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Structural Aspects of the Gastric H,K-ATPase

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The gastric H,K-ATPase is an alpha,beta heterodimer. The large catalytic subunit is composed, in the case of the hog enzyme, of 1033 amino acids, whereas the beta subunit is composed of about 291 amino acids and is heavily glycosylated. The membrane topology of the alpha subunit is difficult to predict using hydropathy analysis. Tryptic hydrolysis of intact, inside out vesicles followed by cysteine labelling with fluorescein-5-maleimide provided experimental evidence for an 8 membrane spanning model for the alpha subunit, between residues 104 and 162 (M1/M2), 291 and 358 (M3/M4), 776 and 835 (M5/M6), and 853 and 946 (M7/M8). No evidence was found for a pair of segments (M9/M10) towards the C terminal end of the molecule, contrary to predictions for the Na,K- and Ca-ATPases. Iodination of intact vesicles followed by carboxypeptidase Y cleavage of the C terminal tyrosines showed that the C terminal end of the alpha subunit was cytoplasmic. The epitope for antibody 146 was extracytoplasmic and located between residues 871 to 874 between M7/M8. The binding site of the K competitive imidazo-pyridine, SCH28080, was to the extracytoplasmic loop between M1 and M2, whereas the binding of the covalent SH reagent generated from acid activation of omeprazole in acid transporting vesicles was to 2 cysteines at positions 813 (or 822) and 892 predicted to be in the extracytoplasmic loops connecting M5/M6 and M7/M8, respectively. The beta subunit was only hydrolysed in broken vesicles. A fragment beginning at position 236 was liberated under these conditions only in the presence of reducing agents, showing that cysteine 210 and 263 were disulfide linked. It seems that this subunit has only a single membrane spanning segment as predicted by hydrophobicity. Binding of either SCH28080 or omeprazole to the extracytoplasmic face of the enzyme affected cytoplasmic conformational changes, showing that there was transmembranal transmission of changes of shape of the protein.

KEY WORDS: Gastric; H,K-ATPase; omeprazole; SCH28080; topology; alpha subunit; beta subunit.

INTRODUCTION

The gastric H,K-ATPase is an α,β heterodimeric member of the phosphorylating ion-motive ATPase group. It is responsible for the secretion of gastric HCl, and does so by an H for K exchange mechanism. The final gradient of H is about 4×10^6 and of K about 10^1 in the other direction. It would therefore appear to be the most powerful ion-motive device in this class of enzyme thus far described.

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PRIMARY STRUCTURE

The α subunit of this ATPase has been cloned from various species; man, dog, hog, rabbit, and rat to name some (Shull and Lingrel, 1986). The β subunit has been cloned from rat, rabbit, hog, and man (Reuben *et al.*, 1990). The homology between the α subunit of the H,K and the α_1 of the Na,K subunit is of the order of 65% and between β subunits of the order of 35%.

There is close association between the two subunits since they are readily cross-linked with glutaraldehyde. It appears that there are in the mature enzyme, as isolated from hog mucosa, 2 MWt classes

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of the β subunit, of unknown significance (Rabon *et al.*, 1991a).

The close association appears to play a role in the stabilization of the α product, in that cooexpression of the Na pump α and β stabilized the appearance of the α protein in *Xenopus* oocytes (Geering, 1990). The β subunit of the H,K-ATPase was able to act as a surrogate for the β subunit of the Na,K-ATPase (Horisberger *et al.*, 1991). Thus far, no enzymic function has been established for the β subunit.

The α subunit is composed of about 1000 amino acids, and the β subunit of about 300 amino acids. The β subunit has six glycosylation consensus sequences and is clearly glycosylated on its extracytoplasmic face (Hall *et al.*, 1990). The α subunit has three glycosylation consensus sequences, and there is some evidence that there is partial glycosylation of this subunit also (Tai *et al.*, 1989).

