Stabilization of the H,K-ATPase M5M6 Membrane Hairpin by K⁺ Ions

MECHANISTIC SIGNIFICANCE FOR P₂-TYPE ATPases*

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The integral membrane protein, the gastric H,K-ATPase, is an α-β heterodimer, with 10 putative transmembrane segments in the α-subunit and one such segment in the β-subunit. All transmembrane segments remain within the membrane domain following trypsinization of the intact gastric H,K-ATPase in the presence of K⁺ ions, identified as M1M2, M3M4, M5M6, and M7, M8, M9, and M10. Removal of K⁺ ions from this digested preparation results in the selective loss of the M5M6 hairpin from the membrane. The release of the M5M6 fragment is directed to the extracellular phase or aqueous compartment. Occlusion was also recently shown to be retained in a post-tryptic preparation of the Na,K-ATPase composed of the transmembrane segments and lacking most of the cytosolically exposed protein (5).

Chemical modification and site-directed mutagenesis studies of expressed P₂-ATPases have indicated that the M5M6 transmembrane region may have a special importance in cation occlusion and transport (6–10). In studies on tryptic membrane preparations of the Na,K-ATPase obtained in the presence of K⁺, mere removal of K⁺ ions resulted in the selective release of the M5M6 hairpin from the membrane to the aqueous phase (11). This membrane stabilization of the M5M6 region by the counter-transported cation suggested that the organization and folding of the M5M6 hairpin and the interactions between M5M6 and the rest of the protein may be associated with the transport function (11). The uniqueness of this region of the membrane is also shown by the failure of the M5M6 hairpin to behave as an independent unit in the process of synthesis and insertion in in vitro translation studies of the H,K-ATPase (12). Similar conclusions were derived from Cos-1 cell translation of segments of the Na,K-ATPase and from in vitro translation of the endoplasmic reticulum Ca-ATPase (13, 14), suggesting a general property of this membrane domain of the P₂-type ATPases.

The present studies were undertaken to determine whether or not cation-dependent stabilization of the M5M6 hairpin in the membrane was also a feature of the H,K-ATPase, another P₂-ATPase. Because this protein can be obtained in a sealed vesicular inside out orientation, the directionality of the release of the M5M6 hairpin from the membrane (toward the cytoplasmic or extracellular space) following K⁺ removal could also be determined, unlike right side out oriented vesicles achieved in microsomal preparations of Na,K-ATPase (15). A preliminary report of this work has been presented (16).

EXPERIMENTAL PROCEDURES

Materials—TPCK-treated¹ trypsin, NaCl, KCl, Na₂ATP, bovine serum albumin, sucrose, ultra pure urea, Trizma (Tris) base, and Tricine were purchased from Sigma. β-Mercaptoethanol, SDS, ammonium per sulfate, and Coomassie Brilliant Blue R-250 were from Bio-Rad. 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was from ICN. Acrylamide and bisacrylamide were from Roche Molecular Biochemicals. Rainbow gel electrophoresis standards were from Amersharm Pharmacia Biotech. 7-Diethyilamino-3-(4'-maleimidylphenyl)-4-methyl coumarin (CFM) was from Molecular Probes. Polyvinylidene difluoride (PVDF) electrobolting membrane was from Millipore.

Enzyme Preparation and Activity Assay—The H,K-ATPase was isolated from hog gastric mucosa (17). Protein was determined by the method of Lowry et al. (18). Vesicles were aliquoted and stored (3–5 mg/ml protein) in a 34% sucrose solution buffered with 50 mM PIPES

P-type ATPases can be classified into two subgroups based upon their function and structure (3). A mechanistic aspect of most P₂-type ATPases is occlusion of the transported cations during the reaction cycle (4). Such occlusion is pictured as an intermediate state where cations are bound within the transmembrane segments of the protein without free access to either aqueous compartment. Occlusion was also recently shown to be retained in a post-tryptic preparation of the Na,K-ATPase composed of the transmembrane segments and lacking most of the cytosolically exposed protein (5).

