle of ATP hydrolysis and cation translocation. Among this class of ATPases are the Ca^{2+}-ATPases of the plasma membrane, sarcoplasmic reticulum and endoplasmic reticulum, the H^+-ATPase of yeast and plants, the K^+-ATPase of bacteria, the Na^+/K^+-ATPase of animal cell plasma membranes and the H^+/K^+-ATPase of gastric parietal cells. Because of the formation of phosphoenzyme intermediates, the enzymatic cycle of P-ATPases can be divided into steps that include a kinase activity, by which an aspartate residue on the enzyme is phosphorylated, and a phosphatase activity, by which the phosphoenzyme is dephosphorylated. Another common feature of these ATPases is their inhibition by submicromolar concentrations of vanadate, acting as a tightly binding phosphate analog (Cantley *et al.* 1977; O'Neal *et al.* 1979; Faller *et al.* 1983). Furthermore, during the enzymatic cycle, P-ATPases characteristically exhibit two phenomenologically and structurally distinct conformations, E_1 and E_2, which have distinct kinetic variables, e.g. affinities for substrates (Jorgensen and Andersen, 1988). For this reason, the P-ATPases are also called E_1–E_2 ATPases. Identification and probing of these various features have provided significant insight into the detailed mechanistic operation of the P-ATPases (Jorgensen and Andersen, 1988; de Meis and Vianna, 1979).

In addition to their functional similarities, P-ATPases have a number of structural homologies, belonging to a common large gene family. All members have a principal peptide of approximately 100kDa, designated as the catalytic subunit because it contains the site for ATP binding and phosphorylation. The high degree of sequence homology for amino acids within the ATP binding site and the phosphorylation site attests to the highly conserved nature of protein domains that interact with ATP and has been used to design nucleic acid probes for cloning other members of the gene family (Shull and Lingrel, 1986). Amino acid sequence analysis suggests that all P-type catalytic subunits have similar topological and domain organization. Multiple transmembrane segments occur within both the N-terminal third and C-terminal third of the peptide. A large cytoplasmic domain in the middle of the peptide contains the nucleotide binding and phosphorylation sites (Fig. 1). Some disagreement remains as to the exact location, and even the number, of transmembrane segments (Inesi and Kirtley, 1992); the specific locations for the binding of cations and the paths for their transport are far from resolved.

Na^+/K^+-ATPase and H^+/K^+-ATPase: cation exchangers

Among the P-ATPases, the ubiquitous Na^+/K^+-ATPase and the gastric H^+/K^+-ATPase share a number of common features; this review will primarily address these structural and functional homologies. Both ATPases are cation exchangers, involving the cellular uptake of K^+ in exchange for the export

Fig. 1. Schematic representation of the organization within a membrane of a heterodimeric cation-exchange subfamily of P-ATPase (including Na^+/K^+-ATPase and H^+/K^+-ATPase). The scheme points out the multiple transmembrane segments of the α-subunit with its principal amino acid mass on the cytoplasmic side containing several important catalytic functions as well as the N-terminal and C-terminal segments. The β-subunit has a single transmembrane segment and most of its mass, which is highly glycosylated and contains three structurally important disulfide bonds, is on the extracellular side of the membrane. The diagram also suggests that K^+ binding sites may be a structure/function cooperation between α- and β-subunits. An inhibitory site (O) is illustrated on the extracellular domain of the α-subunit (the ouabain binding site for Na^+/K^+-ATPase and the omeprazole binding site for H^+/K^+-ATPase). The ouabain binding site has been shown to involve regions within 200 amino acids of the N terminus and within the C-terminal half of the Na^+/K^+ α-subunit (Ishii *et al.* 1994; Canessa *et al.* 1992; Feng and Lingrel, 1994). Omeprazole has been shown to label covalently the Cys-813 and Cys-892 of the H^+/K^+ α-subunit (Besancon *et al.* 1993).
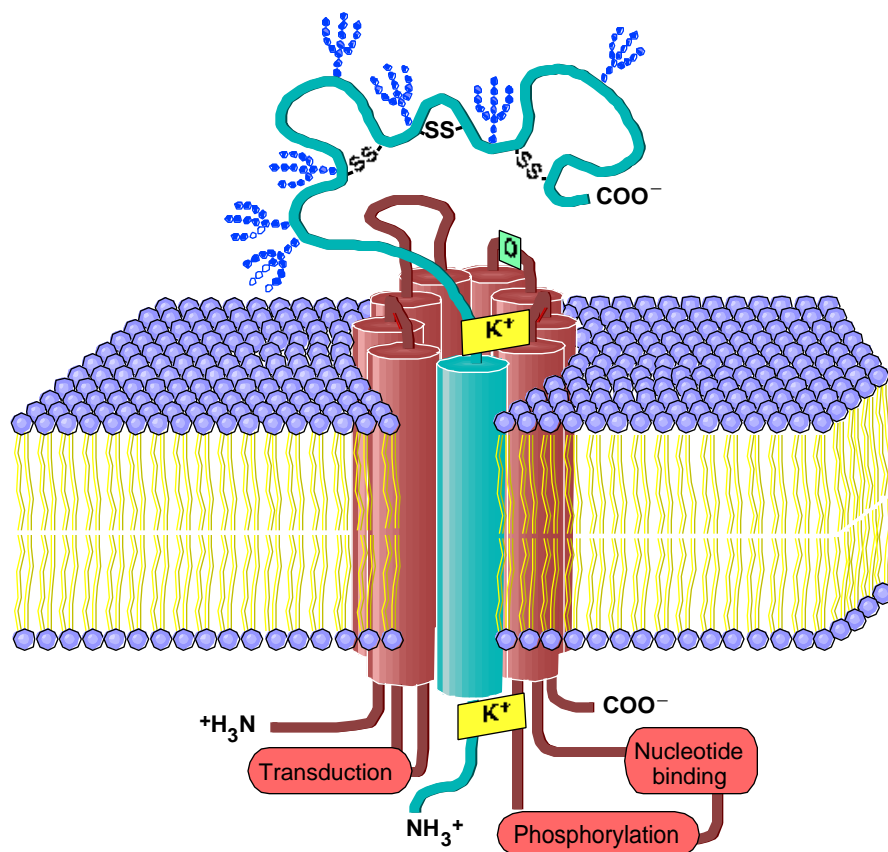
of Na^+ or H^+. In addition to amino acid sequence homology, both Na^+/K^+-ATPase and gastric H^+/K^+-ATPase are heterodimers with the minimal functional complex containing the approximately 100kDa α-subunit and a glycosylated β-subunit. The apparent molecular mass of the β-subunit on SDS–PAGE is highly variable, depending upon the degree of glycosylation, but the deglycosylated core protein is always about the same size (approximately 34kDa). Thus, many structural and functional similarities, including K^+ exchange and the functional requirement for a β-subunit, place Na^+/K^+-ATPase and H^+/K^+-ATPase within a subfamily of P-ATPases. A schematic representation of the α/β-heterodimeric organization for this P-ATPase subfamily is shown in Fig. 1.

The Na^+/K^+-ATPase is known to occur in a number of isoforms, both for the α-subunit and for the β-subunit. For example, various α-subunit isoforms have been designated ($α_1$, $α_2$, $α_3$), differing somewhat with respect to tissue distribution and affinity for ligands as well as primary structure (Lingrel *et al.* 1990); however, the physiological significance of the Na^+/K^+-ATPase isoforms is unknown. There may also be isoforms for the gastric H^+/K^+-ATPase. For example, a new member of the Na^+/K^+-, H^+/K^+-ATPase subfamily has been identified in toad urinary bladder and has been postulated to be an H^+/K^+-ATPase isoform performing H^+ and K^+ homeostasis specific to the urinary tract (Jaisser *et al.* 1993*b*). The α-subunit of this putative isoform, designated as $α_{bl}$, has approximately 69% amino acid identity with the α-subunit of the gastric H^+/K^+-ATPase and approximately 67% identity with $α_1$ of Na^+/K^+-ATPase. A novel β-subunit ($β_{bl}$) has also been cloned from toad bladder epithelial cells (Jaisser *et al.* 1993*c*). Expression of $α_{bl}$ in *Xenopus* oocytes requires co-expression of $β_{bl}$ for functional H^+ and K^+ exchange activity, further supporting the α/β-heterodimeric nature of the ion-exchange ATPase subfamily. A recently cloned P-ATPase, localized to surface epithelial cells of the distal colon, may be yet another isoform of the H^+/K^+-ATPase, sharing 76% identity with rat $α_1$ and 75% identity with $α_{bl}$ (Jaisser *et al.* 1993*a*). Although the distal colon is known to carry out active K^+ absorption *via* K^+/H^+ exchange, the colonic H^+/K^+-ATPase has not been functionally expressed and there is no evidence yet for a corresponding β-subunit.