SECONDARY STRUCTURE

The development of routine methods that give direct information as to the secondary structure of this class of enzyme has been slow. A particular focus has been the membrane arrangment of the class. This problem is highlighted by the hydropathy profiles for the two subunits as displayed in Figs. 1 and 2 for both the α and the β subunits. It is usual to consider that only α helices tranverse the membrane, and that alternative structures such as β sheets remain extramembranal. The basis for this is that so far only α helices have been seen in membrane-embedded proteins (with the exception of porin). Until many more crystal structures have been studied, one cannot be certain that this assumption is correct.

If we use this hydropathy profile as the sole method for establishing membrane distribution of the α protein, it can be seen that there is likely to be four membrane-spanning segments in the N terminal one-third of the protein. In the C terminal one-third of the protein, the prediction from hydrophobicity is much more difficult, both odd and even numbers being possible, ranging from 3 to 5, by inspection. Further, there are also regions in the middle third of the protein that have almost the same hydrophobicity as in the C terminal one-third. It is also not essential for every region that is hydrophobic to be membrane spanning. Conversely, hydrophilic regions may also be membrane spanning.

The β subunit has a single relatively large region of hydrophobicity and another hydrophobic region a few amino acids proximal to the C terminal region that is also devoid of glycosylation consensus sequences. The natural interpretation for the membrane arrangement for this sequence is that there is a single membrane-spanning segment, with the majority of the protein being placed extracytoplasmically. However, there has been speculation for the β subunit of the Na,K-ATPase, that this might be an oversimplification (Capasso *et al.*, 1992).

It is therefore necessary to determine the membrane location of every possible membrane-embedded segment in this enzyme and not rely entirely on perhaps unreliable hydrophobic scales for amino acids.

DETERMINATION OF MEMBRANE ORIENTATION

α Subunit

The gastric enzyme is readily isolated as intact, ion-tight, inside-out vesicles. With therefore a sided preparation, it would seem relatively straightforward to use a set of techniques that would establish unequivocally the location of each region of the enzyme. The techniques available are relatively standard and include protease cleavage of cytoplasmic residues, location of sites of reaction of sided ligands such as ATP, FITC, or pyridoxal phosphate which would be cytoplasmic, iodination of C terminal residues followed by carboxypeptidase cleavage, the mapping of antibody epitopes, the generation of sided reagents similar to ouabain and determination of their site of action, and the use of fusion protein technology to determine the presence or absence of signal start and stop sequences in the protein. Another approach that has been used with interesting results in the Ca-ATPases is mutagenesis of residues thought to be intramembranal and part of the hydrophilic pathway through the membrane sector (Clarke *et al.*, 1990a).

Tryptic Cleavage of the α Subunit

The assumption of this approach is that, provided care is taken to maintain vesicle integrity, protease cleavage of such vesicles specifies the cytoplasmic location of these sites. It is of course necessary to select for the intramembranal segments so as not to be confused by the possible 97 tryptic fragments, for example, that can originate from the α subunit. This is achieved in part by washing the hydrolyzed membranes free of dissociable peptide, and in part by