¹ The abbreviations used are: TPCK, t-1-tosylamido-2-phenylethyl chloromethyl ketone; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methyl coumarin; PVDF, polyvinylidene difluoride; PIPES, 1,4-piperazinedithanesulfonic acid; CAPS, 3-cyclohexylamino)propanesulfonic acid.

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Fig. 1. Scheme showing the methods used to determine the directionality of the M5M6 hairpin loss from a post-tryptic membrane preparation of porcine gastric H,K-ATPase. 1, intact cytoplasmic side out vesicle preparation containing intact gastric H,K-ATPase α and β subunits. 2, post-tryptic membrane preparation of H,K-ATPase showing the remaining segments: M1M2, M3M4, M5M6, and M7–M10, as well as an essentially intact β-subunit. 3, the two potential outcomes depending on the directionality of release. a, expected result if M5M6 release is to the cytoplasmic side; b, expected result if M5M6 is released to the extracellular side. 4, if the M5M6 segment is released to the extracellular side (intravesicular side) then permeabilization of the vesicles is required to release the “trapped” peptide.

K⁺ Dependence of Transmembrane Mobility in H,K-ATPase

Results

The two specific questions addressed by our studies were (i) in the H,K-ATPase post-tryptic preparation does the removal of K⁺ ions lead to the destabilization and selective release of the M5M6 hairpin from the membrane and (ii) if such release occurs, is the hairpin released to the cytoplasmic or extracellular compartment?

SDS Treatment of the H,K-ATPase Vesicle Preparation—As shown in Fig. 1, SDS treatment of the vesicle preparation is used to gain access to the intravesicular (extracellular) space. If release of M5M6 occurs to the extracellular space the vesicles will need to be disrupted in order for the M5M6 hairpin to be seen in the supernatant; if release is to the cytoplasmic space, disruption of the vesicles by detergent will not be required, and centrifugation alone will separate the released hairpin from the vesicles. It was necessary first to determine an appropriate SDS concentration that would permeabilize the vesicles but not denature or disrupt the H,K-ATPase. To do this, we made use of the fact that for maximal H,K-ATPase activity K⁺ ions must gain access to the intravesicular space. Fig. 2 demonstrates that SDS ratios ≥0.1% (w/v) were able to disrupt vesicle integrity without denaturing the H,K-ATPase.

Directionality of Release of the M5M6 Hairpin—Just as was originally reported for the Na,K-ATPase (5), extensive tryptic digestion of the H,K-ATPase in the presence of K⁺ ions produces a membrane residue that contains the β-subunit (largely undigested) and four sets of transmembrane segments, M1M2, M3M4, M5M6, and M7–M10 (22). The post-tryptic preparations of both the Na,K- and H,K-ATPases retained the ability to occlude K⁺ ions (5, 23). In the Na,K-ATPase, removal of K⁺ ions from this preparation resulted in a loss of the ability to occlude K⁺ ions associated with the release of the M5M6 hairpin from the membrane to the aqueous phase (11). Fig. 3 shows a single fluorescently labeled peptide (~10 kDa) that was released from the post-tryptic H,K-ATPase preparation upon the removal of K⁺ ions. N-terminal amino acid analysis gave a single sequence (NAADML. . .) that corresponds to the residues in the M5M6 hairpin (Table 1). It was shown earlier that both the N and C termini of this fragment are cytoplasmic (24), whereas either (or both) cysteine 813 and 822 within this stretch are located at the extracytoplasmic side (25). These observations demonstrated that the M5M6 segment indeed spans the membrane twice. Furthermore, this peptide was not released to the supernatant until after SDS treatment of the proteolyzed vesicles, thus it is released toward the intravesicular (extracellular) space (Fig. 3A). In some experiments, a faint