Despite their many common features, there are some notable and instructive differences between the Na^+/K^+-ATPase and the H^+/K^+-ATPase. Cardiac glycosides, such as ouabain, are well-known specific inhibitors of Na^+/K^+-ATPase activity, but do not inhibit gastric H^+/K^+-ATPase. Other compounds, such as SCH 28080 and omeprazole (see Sachs *et al.* 1989), effectively inhibit H^+/K^+-ATPase with no effect on Na^+/K^+-ATPase. In fact, this latter specificity forms the basis for using omeprazole-related compounds as gastric proton pump inhibitors for clinical treatment of hyperacidity and peptic ulcer control. Functional expression of the toad bladder H^+/K^+-ATPase isoform, *via* $α_{bl}β_{bl}$ co-expression in oocytes, revealed H^+/K^+ exchange transport activity that was sensitive to both SCH 28080 and ouabain, although less sensitive than is typical of gastric H^+/K^+-ATPase and Na^+/K^+-ATPase.

The two exchange pumps also differ in some physical characteristics, e.g. generated ionic gradients and turnover stoichiometry are markedly different. The Na^+/K^+-ATPase typically transports Na^+ against a 5- to 15-fold Na^+ concentration gradient, whereas the gastric H^+/K^+-ATPase will operate in a steady state against a proton gradient greater than 10^6. For turnover of the Na^+/K^+-ATPase, the transport stoichiometry is electrogenic, $3Na^+/2K^+$ (Post and Jolly, 1957; Clarke *et al.* 1989); whereas for turnover of H^+/K^+-ATPase the stoichiometry is electroneutral, $1H^+/1K^+$ (Sachs *et al.* 1976). These physical differences may underlie a common principal of operation that is inherent in the thermodynamic efficiency for all P-ATPases. The work (W_p) involved in the turnover of an ion pump is the sum of the energy requirements for all the transported species, which is a function of the electrochemical potential gradient for each ion ($\Delta\bar{\mu}_k$) and the stoichiometric number of ions moved (n_k):

$$W_p = \Sigma n_k \Delta\bar{\mu}_k,$$

where:

$$\Delta\bar{\mu}_k = \boldsymbol{R}T\ln\frac{[c_k]_o}{[c_k]_i} + zF\Delta\Psi,$$

where $[c_k]$ represents the molar concentration of the ion with the subscripts i and o referring to the inside (cytosolic) and outside (extracellular) solutions, $\Delta\Psi$ is the transmembrane electrical potential difference, and $\boldsymbol{R}$, T, z and F have their usual meanings. The electrochemical gradients for operating the Na^+/K^+-ATPase are not very high but, taking into account the stoichiometry of three Na^+ and two K^+ per turnover (i.e. per ATP utilized), the pump demands about 42kJmol^{-1} per turnover. In the case of the H^+/K^+-ATPase, the proton gradient is huge but, since transport involves only one H^+ and one K^+ per turnover, the total work of the gastric H^+ pump turns out to be nearly the same as that of the Na^+ pump; that is, about 42–46kJmol^{-1} per turnover. [For a general case with a $\Delta\Psi$ of about -0.05V, intracellular concentrations of 11mmoll^{-1} Na^+ and 140mmoll^{-1} K^+, and extracellular concentrations of 140mmoll^{-1} Na^+ and 5mmoll^{-1} K^+, the minimum energy requirement is 11.4kJmol^{-1} for Na^+ efflux and 3.8kJmol^{-1} for K^+ influx. From the stoichiometry of three Na^+ (3×11.4) and two K^+ (2×3.8) per pump turnover, a minimum of approximately 42kJ per Na^+/K^+ pump turnover is predicted. To operate the gastric H^+/K^+ pump, with a luminal pH of 0.8 and a cell pH of 7.0, approximately 42kJmol^{-1} would be required for H^+ secretion; electrochemical gradient conditions predict approximately 2.5kJmol^{-1} for K^+ influx. Since the stoichiometry of H^+/K^+-ATPase is one H^+ per one K^+, a minimum of approximately 44kJ is required per pump turnover.] Similar considerations applied to other P-ATPases (e.g. Ca^{2+}-ATPase) give about the same energy requirements per molar turnover. The free energy available from ATP hydrolysis under typical cellular conditions is about -42 to -54kJmol^{-1}. Thus, for the sake of energetic efficiency, evolution of the specific number of ion binding sites for a given pump protein may have conformed to the relationship between

the electrochemical gradient of transport and the availability of phosphate bond energy for conformational rearrangement.

Features of β-subunits of the cation exchange ATPase subfamily

The β-subunit of the Na$^+$/K$^+$-ATPase was initially identified as a glycoprotein associated with the α-subunit in purified functional enzyme preparations (Brotherus *et al.* 1983). The association between α- and β-subunits is relatively strong and remains stable in most non-ionic detergents. The β-subunit of the Na$^+$/K$^+$-ATPase, like the α-subunit, has several isoforms, designated β$_1$, β$_2$, β$_3$ (see Horisberger *et al.* 1991*b*). Among these isoforms, there are various degrees of difference and, although all β-subunits are glycosylated, the number of glycosylations varies with the isoform.

The β-subunit of the gastric H$^+$/K$^+$-ATPase is also a highly glycosylated protein, so much so that it appears on SDS–PAGE as a broad 60–80kDa band that is weakly stained by Coomassie Blue. The β-subunit of the H$^+$/K$^+$-ATPase had eluded confirmation until it was shown that the 60–80kDa glycoprotein remained stably associated with the α-subunit in non-ionic detergents and could be deglycosylated to a 34kDa core peptide similar to Na$^+$/K$^+$-ATPase (Okamoto *et al.* 1989). Amino acid sequences from several species were published within months of this finding (Canfield *et al.* 1990; Reuben *et al.* 1990; Shull, 1990; Toh *et al.* 1990).

Sequences for β-subunit isoforms for the subfamily of Na$^+$/K$^+$- and H$^+$/K$^+$-ATPases are aligned in Fig. 2. Although drawn from diverse animal species, at least 15% of the 300 amino acids in the core are identical, along with a great deal of conservative substitution. All of the known β-subunit species and isoforms share a common domain structure: a short N-terminal cytoplasmic piece, a single transmembrane segment and a large extracellular C-terminal domain containing six extracellular cysteine residues, whose locations are completely conserved among the isoforms. For internal consistency, the numbering system given in Fig. 2 is used throughout the text.

The amino acid sequence data for 24 β-subunits were analyzed by a multiple sequence alignment program to show clustering relationships within the group. The dendrogram shown in Fig. 3 plots the similarity and shared relationships between sequences. Although it is not a direct phylogenetic reconstruction, the horizontal branch lengths are related to the evolutionary development of the peptide. Thus, the Na$^+$/K$^+$-ATPase β-subunit from the shrimp, for which there is no isoform assignment, shows the least similarity, but appears to have precursor characteristics for all the isoforms. The analysis objectively grouped the Na$^+$/K$^+$ β$_1$ and H$^+$/K$^+$ β isoforms into two distinct clusters, indicating that they are monophyletic; however, division of the Na$^+$/K$^+$ β$_2$ and β$_3$ isoforms is more tenuous. As pointed out by Yu *et al.* (1994), it is not certain to what extent β$_2$/β$_3$ distinctions might be related to phylogenetic variance. When β-subunit sequences are compared for multiple consensus similarity, three relatively conserved regions appear,

as shown in Fig. 4A: (i) about 65 amino acids in the N-terminal region from Trp-22 to Pro-86, including the transmembrane segment of Trp-37 to Ile-66; (ii) the region from Phe-159 to Arg-190; and (iii) the region from Gly-246 to the C-terminal end (with the exception of the highly unusual 10 amino acid C-terminal tail on the chicken H$^+$/K$^+$ β-subunit). There are also regions of great dissimilarity in the comparison across all β-subunit isoforms (shaded regions in Fig. 4A), indicating flexibility for evolutionary variation within these regions. In general, the same regions of similarity and dissimilarity are evident when the analysis is limited to the isoform subclasses of β-subunits (Fig. 4B–D), suggesting that structural and functional similarities transcend the isoforms. These comparative similarities are consistent with expression studies showing that the H$^+$/K$^+$-ATPase β-subunit can act as a surrogate for the Na$^+$/K$^+$-ATPase α-subunit in functionally supporting the activities of the Na$^+$/K$^+$ pump (Horisberger *et al.* 1991*a*).