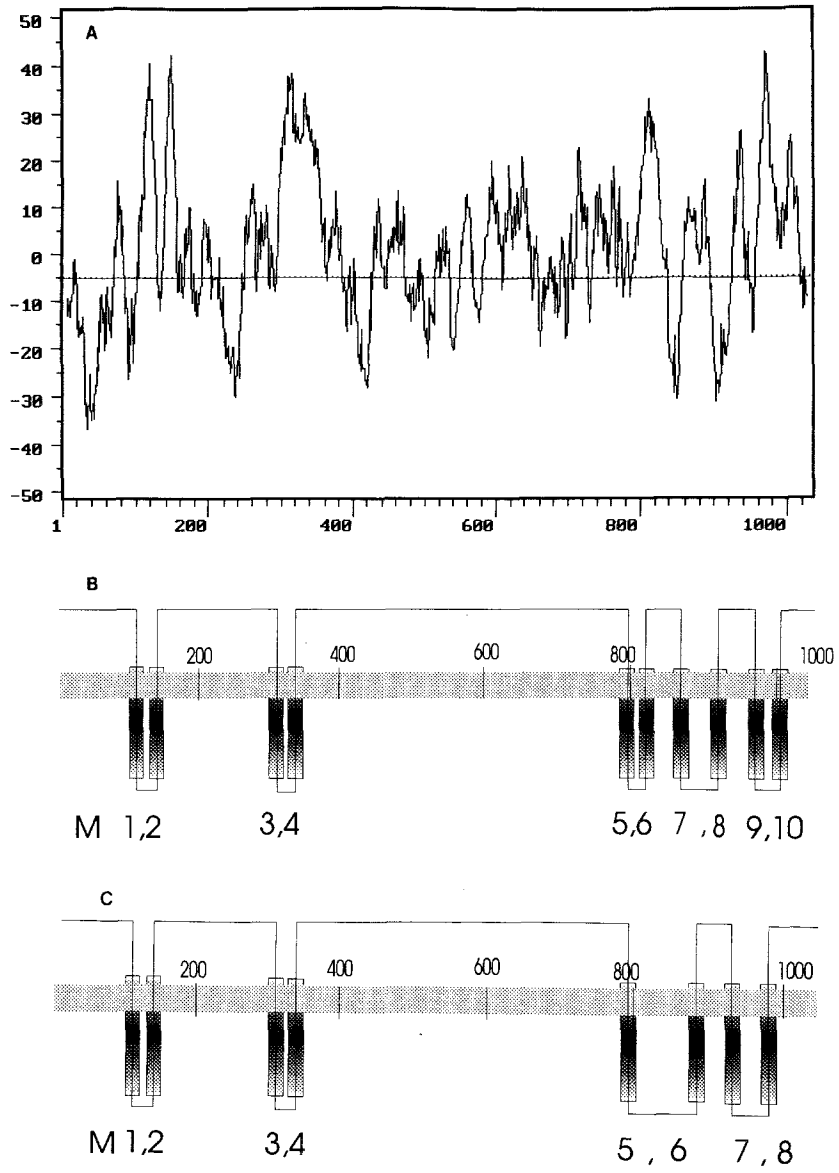

Fig. 1. (A) The hydropathy profile of the α subunit of the H,K-ATPase is presented, using a moving average of 15 amino acids. Hydropathic index of ATHA$PIG from amino acid 1 to amino acid 1034. Computed using an interval of 15 amino acids (GRAVY = 0.54). (B) The 10 membrane segment model thought to be justified by present data. (C) The model originally proposed for the membrane topology of this subunit (Shull and Lingrel, 1986).

relatively short incubation times with relatively high concentrations of trypsin.

In the particular instance of the H,K-ATPase, inspecting putative membrane-spanning sequences, in an 8- or 10-membrane segment model, we saw that each membrane pair had at least 1 cysteine, which would then allow fluorescent labeling of the cysteines left after complete cleavage of the cytoplasmic domain. When this is done using fluorescein-5-maleimide, four fluorescent peptides are detected, representing the first four membrane pairs of the model of the 10 segment arrangement of Fig. 1.