FIG. 1 . Scheme showing the methods used to determine the directionality of the M5M6 hairpin loss from a post-tryptic membrane preparation of porcine gastric H,K-ATPase. 1, intact cytoplasmic side out vesicle preparation containing intact gastric H,K-ATPase α and β subunits. 2, post-tryptic membrane preparation of H,K-ATPase showing the remaining segments: M1M2, M3M4, M5M6, and M7–M10, as well as an essentially intact β-subunit. 3, the two potential outcomes depending on the directionality of release. a, expected result if M5M6 release is to the cytoplasmic side; b, expected result if M5M6 is released to the extracellular side. 4, if the M5M6 segment is released to the extracellular side (intravesicular side) then permeabilization of the vesicles is required to release the “trapped” peptide.
band was apparent (~10 kDa) in the supernatant prior to SDS treatment (data not shown). This peptide was most likely the M5M6 hairpin, because it was only observed when K⁺ ions were not present in the trypsinized preparation (i.e. treatments outlined in 3a of Fig. 1). Additionally, because the intensity of this band appeared to vary inversely with the fraction of tight vesicles (as determined by K⁺ ionophore stimulated ATPase activity), we conclude that the M5M6 hairpin is released to the intravesicular space and then exits from leaky vesicles. Consistent with this conclusion is that SDS permeabilization of these preparations subsequently revealed a vivid band at 10 kDa that subsequent amino acid sequencing confirmed as M5M6 (Table 1).

These findings cannot directly rule out an SDS effect on the post-tryptic H,K-ATPase preparation. That is, does SDS itself promote the release of the M5M6 fragment? To answer this question, we also treated post-tryptic H,K-ATPase vesicles with SDS in the presence of 200 mM K⁺. Under these conditions, we observed no peptide release (Fig. 3B, lane 1). However, when K⁺ was subsequently removed from these permeabilized vesicles the M5M6 hairpin appeared in the supernatant (Fig. 3B, lane 2).

In the experiments discussed above, the post-tryptic membrane preparation was obtained in the presence of 200 mM KCl. In two initial experiments, when trypsin digestion was performed in the presence of ~20 mM KCl, we obtained two separate peptide sequences from the released fragment (~10 kDa; Table 1). The first sequence corresponded to M5M6, whereas the second sequence corresponded to the M7M8 transmembrane pair (Table 1). It has been previously shown that in the absence of K⁺ ions an additional trypsin cleavage takes place in the H,K-ATPase between M8 and M9 (22). It seems that this cleavage also takes place at low K⁺ (i.e. ~20 mM), thus separating the M7M8 hairpin from the ~21-kDa C terminus.

**DISCUSSION**

The membrane domain of the P₂-type ATPases contains the ion transport pathway, and the results of a large number of studies have been interpreted as showing involvement of M4, M5, M6, and perhaps M8 in ion translocation and ion occlusion (2, 7, 26). Various methods including sequencing of membrane-embedded tryptic fragments (5, 22), labeling with sited thio-philic reagents (23), or extracytoplasmic photoactivatable re-agents (27) and in vitro translation provided strong evidence for 10 membrane segments in the α-subunit of the P₂-type ATPases.

Hydropathy algorithms of P₂-type ATPases all predict four membrane sequences in the N-terminal sector but predict only a single sequence in the M5M6 region and vary in terms of prediction of the last four segments (3). In accord with the ambiguity of these predictions, in vitro translation of individual segments or truncated constructs of the H,K-ATPase were not able to demonstrate transmembrane insertion of M5, M6, and M7 (13). Expression of segments or truncated constructs of the Na,K-ATPase also showed that M5 and M6 were not membrane inserted (14, 28). These results suggested that peptide-peptide interactions were more significant for these segments as compared with the N-terminal segments. Indeed, it was suggested earlier that the M5M6 hairpin of the Na,K-ATPase was involved in protein-protein interactions with other transmembrane segments within the lipid portion (11). Furthermore, our results (Ref. 11 and this work) suggest that cations are also required for stabilization in the membrane of the M5M6 hairpin of P₂-type ATPases.