The carbohydrate moiety of the β-subunit

Glycosylation is a prominent feature of all β-subunit isoforms and, accordingly, the apparent molecular mass on SDS–PAGE is greater than predicted from the core peptide. On the basis of sequence data, there are significant variations in the predicted number of glycosylation sites between different isoforms, even between the same isoform from different species (see Fig. 2). The β$_1$ isoform of Na$^+$/K$^+$-ATPase, which is consistently predicted to have three N-linked glycosylation sites, has been most extensively studied. All three consensus sites of β$_1$ are glycosylated (Tamkun and Fambrough, 1986; Miller and Farley, 1988), and the oligosaccharides are terminally sialylated. Recently, Treuheit *et al.* (1993), using mass spectrometry, showed that the oligosaccharides of the β-subunit from dog and lamb kidney are of tetra-antennary structure with extensions of 2–4 *N*-acetyllactosamine units, and the units of extension seemed to differ between the dog and the lamb β-subunits. Detailed information is not available for oligosaccharides of the other isoforms, but it seems clear that there is a high degree of species variability for the β$_2$ isoform. The Na$^+$/K$^+$-ATPase β$_2$ isoform in chicken has four potential sites for glycosylation, yet the same isoform in rat has eight potential sites and in the mouse there are nine potential sites for glycosylation. This high degree of oligosaccharide variation suggests that carbohydrates may not be essential for primary transport function. This view is supported by observations that Na$^+$/K$^+$-ATPase, without oligosaccharides or with incompletely processed oligosaccharides, can function properly for ATP hydrolysis, ouabain binding (Zamofing *et al.* 1988) and protein trafficking (Tamkun and Fambrough, 1986). However, without core glycosylation of the β-subunit, the enzyme is more sensitive to trypsinolysis, suggesting that the glycosylation may play some role in protein processing and folding. As with other glycoproteins, β-subunit glycosylation may be important in intact tissues for various extracellular surface properties, e.g. the mouse Na$^+$/K$^+$ β$_2$ isoform has been

identified as the protein formerly known as AMOG, the adhesion molecule on glial cells (Schmalzing *et al.* 1992).

For the H$^+$/K$^+$-ATPase, all β-subunits contain seven potential N-linked glycosylation sites, except that from the pig where only six such sites have been identified (see Fig. 2). The nascent β-subunit of rabbit H$^+$/K$^+$-ATPase is cotranslationally glycosylated with high-mannose core oligosaccharides at all seven consensus N-linked sites (Chow and Forte, 1993). Fully mature oligosaccharides on the β-subunit of H$^+$/K$^+$-ATPase are relatively bulky, as shown by the high apparent molecular mass on SDS–PAGE and, like Na$^+$/K$^+$-ATPase, the structure is tri- or tetra-antennary (Okamoto *et al.* 1990; Toh *et al.* 1990) with an abundance of *N*-acetyllactosamine units (Weitzhandler *et al.* 1993). However, in contrast to Na$^+$/K$^+$-ATPase, the β-subunit of H$^+$/K$^+$-ATPase is devoid of sialic acid (Weitzhandler *et al.* 1993). It has been suggested that the H$^+$/K$^+$ β-subunit may initially be sialylated, and later desialylated within the highly acidic luminal space. However, it is also possible that the β-subunit is never sialylated, the post-translational pathway either lacking sialyl transferase or providing alternative linkages, e.g. α-galactose linkages or terminal fucosylation.

The function of oligosaccharides on the H$^+$/K$^+$-ATPase β-subunit, as for the Na$^+$/K$^+$-ATPase, is unclear. An early hypothesis proposed that glycosylation on the β-subunit (known then as an accessory glycoprotein for the H$^+$/K$^+$-ATPase) provided protection against acidic and autodigestive extracellular conditions (Forte and Forte, 1970). A preliminary study indicates that oligosaccharides on the H$^+$/K$^+$ β-subunit afford resistance against pepsinolysis *in vitro* (Chow *et al.* 1993), but to what extent this operates *in situ* remains uncertain.

Disulfide bonds in the β-subunit

The six cysteine residues in the extracellular domain of the β-subunit are 100% conserved between all known isoforms of both Na$^+$/K$^+$-ATPase and H$^+$/K$^+$-ATPase. In contrast, cysteine residues within the intracellular and transmembrane domains vary widely. Kirley (1989) and Miller and Farley (1990) showed that the six extracellular cysteines of the Na$^+$/K$^+$-ATPase β$_1$-subunit form three disulfide bonds in a sequential pattern. For the β-subunit of H$^+$/K$^+$-ATPase, six of the nine cysteines are in the oxidized state (Chow *et al.* 1992). By analogy with Na$^+$/K$^+$ β$_1$-subunit, we suggest three extracellular disulfide bonds in the H$^+$/K$^+$ β-subunit in the same sequential pattern.

Functional activities of the β-subunit

Despite the requirement of an α/β-heterodimer as the minimal functional complex, most functional activity of the Na$^+$/K$^+$-ATPase, e.g. phosphorylation, ATP binding and inhibitor binding, had been identified within the α-subunit. Defining a functional role for the β-subunit had been hindered by the inability to reassemble a functional α/β complex from solubilized monomeric subunits. However, several recent studies provide direct and indirect evidence of the important functional properties of the β-subunit, including an essential role in the stabilization, maturation and enzymatic activity of both Na$^+$/K$^+$-ATPase and H$^+$/K$^+$-ATPase.

The importance of the β-subunit for functional integrity was first observed for Na$^+$/K$^+$-ATPase by Kawamura and Nagano (1984), who showed that reduction of β-subunit disulfide bonds was correlated with a loss of activity. Disulfide bond reduction required strong reducing conditions and could be attenuated by the presence of K$^+$ or Na$^+$, suggesting a possible role for the β-subunit in binding these cations (Kawamura *et al.* 1985; Kirley, 1990). H$^+$/K$^+$-ATPase activity is also inactivated by reduction of β-subunit disulfide bonds; however, the protective effect of cations in preventing reduction and sustaining enzymatic activity is specific for K$^+$ and its congeners, Rb$^+$ and Tl$^+$ (Chow *et al.* 1992). Furthermore, the ability of K$^+$ congeners to protect the enzyme, i.e. the measured EC$_{50}$, was highly correlated with the K_m of these cations for stimulating *p*-nitrophenylphosphatase (*p*NPPase) activity. Since *p*NPPase activity is a model for the phosphatase step of the ATPase cycle and stimulation of *p*NPPase is related to the E$_2 \cdot$K$^+$ conformation, it was proposed that the strength of disulfide bonds in the β-subunit increased as the enzyme shifted from the E$_1$ to the E$_2 \cdot$K$^+$ conformation (Chow *et al.* 1992). Na$^+$ is known to be antagonistic to K$^+$, and high concentrations of Na$^+$ shift the Na$^+$/K$^+$-ATPase or H$^+$/K$^+$-ATPase towards the E$_1$ conformer (Skou, 1982). High concentrations of Na$^+$ made the H$^+$/K$^+$-ATPase more susceptible to reduction in the presence of K$^+$ (Chow *et al.* 1992). Thus, it appears that, as the conformation of the β-subunit tightens (as indicated by an increase in strength of the disulfide bonds), the enzyme shifts from the E$_1$ to the E$_2 \cdot$K$^+$ state. The increase in strength of the disulfide bonds in the β-subunit is correlated with increased stability of the holoenzyme; both Na$^+$/K$^+$-ATPase and H$^+$/K$^+$-ATPase are in a more stable conformation in the E$_2 \cdot$K$^+$ state.