Progressive tryptic hydrolysis using lower protein/trypsin ratios gives similar types of conclusions, but without the clear results of the combination of high ratios and fluorescent SH labelling. When trypsin/

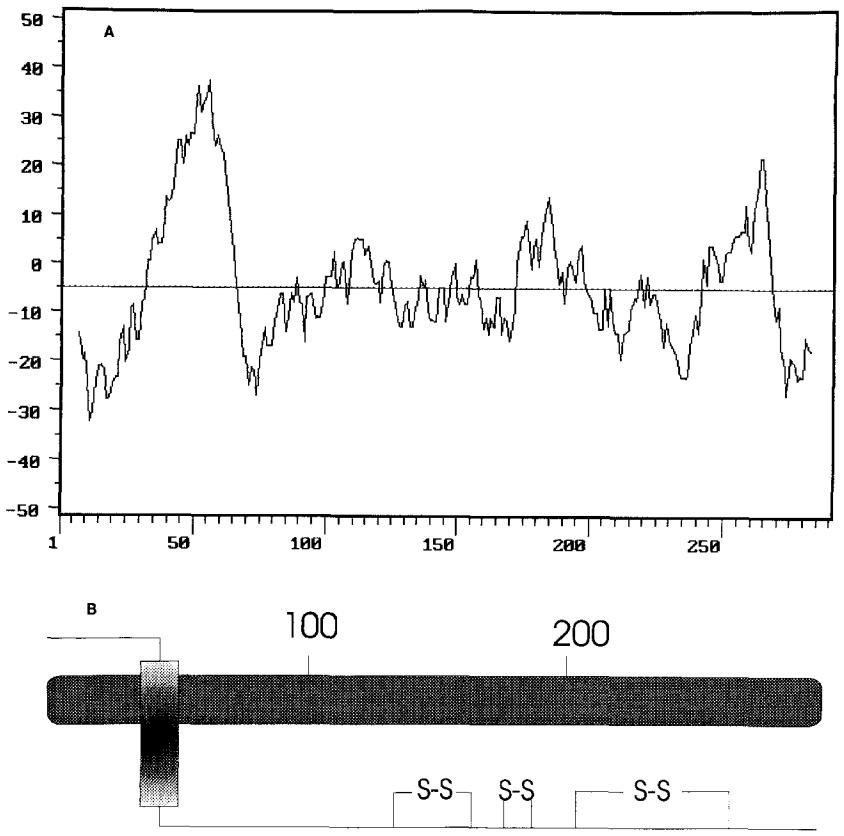

Fig. 2. (A) The hydropathy profile of the β subunit, again using a moving average of 15 amino acids. Hydropathic index of ATHA$PIG from amino acid 1 to amino acid 290. Computed using an interval of 15 amino acids (GRAVY = 0.31). (B) The single membrane spanning model justified by the present data, showing in addition the disulfide linkage in the extracytoplasmic domain.

protein ratios of between 1/75 and 1/30 are used, it is necessary to sequence multiple peptides before an overall picture can emerge of the membrane arrangement. With the fluorescent method the membrane segments are immediately obvious, but, as noted, this is dependent on the presence of residual, reactive SH groups in all the pairs left in the membrane.

From these data there is substantial evidence for at least eight membrane-spanning segments, as defined by the presence of cysteine in pairs left in the membrane after rapid, severe trypsinization. The last pair might have disulfide-linked cysteines requiring reduction before labelling. The hydrophobicity in this region is sufficiently compelling to continue to place an M9/M10 pair in the membrane, to give a total of 10 membrane segments. It should be noted that this method gives clear fluorescent bands not detected by Coomassie staining, since the latter is less effective in staining small hydrophobic peptides.

Ligand Sites

Since ATP is cytoplasmic, it is certain that the site of phosphorylation by ATP is also cytoplasmic. In the H,-K-ATPase this is asp^{385}. Another assumption is that sites protected by ATP against reaction are also cytoplasmic. This is likely to be true, but not certain, since conformations can be transmitted over the membrane-spanning segments. Nevertheless, it would appear likely that FITC binding to lys^{516} (Walderhaug *et al.*, 1985) and pyridoxal phosphate binding to lys^{496} (Tamura *et al.*, 1989) do show that these residues are indeed cytoplasmic. On the other hand, a monoclonal antibody, 95-111, has its epitope between ser^{531} and arg^{560}. This antibody inhibits the ATPase in intact vesicles and is K competitive in the presence of nigericin, showing conformational interaction across the membrane between the epitope and K activation of ATPase activity (D. Bayle, M. F. M. Lewin, and A. Soumermon, personal communication).