In the case of the P₂-type ATPases, results from chemical modification (8) and mutagenesis (6–10) have suggested that amino acid residues in M5M6 are intimately associated with cation occlusion and transport, and this hairpin is now believed to play a central role in cation binding and complexation. The loss of the M5M6 hairpin from the membrane and its prevention by K⁺ (Ref. 11 and this work) suggest that the occluded K⁺ ions play a role in causing a rearrangement of these membrane segments. One plausible mechanism for the action may be the neutralization of repulsive negative charges in Asp residues of M6 and the Glu in M5. Cation complexation by the electron-donating residues of M5M6 substitute for hydration and stabilize the presence of the charged cation in the membrane. At the same time the positive cation apparently serves to stabilize the anionic and hydrophilic transmembrane segments of M5 and M6 within the membrane.

The finding that the M5M6 hairpin of about 10–11 kDa (or about 90 amino acid residues) is lost to the extracellular space shows that this redistribution involves the mobilization across the membrane of a considerable number of charged and hydrophilic amino acid residues. We believe that M5M6 is probably surrounded by other protein helices rather than the membrane lipid (3, 11). In this way protein-protein interactions would be greatly modified as cations (e.g. K⁺) are bound to and released.
by the pump. Another line of evidence suggested mobility in this region of the enzyme. Pantoprazole (5-difluoromethoxy-2-[3,4-methoxy-2-pyridyl)methylsulfinyl]-1H-benzimidazole) converts to a cationic thiolic sulfenamide and binds to Cys\(^{813}\) and Cys\(^{822}\) in the loop joining M5 and M6 forming disulfide bonds. Prior to this covalent reaction tryptic cleavage occurs largely at Lys\(^{791}\) (29). After derivatization, cleavage is found only at Lys\(^{791}\), showing that formation of disulfides within the connecting loop of M5 and M6 results in a change in accessibility of the N-terminal region of M5 to trypsin. Furthermore, the exposure of Cys\(^{883}\) in M10 of the Na,K-ATPase to sulfhydryl-reactive probes (but not of Cys residues in M1, M2, or M4) following the loss of the M5M6 hairpin also provides evidence for interactions between C-terminal segments and M5M6 (15). Similar conclusions have been reached from \textit{in vitro} translation studies of both the Na,K-ATPase and the Ca-ATPase (13, 14).

In addition to the release of the M5M6 hairpin, the M7M8 transmembrane pair was also released from the membrane following digestion by trypsin in low K\(^+\) conditions (Table I) where the 21-kDa fragment is cleaved between M7M8 and M9M10, also suggesting a weak interaction between the bilayer and this pair of transmembrane segments. Prior to cleavage between M8 and M9, the M5M6 tryptic fragment remains largely at Arg\(^{775}\), when cleavage occurs between M8 and M9 the hairpin and this pair of transmembrane segments. Prior to cleavage of the connecting cytoplasmic linkage.

TABLE I

| Experiment number | Sequence       | H,K-ATPase region | Size  | [KCl] during digestion |
|-------------------|----------------|-------------------|-------|------------------------|
| 1                 | NAADM1LDDD     | M5M6              | –10   | 200                    |
| 2                 | NAADM1L        | M5M6              | –10   | 200                    |
| 3                 | NAADM1L        | M5M6              | –10   | 200                    |
| 4                 | NAADM1L        | M5M6              | –10   | 200                    |
| 5                 | LVNEPLAAYS     | M7M8              | –10   | 20                    |
| 6                 | NAADM1L        | M5M6              | –10   | 20                    |
| 7                 | LVNEP1L        | M7M8              | –10   | 20                    |

The striking mobility of the M5M6 hairpin with respect to the membrane that is modified by the presence of the occluded and transported cation plays a significant role in the transport cycle. Indeed, the direct link between the M5M6 hairpin and the large intracellular loop, shown to contain the ATP binding site (33–35), is consistent with a role in coupling ATP hydrolysis with cation transport. We have speculated earlier (11) that movements of M5M6 in the Na,K-ATPase protein in a direction that is perpendicular to the plane of the membrane may play a role in active transport; on the basis of the present results such a mechanism may apply to other P\(_2\)-ATPases.

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