Another way to study relationships between β-subunit disulfide bond stability and general conformational stability is to monitor the strength of the disulfide bonds under the influence of ligands and of denaturants (Browning *et al.* 1992). Dose–response curves for the inactivation of H$^+$/K$^+$-ATPase by 2-mercaptoethanol (2ME) under differing conformational states are shown in Fig. 5A. The lability of H$^+$/K$^+$-ATPase activity to 2ME is increased by ethanol (curve shifted to the left), whereas K$^+$ stabilizes H$^+$/K$^+$-ATPase against 2ME (curve shifted to the right). For all conditions, inactivation occurs abruptly over a narrow range of 2ME concentration, a typical feature of cooperativity among various interactions stabilizing a protein (Creighton, 1993). The correlation between disulfide bond content and H$^+$/K$^+$-ATPase activity shown in Fig. 5B confirms the importance of β-subunit disulfide bonds. Furthermore, the sensitivity of these disulfide bonds to agents that alter holoenzyme stability (stabilized by K$^+$ and labilized by denaturants such as organic solvents and detergents) suggests that there is cooperativity between disulfide bonds
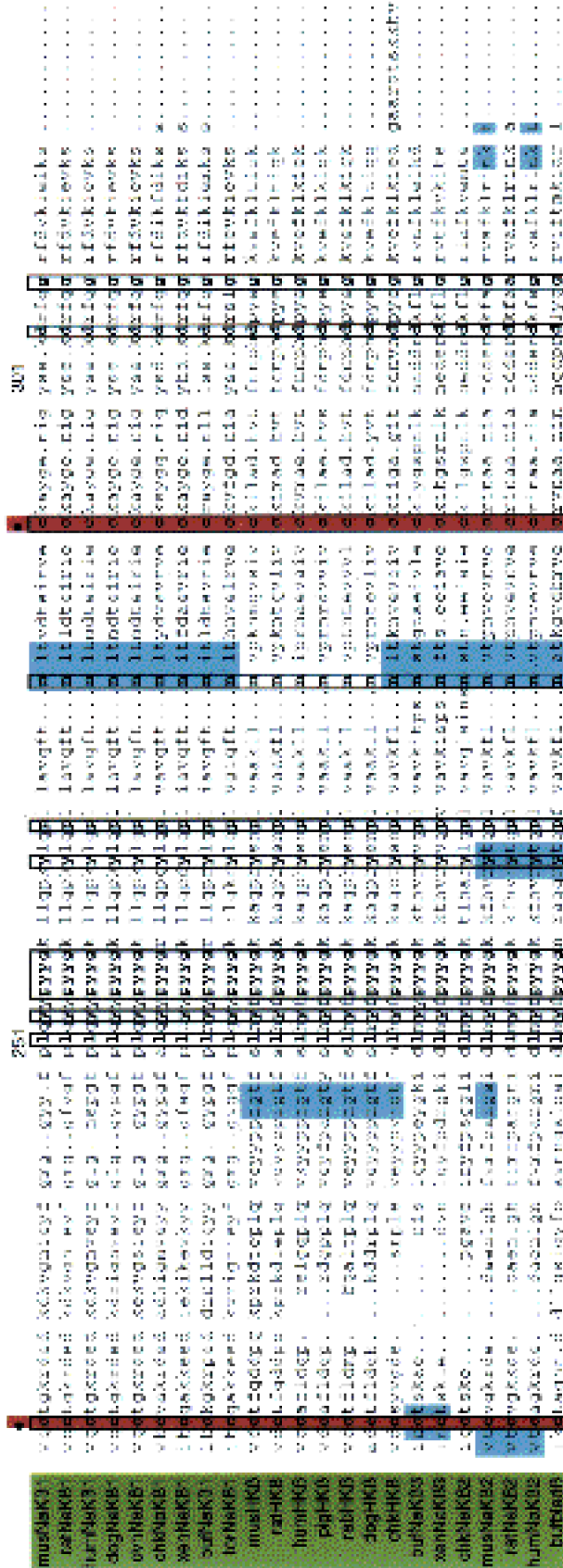
Fig. 2. Multiple sequence alignment of a variety of β-subunits belonging to the cation exchange subfamily of P-ATPases. For Na⁺/K⁺-ATPase, β₁, β₂ and β₃ isoforms are shown (NaKβ1, NaKβ2 and NaKβ3, respectively), with the animal species being given by the prefix: mouse (mus), rat (rat), human (hum), dog (dog), sheep (ovn), chicken (chk), *Xenopus laevis* (xen), toad (buf) and *Torpedo* (tor). The H⁺/K⁺-ATPase is indicated as HKβ, using the same species prefix notation, with the inclusion of rabbit (rabHKβ). A β-subunit cloned by polymerase chain reaction from toad bladder cells has not been definitively assigned to a particular isoform and is shown as bufbladβ. The alignment was performed with the PILEUP program from the Unix version of sequence analysis software by Genetics Computer Group (GCG). Minor adjustments were introduced to guarantee alignment of the N-terminal Met and the six extracellular Cys residues, which are indicated by a black dot. The sequence for the shrimp Na⁺/K⁺-ATPase was omitted for the sake of clarity. The region of the single membrane-spanning segment is shown in yellow. Identical amino acids among all 23 isoforms are indicated in bold type. Potential N-glycosylation sites are outlined. Amino acid numbering shown here is used throughout the text. Sources for the sequences: (1) Gloor, 1989; (2) Mercer *et al.* 1986; (3) Kawakami *et al.* 1986; (4) Brown *et al.* 1987; (5) Shull *et al.* 1986; (6) Takeyasu *et al.* 1987; (7) Verrey *et al.* 1989; (8 and 17) Jaisser *et al.* 1992; (9) Noguchi *et al.* 1986; (10) Canfield and Levenson, 1991; (11) Canfield *et al.* 1990; (12) Ma *et al.* 1991; (13) Toh *et al.* 1990; (14) Reuben *et al.* 1990; (15) Song *et al.* 1993; (16) Yu *et al.* 1994; (18) Good *et al.* 1990; (19) Lemas and Fambrough, 1993; (20) Gloor *et al.* 1990; (21 and 22) Martin-Vasallo *et al.* 1989; (23) Jaisser *et al.* 1993c.
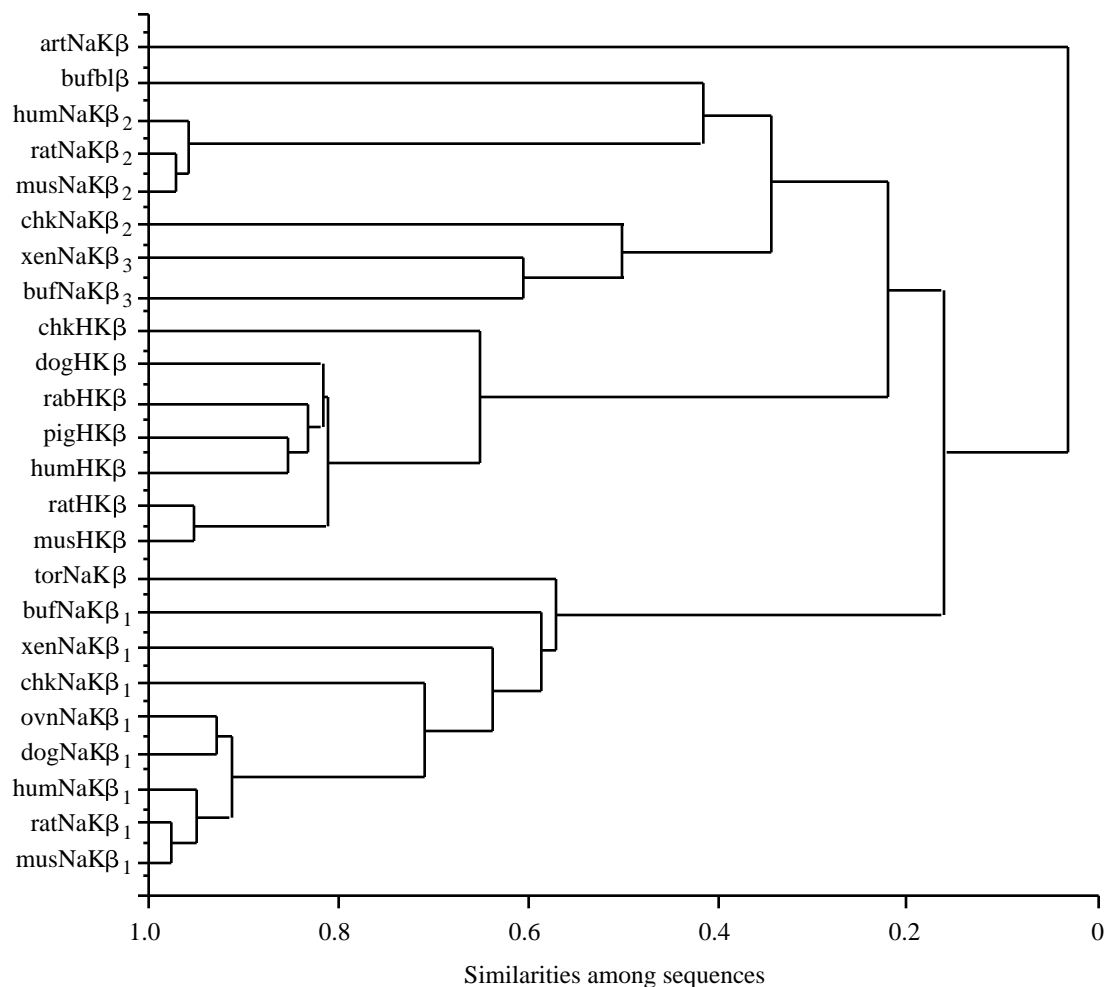
Fig. 3. Dendrogram of 24 β-subunits in the cation exchange subfamily of P-ATPases. Sequences were first aligned as in Fig. 2 and the clustering relationships were determined with the PILEUP routine from the GCG software package using pairwise comparison. The horizontal scale plots the degree of similarity and shared relationships among the sequences. Branch points close to 1.0 are most similar; those approaching zero are least similar. Species and isoform are indicated as described in Fig. 1 with the addition of the shrimp Na^+/K^+-ATPase (artNaKβ) from Bhattacharyya *et al.* (1990). The Na^+/K^+-ATPase β_1-subunit peptides clearly fall into one cluster and those for the H^+/K^+-ATPase β-subunit fall into a second cluster. It is less clear whether the β_2 and β_3 peptides of Na^+/K^+-ATPase fall into two additional groups or whether they should be considered as a single isoform.