Iodination

The C terminal amino acids of the α subunit are tyr-tyr. It has been possible therefore to iodinate the intact vesicles with peroxidase–H_2O_2–^{125}I, and show that of the two subunits, only the α is labelled. Following this, digestion with carboxypeptidase Y released about 28% of the counts incorporated into the α subunit, as would be predicted from a cytoplasmic location of the C terminal tyrosines (Scott *et al.*, 1991). This then shows that the number of membrane-spanning segments in the α subunit is even.

Mapping of Antibody Epitopes

The procedure with antibodies is either to generate antibodies specific for a known sequence in the peptide, or to take known monoclonals that have been well defined and determine their epitope. Once the epitope is known, then knowledge of the sidedness of the epitope again gives information as to the membrane-spanning structure of the protein. Unknown epitopes may be defined by the peptide walk method using successive octapeptides bound to pegs on Elisa plates, by generation of *c*DNA fragments and expression of these in *E. coli* followed by Western blotting, or by analysis of Western blots of trypic digests of the protein, where sequence is known.

Locating epitopes may not be a trivial problem. Various ways have been developed. One method using intact vesicles of known orientation is to compare reactivity on Elisa plates with and without detergent. It is asumed that epitopes inside the vesicle will only react following detergent, and this approach has been taken for the Ca-ATPase (Matthews *et al.*, 1990; Clarke *et al.*, 1990b). Hence epitopes that require detergent would be regarded as extracytoplasmic. However, epitopes that are located at the phospholipid headgroups could be relatively inaccessible to antibody without detergent, and results from this method might be misleading.

Comparing fluorescent reaction of intact and permeable cells is another approach taken when dealing with a plasma-membrane enzyme such as the Na,K-ATPase. However, again what is intact and what is permeable requires careful definition. For a non-plasma membrane enzyme, however, that is part membrane inserted, determination of the sidedness of an epitope by intact cell reaction is possible only for extracytoplasmic epitopes. Permeabilization, which reveals non-membrane-inserted antigen, always enhances the signal independent of the sidedness of the epitope. Determining the sidedness of immunogold particles in cells and/or vesicles is probably the method of choice, using immunoelectron microscopy, provided artefacts are corrected for, such as nonspecific binding and edge effects, and provided adequate post-embedding technique is developed that truly allows comparison between intracellular or intravesicular labelling and extracellular or extravesicular labelling.

In the case of the H,K-ATPase, antibody 146 has been well defined in terms of sidedness of the epitope (Mercier *et al.*, 1989) and in terms of the nature of the epitope itself. The latter was localized generally by analysis of Western blots of fragments of known sequence (Bamberg *et al.*, 1992), and then refined by the Elisa method by determining binding to octapeptides immobilized on Elisa plates (F. Mercier and T. Joys, personal communication). The epitope was defined by the partial digest method to be between positions 855 and 901 (Bamberg *et al.*, 1992), and then by the Elisa shown to be uniquely between 871 and 874, FAGF.

Generation of Sided Reagents

The best-known sided reagent for this class of pump is ouabain, known to bind to the region of the Na,K-ATPase encompassing the putative extracytoplasmic domain between M1 and M2 (Price and Lingrel, 1988). In the case of the gastric H,K-ATPase, two sets of reagents are known that also react exclusively with the extracytoplasmic surface and hence are useful in mapping the membrane position of these sites and in determining the functional implications of binding at these sites.