and the noncovalent bonding forces. Cooperativity has been studied in soluble globular protein as an important feature of conformational stability (see Creighton, 1993). This case for cooperativity of bonding forces within the subfamily of cation exchange P-ATPases is an extension from simple protein models, since the forces of interaction occur within and among two subunit peptides, and they are membrane-bound peptides. Thus, correlations between K^+-dependent conformational shifts, disulfide bond stability and general protein folding demonstrate that the α/β-subunit interactions are extensive and important for enzymatic function of the holoenzyme. A schematic representation of how folding of the protein and disulfide bond formation can be cooperatively linked in a membrane-bound α/β heterodimer is depicted in Fig. 6.

Alternative support for β-subunit participation in enzymatic function comes from studies using a specific inhibitory monoclonal antibody, Mab-2G11, developed against the β-subunit of H^+/K^+-ATPase. Mab-2G11 binds specifically to the cytoplasmic side of gastric secretory membranes with an epitope of interaction within the first 36 N-terminal amino acids of the β-subunit (Chow and Forte, 1993). Mab-2G11 altered the affinity of the cytoplasmic K^+ binding site, further supporting the hypothesis that the β-subunit contributes to the apparent stability of the $E_2 \cdot K^+$ conformer. However, kinetic arguments based on the full catalytic cycle of H^+/K^+-ATPase suggested that the inhibition was not solely due to the lowered K^+ affinity, but to interference with the conformational transition induced by K^+. Thus, the β-subunit is involved in the conformational changes associated with the transport of K^+.

Several recent experiments suggest that the β-subunit may be involved in the well-known ability of Na^+/K^+- and H^+/K^+-

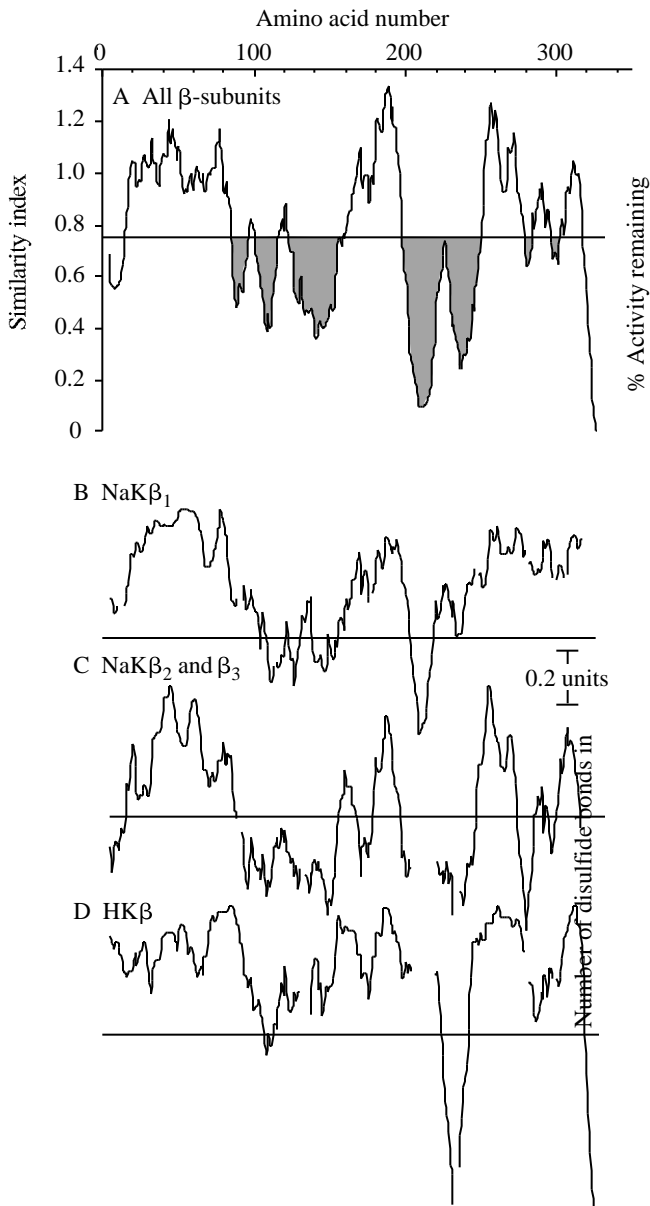Fig. 4. Similarity plots of β-subunit peptides within the subfamily of cation exchange P-ATPases. The similarity plot for the entire 24 isoforms (A, all β-subunits) were generated with the alignment shown in Fig. 2. The similarity plots shown for the subgroups of β-subunit isoforms (B, NaKβ$_1$; C, NaKβ$_2$ and β$_3$; and D, HKβ) were first realigned and then processed. All plots are drawn to the same scale, with zero being least similar and 1.8 most similar; amino acid alignment and insertion of spaces are as described in Fig. 2. The solid horizontal line in A is the average similarity index for all β-subunits. The horizontal dashed lines in B, C and D indicate a similarity index of 1.0 for each of the respective subgroups.

ATPases to occlude K$^+$ (the very slow exit of K$^+$ from E$_2$·K$^+$ suggests that it is not simply an ion-binding step, but is impeded by the enzyme form, hence occluded). After subjecting Na$^+$/K$^+$-ATPase to extensive protease digestion, Capasso *et al.* (1992) found that the membrane-bound remnant
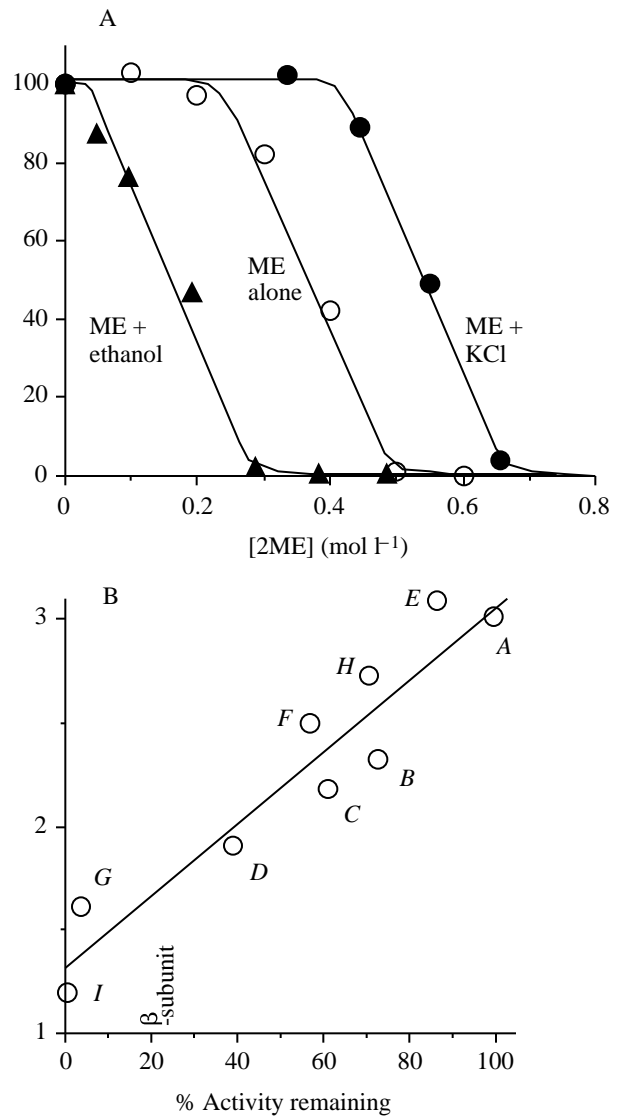

Fig. 5. Demonstration of cooperative inactivation of H$^+$/K$^+$-ATPase by a combination of reducing reagents and non-covalent bond modification. (A) Inactivation of the H$^+$/K$^+$-ATPase by reducing reagents is further influenced by agents that alter protein conformation. The inclusion of denaturants at concentrations that are by themselves harmless (e.g. 0.6 mol l^{-1} ethanol) makes disulfide reduction, and enzyme inhibition, by mercaptoethanol (2ME) more efficient. Ligands such as K$^+$ (15 mmol l^{-1}), which favor a more stable E2·K$^+$ conformation, increase the energy barrier for disulfide reduction. Specific conditions for inactivation were as described by Chow *et al.* (1992). (B) Correlation between the number of disulfide bonds in the β-subunit and the percentage of enzyme activity remaining after H$^+$/K$^+$-ATPase had been treated with combinations of reducing reagent and denaturants. Gastric membranes were incubated at 44 ˚C with 2ME for various periods. In some cases, the incubation also included 0.6 mol l^{-1} ethanol as a labilizer and/or 15 mmol l^{-1} KCl as a stabilizer. *A*, control (no treatment); *B*, 0.45 mol l^{-1} 2ME for 5 min; *C*, 0.45 mol l^{-1} 2ME for 10 min; *D*, 0.45 mol l^{-1} 2ME for 25 min; *E*, 0.45 mol l^{-1} 2ME plus KCl for 25 min; *F*, 0.3 mol l^{-1} 2ME for 20 min; *G*, 0.3 mol l^{-1} 2ME plus ethanol for 20 min; *H*, 0.3 mol l^{-1} 2ME plus ethanol and KCl for 20 min; *I*, 0.6 mol l^{-1} 2ME for 20 min.

-SS- -SS-

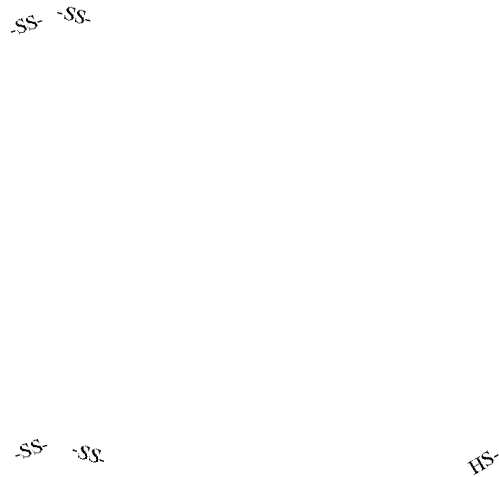-SS-   -SS-   HS-

Fig. 6. Model to account for cooperative linking between protein disulfide bonds and conformational stability in a membrane heterodimeric protein. The protein is illustrated in either the folded or unfolded conformation with four exemplary cysteine residues in one of the subunits that can form two disulfide bonds. Equilibrium constants represent stabilities of the folded conformation, K_F, and of the disulfide bonds, K_{SS}. According to the cooperative linkage relationship, the effects of the folded conformation on the stability of the disulfide bond are mirrored in the effects of the disulfide bond on the stability of the folded conformation, that is to say, $K_F^{SS}/K_F^{SH} = K_{SS}^F/K_{SS}^U$ (adapted from Creighton, 1993).

retained the ability to occlude Rb^+, and the remaining peptides included a relatively intact β-subunit. Later, Lutsenko and Kaplan (1993) reported that reduction of disulfide bonds in the remnant β-subunit correlated with the loss of ability to occlude Rb^+ by the proteolyzed membranes. Furthermore, sites of digestion on the β-subunit were different with and without Rb^+, further strengthening the argument that β-subunit conformations are related to the E_1–E_2 states (Lutsenko and Kaplan, 1994). These data demonstrate that interactions between α- and β-subunits are required for occlusion of K^+.

Experiments with a variety of expression systems reinforce the importance of the β-subunit for functional enzymatic activity of the cation-exchange ATPases. For Na^+/K^+-ATPase, Horowitz et al. (1990) used a yeast expression system to show that ouabain binding and ouabain-inhibitable ATP hydrolysis required expression of both α- and β-subunits. Using the baculovirus expression system, Klaassen et al. (1993) reported that H^+/K^+-ATPase also required both α- and β-subunits for functional activity, as defined by phosphoenzyme formation, though they could not demonstrate the full hydrolytic cycle. Additional insight into α/β interaction comes from hybrid studies testing the complex expression of Na^+/K^+-ATPase α-subunit with various β-subunit isoforms. Jaisser et al. (1992) showed that the $α_1/β_1$ complex had different K^+ transport kinetics from the $α_1/β_3$ complex. The β-subunit of H^+/K^+-ATPase can serve as a substitute in supporting some functional activities when expressed together with the α-subunit of the Na^+/K^+-ATPase in both Xenopus and yeast expression systems

(Horisberger et al. 1991a; Eakle et al. 1992). However, in the yeast system, Eakle et al. (1992) observed that the antagonism between ouabain binding and K^+ is different for the Na^+/K^+ α, Na^+/K^+ β complex than for the Na^+/K^+ α, H^+/K^+ β complex. These observations also link the β-subunit with K^+ binding and the conformational transitions associated with K^+.

Two laboratories have reported on the expression of functional hybrids in which the α-subunit of Na^+/K^+-ATPase was expressed along with β-subunit chimeras fabricated from different halves of Na^+/K^+-ATPase and H^+/K^+-ATPase (Jaunin et al. 1993; Eakle et al. 1994). The chimeras were constructed by exchanging the extracellular domain of one β-subunit with the other, and both were found to assemble with the α-subunit of Na^+/K^+-ATPase to produce functional activity. Furthermore, the measured affinity for K^+ matched the K^+ affinity of the β-subunit that provided the extracellular domain, whereas the transmembrane domain served to determine the strength of interaction with the α-subunit. Thus, the extracellular domain of the β-subunit has a dominant influence on the interaction with K^+.

The evidence reviewed in this section converges into a theme that the β-subunits of Na^+/K^+-ATPase and H^+/K^+-ATPase play some role in the K^+ transport function of the cation-exchange process. Since α- and β-subunits are associated via tight coupling, the transport of cations probably requires the concerted conformational change of both subunits, rather than each subunit being responsible for a given step independently of the other subunit.

Biosynthesis of multimeric P-ATPase: transcription of subunit genes

For both Na^+/K^+-ATPase and H^+/K^+-ATPase, the α- and β-subunits are synthesized by translation of individual mRNA molecules which are, in turn, transcribed from separate genes (Lingrel *et al.* 1990; Song *et al.* 1993; Canfield *et al.* 1990). Transcription of the α- and β-subunits appears to be coordinated to some extent (see review by Lingrel *et al.* 1990). The increase in the amount of renal Na^+/K^+-ATPase produced by thyroid hormone is associated with parallel increases in mRNA level for both subunits. In a variety of cell lines, low extracellular $[K^+]$ has been reported to increase Na^+/K^+-ATPase abundance through increased expression of mRNA for both subunits. For H^+/K^+-ATPase, the information is more confined to the stomach, where high levels of the enzyme occur. Ontogenetic studies show that increased H^+/K^+-ATPase abundance correlated with increased mRNA levels for α- and β-subunits as well as with increased synthesis of both subunits (Crothers *et al.* 1990; Morley *et al.* 1992).

Translation and membrane insertion

As for other integral membrane proteins, the α- and β-subunits of multimeric P-ATPases appear to be cotranslationally inserted into membranes of the rough endoplasmic reticulum (ER) by membrane-bound ribosomes. Neither the α-subunit nor the β-subunit has a cleavable signal sequence (Geering, 1990).

In the lumen of the rough ER, the β-subunits are subjected to two covalent modifications: disulfide bond formation and N-linked core glycosylation. Disulfide bond formation and rearrangement within the β-subunit are presumed to be catalyzed by protein disulfide isomerase, which is generally located in the lumen of the ER. N-linked core glycosylation occurs cotranslationally. The core oligosaccharides undergo a series of further modifications to their final complex form as the β-subunit passes through the Golgi apparatus *en route* to the plasma membrane. The existence of different glycan forms, with selective sensitivity to endoglycosidases, has provided a convenient index to monitor the trafficking of glycoproteins such as the β-subunit. Experimentally, the β-subunit has been observed in two forms: the high-mannose core-glycosylated form, which resides within the ER and is sensitive to endoglycosidase H; and the final complex glycosylated form, after the glycan has been processed through the Golgi apparatus, which is resistant to endoglycosidase H. These data indicate that the β-subunit has a significant dwell time only in the ER, or in its final destination, and that intermediate processing stages within the Golgi are very brief (Tamkun and Fambrough, 1986; Chow and Forte, 1993).