SCH28080 and its closely related photoaffinity derivative, MeDAZIP$^+$ (Fig. 3), have been shown to bind to the same general region of the H,K-ATPase that ouabain binds to in the Na,K-ATPase (Munson *et al.*, 1991). The technique used was to photolyze radioactive MeDAZIP$^+$, in the presence or absence of K^+, and isolate and sequence the radioactive peptides after protease digestion of the labelled vesicles. Figure 4 illustrates the results achieved. The inhibitors are strictly K competitive and stabilize the E_2P form of the enzyme. Indeed, another class of K-competitive inhibitor, MDPQ, an arylquinoline, shows that the region of binding undergoes conformational change with phosphorylation that is consistent with a motion of the binding site toward a hydrophobic region. Modelling the binding site, based on the known neces-

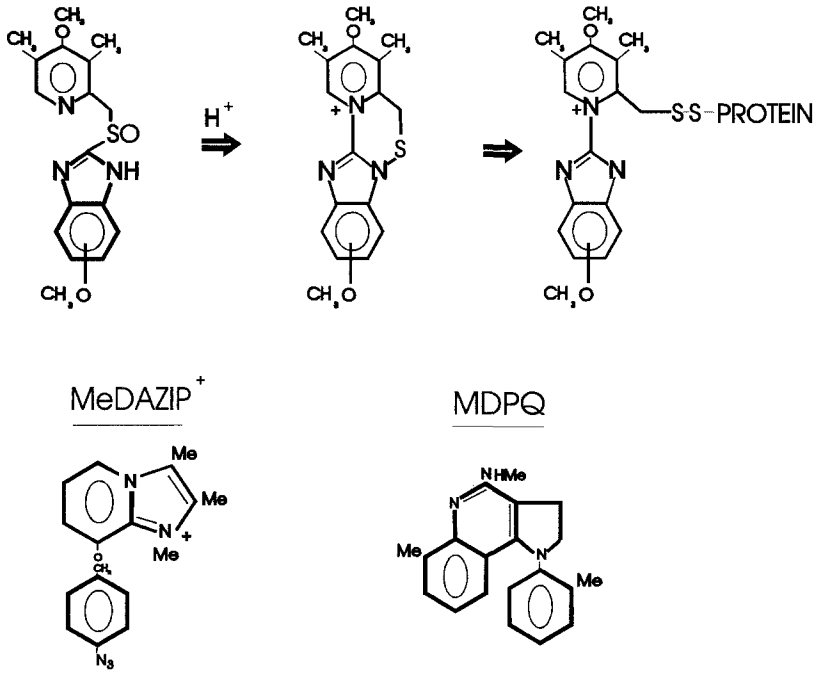

Fig. 3. The structure of the inhibitors used to define extracytoplasmic sites in the H,K-ATPase. Above is the reaction pathway of the acid-activated inhibitor, omeprazole. Below are the K competitive photoaffinity ligand, MeDAZIP$^+$, and the fluorescent K competitive compound, MDPQ.

sary conformation of these inhibitors, suggests that phe[124] and asp[136] interact with the inhibitors. This region is predicted to be just at the membrane interface.

Since the inhibitor is effective only from the extracytoplasmic face, this allows placement of the first membrane pair of the H,K-ATPase, much as the ouabain binding site allows placement of the first membrane pair of the Na,K-ATPase.

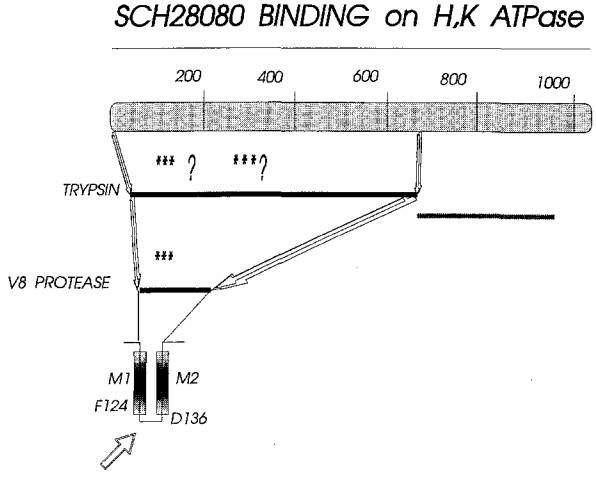

Fig. 4. A summary of the trypsin and V8 protease digestion results which allowed the conclusion that the M1/M2 region was the one reacting wtih MeDAZIP$^+$.