Assembly of α- and β-subunits

To date, assembly of the α/β-heterodimeric complex has only been effected through cellular and/or synthetic pathways; it has not been possible to re-associate monomers that have been isolated from membranes by more aggressive detergent extraction. Evidence for functional association between α- and β-subunits is usually obtained by measuring the stability of the heterodimeric complex in mild non-ionic detergents, e.g. co-immunoprecipitation in Triton X-100 or NP-40.

Early expression studies using a cell-free translation system suggested that the α- and β-subunits of Na^+/K^+-ATPase could associate in the rough ER, possibly cotranslationally (Hiatt *et al.* 1984). Cotranslational α/β association is supported by cell expression studies demonstrating co-immunoprecitation of radiolabeled α-subunit with β-subunit within a 15min pulse-chase (Tamkun and Fambrough, 1986). Can association between α- and β-subunits occur long after translation? Noguchi *et al.* (1990*a,b*) used a strategy of injecting mRNA for Na^+/K^+-ATPase subunits from *Torpedo californica* into *Xenopus* oocytes in different sequences and at different intervals. They showed functional α/β complex formation when the β-subunit was presynthesized, but not when the α-subunit was presynthesized, and speculated that the β-subunit may serve as a receptor for inserting the α-subunit into the membrane. Ackermann and Geering (1992) employed a similar strategy, except that they injected homologous *Xenopus* mRNA transcribing α- and β-subunits into *Xenopus* oocytes. In this case, presynthesized *Xenopus* α-subunit was able to associate with newly synthesized *Xenopus* $β_1$- or $β_3$-subunit, and the presynthesized β-subunits were able to associate with the newly synthesized α-subunit. Discrepancies between these data may due to the lability of exogenous α-subunit in *Xenopus* oocytes. Therefore, it appears that presynthesized subunits retain the capability of association with the other subunit. The discrepancy seen in these observations emphasizes a recurrent difficulty. When heterologous systems are used for expression studies, one must be circumspect in intepreting and generalizing the data.

Important protein domains for α/β association

Some information about the regions of α/β interaction can be inferred from the study showing that remnant material from extensively proteolyzed Na^+/K^+-ATPase retained the functional capability of cation occlusion (Beauge and Glynn, 1979). The residual membranous fragments contained most of the β-subunit and a 19kDa C-terminal piece of α-subunit, suggesting some interaction between the β-subunit and the C terminus of the α-subunit. An analogous approach using proteolyzed H^+/K^+-ATPase suggests that the β-subunit specifically interacts with the α-subunit C-terminus, which includes the seventh and eighth membrane-spanning segments (Shin and Sachs, 1994).

When α-subunit chimeras were constructed from the Na^+/K^+-ATPase and the Ca^{2+}-ATPase, as a monomeric counterpart, the C-terminal half of the α-subunit was shown to be sufficient for α/β association. As little as 26 amino acids in the extracellular domain proximal to the C terminus are sufficient for α/β association (Lemas *et al.* 1992, 1994; Luckie *et al.* 1992; Fambrough *et al.* 1994). For chimeras of Na^+/K^+-

ATPase and H$^+$/K$^+$-ATPase, Gottardi and Caplan (1993a) showed that the C-terminal half of the α-subunit dictates the assembly with the respective β-subunit. Furthermore, the complex of α-chimera and β-subunit appears to be functional (Blostein *et al.* 1993).

For deletion mutations made within the β-subunit of Na$^+$/K$^+$-ATPase, mutant subunits lacking the entire cytoplasmic piece, including portions of the transmembrane domain, or almost half of the extracytoplasmic C-terminal domain, could associate with α-subunits (Hamrick *et al.* 1993, Renaud *et al.* 1991). These data suggested that the extracellular segment of about 100 amino acids of the β-subunit immediately adjacent to the membrane is responsible for α/β association. However, the chimera of this 100-amino-acid segment, fused with the cytoplasmic and transmembrane piece of a totally independent membrane protein, failed to associate with the α-subunit, whereas the analogous chimera using the whole extracellular piece could associate with the α-subunit (Hamrick *et al.* 1993). Intepretation of these results is further complicated by results from point mutation studies. Mutation of Pro-256, or of hydrophobic amino acids near the C terminus, prevents the formation of a functional α/β complex on the plasma membrane (Beggah *et al.* 1993; Geering *et al.* 1993). In fact, the entire region from Leu-252 to Lys-260 is highly conserved among all subfamily members (see Fig. 2). Noguchi *et al.* (1994) recently reported that mutation of the first two extracellular cysteine residues on the β-subunit, Cys-134 and Cys-157, will generate an inactive α/β complex and that mutation of the other four extracellular cysteines prevents assembly of the α/β complex. To reconcile apparent inconsistencies between the regional deletion data and point mutation studies, one could speculate that, while there are very specific regions of subunit interplay, assembly of the α/β complex relies on multiple cooperative regional interactions, such that misfolding of one region would alter appropriate bonding within other regions. This may also explain the difficulties of *in vitro* reassembly of the α/β complex from separated subunits. Chimeras of β-subunit constructed from complementary portions of Na$^+$/K$^+$- and H$^+$/K$^+$-ATPase β-subunits have been used to evaluate relative domain participation in the α/β association (Jaunin *et al.* 1993; Eakle *et al.* 1994). These chimeras were constructed with the entire cytoplasmic domain plus the transmembrane domain from one β-subunit and the extracytoplasmic domain from the other. All β-subunit chimeras were capable of assembly with the Na$^+$/K$^+$-ATPase α-subunit to provide functional pumps, with some differences in specific functional properties of the resulting complexes. Chimeras with the N-terminal piece from Na$^+$/K$^+$-ATPase β-subunit had the highest efficiency in stabilizing the α/β complex. Thus, multiple regions within the β-subunit participate in the interactive and functional activities of the holoenzyme.

Functional maturation of the nascent α/β complex

Several lines of evidence suggest that the α/β complex is functional soon after it is formed: (1) within 15min of synthesis, association between subunits can be measured (Tamkun and Fambrough, 1986); (2) within 10–15min of α-subunit synthesis, the Na$^+$/K$^+$-ATPase appears to be capable of binding ouabain (Caplan *et al.* 1990); and (3) within 15min of synthesis, a detectable fraction of newly synthesized Na$^+$/K$^+$-ATPase α-subunit is converted from a trypsin-sensitive form to a trypsin-resistant form consistent with conformational rearrangement (Geering *et al.* 1987). In the above systems, protein transport from ER to plasma membrane requires at least 40–60min; thus, Na$^+$/K$^+$-ATPase must be functional before it reaches the plasma membrane.

For the H$^+$/K$^+$-ATPase, identification of an associated complex between the α-subunit and the 52kDa immature β-subunit (core-glycosylated form) indicated that α/β assembly of the H$^+$/K$^+$-ATPase commences in the ER, probably cotranslationally. Within 15min of radiolabeled amino acid incorporation, abundant radiolabeled α-subunit was seen within cell fractions enriched in ER but was barely detectable in fractions corresponding to normal functioning parietal cell tubulovesicles (Crothers *et al.* 1993). After 30min, the amount of newly synthesized α-subunit in the mature tubulovesicular fraction was significantly increased. Thus, the pattern of functional maturation for the H$^+$/K$^+$-ATPase appears to be similar to that of the Na$^+$/K$^+$-ATPase.

Fate of unassociated subunits

There appear to be conflicting data describing the fate of unassociated subunits. An early model proposed that unassociated α- and β-subunits are retained within the ER until they associate by collision. They then supposedly exit the ER as the heterodimer or are eventually degraded by the ER quality control mechanisms. This model was based on the observations that (1) when α-subunit was overexpressed in a mouse cell line, it was almost exclusively located intracellularly, probably in the ER (Takeyasu *et al.* 1988), and (2) when the β-subunit was overexpressed in *Xenopus* oocytes, it was maintained in the core-glycosylated form, indicative of residence within the ER. Exceptions to this model can be found. Hundal *et al.* (1992) showed, in muscle, that insulin stimulated the translocation of the α$_2$-subunit from an intracellular pool to the plasma membrane and the translocation of the β$_1$-subunit from a different intracellular pool to the plasma membrane. This exception implies that assembly of α$_2$/β$_1$ might occur outside the ER under insulin regulation, but alternative explanations are possible. Insect cells transfected with baculovirus carrying either α- or β-subunit genes express the subunits to their plasma membrane independent of each other (DeTomaso *et al.* 1992). An important feature of these baculovirus-transfected insect cells is that they can be induced to fuse and form syncytia, such that plasma membrane proteins can redistribute but the membrane proteins within the intracellular pool cannot. DeTomaso and Mercer (1993) recently exploited this property to demonstrate that assembly of functional Na$^+$/K$^+$-ATPase α/β complex on

the plasma membrane of the fused syncytium originated from cells independently expressing either the α-subunit or the β-subunit of the Na⁺/K⁺-ATPase. In contrast, assembly of hybrid Na⁺/K⁺-ATPase α-subunit and β-subunit of the H⁺/K⁺-ATPase did not occur in a fusion experiment, but did occur when the cells were cotransfected, suggesting that the hybrid could assemble only within the ER. This result provides a basis for the possibility of α/β assembly outside the ER. In vertebrate cells, the fact that α/β assembly appears to occur predominantly, or exclusively, within the ER may simply be due to the inability of the α-subunit to exit the ER. In addition, we point out that α/β assembly does not guarantee appropriate trafficking. In their study of deletion mutants, Fambrough's group showed that removal of four amino acids from the C terminus or five amino acids from the transmembrane segment did not prevent subunit assembly, but profoundly compromised the ability of the α/β complex to move out of the ER (Hamrick *et al.* 1993; Renaud *et al.* 1991).

α/β association of gastric H⁺/K⁺-ATPase, like that of Na⁺/K⁺-ATPase, appears to begin within the ER (Chow *et al.* 1994). Using the omeprazole-treated stomach model, Crothers *et al.* (1993) found that degeneration of H⁺/K⁺-ATPase was due to a parallel degradation of both α- and β-subunits. There has been one report that some mature β-subunit, independent of α-subunit, can be recovered from parietal cells (Baldwin, 1990); however, this recovery may be due to the exit of some unassociated β-subunit from the ER. In support of this hypothesis, for all reported expression systems, the β-subunit of the H⁺/K⁺-ATPase can exit the ER without a corresponding α-subunit, whereas the α-subunit of the H⁺/K⁺-ATPase appears to require α/β association before exiting the ER (Gottardi and Caplan, 1993*a*; Jaunin *et al.* 1993).

Trafficking in polarized cells

In polarized epithelial cells, ion translocators tend to be restricted to either the apical or basolateral membrane. Na⁺/K⁺-ATPase is localized to the basolateral membrane in most epithelial cells. Proper location of the Na⁺/K⁺-ATPase is essential for effective physiological activity; misdelivery to the apical membrane is an implied cause of polycystic kidney disease (Wilson *et al.* 1991; Avner *et al.* 1992). In total contrast to Na⁺/K⁺-ATPase, current pharmacological and physiological evidence suggests that all putative isoforms of H⁺/K⁺-ATPase reside on the apical membrane of the epithelial cells in which they are expressed (Wingo and Cain, 1993).

The detailed mechanisms for delivery of Na⁺/K⁺-ATPase to a specific membrane domain remain elusive. Studies in MDCK cells have led to conflicting conclusions: one study proposes that the enzyme is delivered exclusively to the basolateral membrane (Caplan *et al.* 1986); another proposes delivery to both apical and basolateral membranes, but that the enzyme is retained and stabilized in the basolateral membrane (Hammerton *et al.* 1991). The conflict may be due to different transport properties in the specific MDCK cell lines used by the different laboratories (Gottardi and Caplan, 1993*b*). In any event, such experimental differences in trafficking may by exploited to understand more fully the intracellular transport mechanisms of polarized membrane proteins.

What are the signals for intracellular transport of the α/β complex and the unassociated subunits of Na⁺/K⁺-ATPase and H⁺/K⁺-ATPase? In contrast to the β-subunit of the Na⁺/K⁺-ATPase, the β-subunit of the H⁺/K⁺-ATPase, expressed in either *Xenopus* oocytes or LLC-PK cells, exits the ER and is targeted to the apical plasma membrane even without an accompanying α-subunit (Jaunin *et al.* 1993; Gottardi and

Fig. 7. Hypothetical scheme for major pathways in the assembly and trafficking of Na⁺/K⁺-ATPase and H⁺/K⁺-ATPase. Individual α- and β-subunits are synthesized in the endoplasmic reticulum (ER), where β-subunits are core-glycosylated. Under physiological conditions, assembly of α- and β-subunits occurs in the ER. The assembled α/β-heterodimers move through the Golgi network, where further post-assembly processing takes place (sugar modification) and distinct paths are set up for trafficking of the Na⁺/K⁺-ATPase to the basolateral plasma membrane and the H⁺/K⁺-ATPase to the apical plasma membrane. Under certain specialized conditions, e.g. overexpression in cell lines, the β-subunit of H⁺/K⁺-ATPase (HKβ) can be individually processed and moves to the apical membrane and the α- and β-subunits can cross-assemble in the ER. Further trafficking beyond the ER of monomers of NaKα, HKα and NaKβ rarely occurs.
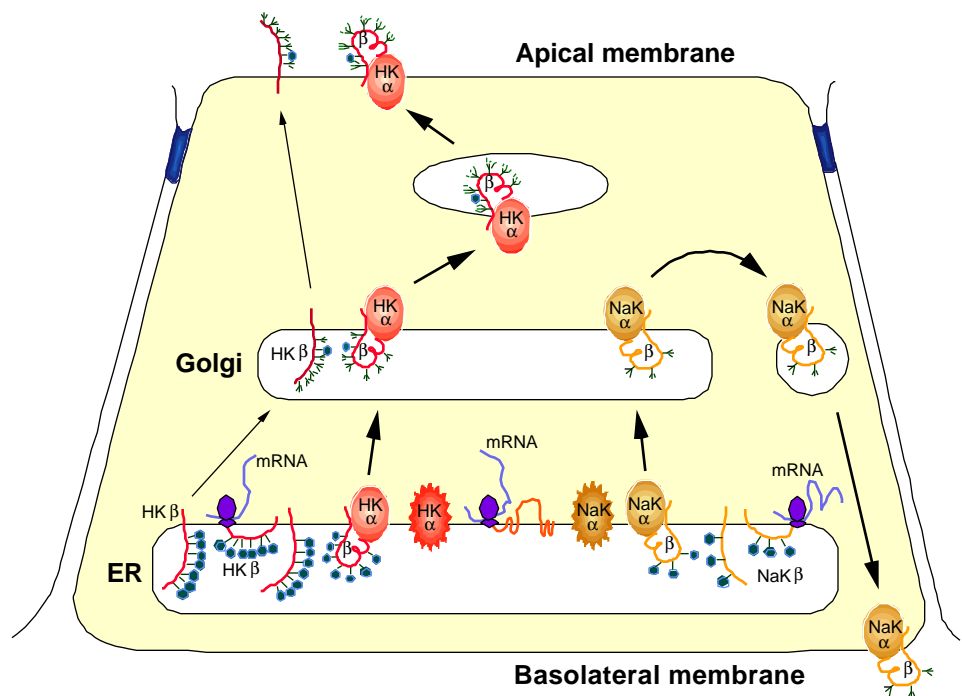
Caplan, 1993*c*). Furthermore, in the polarized LLC-PK cells, the H⁺/K⁺-ATPase β-subunit is delivered to the apical membrane, whereas Na⁺/K⁺-ATPase is delivered to the basolateral membrane. Chimeras constructed of the N-terminal half of the H⁺/K⁺-ATPase α-subunit and the C-terminal half of the Na⁺/K⁺-ATPase α-subunit not only assemble with Na⁺/K⁺-ATPase β-subunit, but the complex is also delivered to the apical membrane. This targeting suggests that both the α- and β-subunits of H⁺/K⁺-ATPase carry a signal for apical membrane delivery. The α-subunit of H⁺/K⁺-ATPase does require association with the β-subunit for efficient delivery to the apical membrane. Detection of the 52kDa core-glycosylated H⁺/K⁺-ATPase β-subunit in ER-enriched fractions from gastric homogenates is consistent with the β-subunit pausing within the ER for some processing activity, such as α/β-subunit assembly and functional maturation. A schematic model depicting the translation and assembly of the H⁺/K⁺-ATPase and Na⁺/K⁺-ATPase subunits and their trafficking to respective apical and basolateral membranes is shown in Fig. 7.

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