A second class of inhibitor typified by omeprazole is covalent. The reaction of this inhibitor depends on the trapping of a weak base in an acid space generated by the enzyme, followed by acid catalyzed conversion to a cationic sulfenamide, which then reacts with available cysteines to form disulfides as shown in Fig. 3 for the inhibitor omeprazole. When the reaction is carried out in acid-transporting vesicles, two cysteines are reacted at full enzyme inhibition (Lorentzon *et al.*, 1987). Tryptic or V8 protease digestion followed by separation of fragments and sequencing have shown that the cysteines that react are in fact the only cysteines predicted to be in the extracytoplasmic domain in the 10-membrane segment model, namely cys[813/822] and cys[892] (Besancon *et al.*, 1992). The results are summarized in Fig. 5. Since there are tryptic cleavage sites immediately proximal to these cysteines in intact vesicles, these two regions of labeling provide evidence that the protein crosses the membrane twice between these sites.

Not shown in Fig. 5 is the additional finding, using the 1/4 trypsin/protein ratio, that, of the four

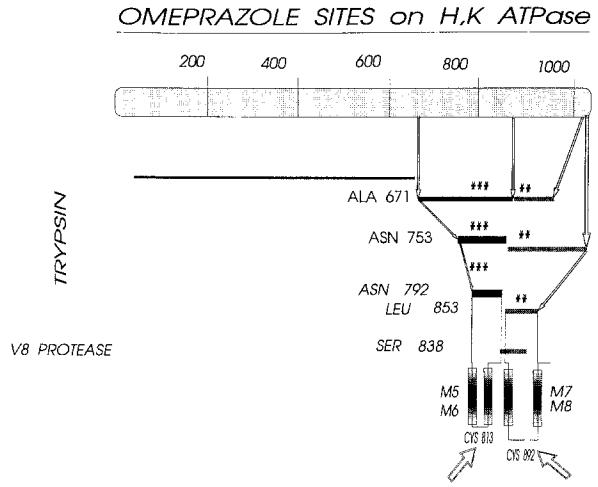

Fig. 5. A summary of the trypsin and V8 protease digestion results which showed that the cysteines reacting with omeprazole sulfenamide on the interior of gastric vesicles were cys^{813} (although cys^{822} has not been excluded) and cys^{892}.

bands labelled with fluorescein-5-maleimide, only two retained omeprazole radioactivity, those corresponding to M5/M6 and M7/M8 as concluded from the more limited digest procedure. The advantage of the procedure was greater retention of radiolabel in the latter band as compared to the lower trypsin/protein ratios.

Fusion Proteins

A method has been developed in *E. coli* that allows detection of membrane-inserted regions, whereby a fragment of *c*DNA containing one or more putative membrane segments is fused at the C terminal end with a signalling protein such as phosphatase, and the sidedness of appearance of this phosphatase allows determination of the number of membrane segments present in the construct. This technique can also be extended to *in vitro* translation using microsomes, with a glycosylated protein as the signalling system. Thus far, it does not appear to have been applied to the H,K-ATPase, but the presence or absence of membrane binding of mRNA fragments *in vitro* has been used to show partial membrane topology of the Na,K-ATPase (Homareda *et al.*, 1989).

β Subunit

The hydropathy profile of the β subunit appears less ambiguous than that of the α subunit. However, it is again necessary to carry out experiments to reveal the membrane arrangement (Fig. 1).

There are six cysteines predicted in the extracytoplasmic domain. Since there is no stable reaction with the omeprazole sulfenamide, it is likely that these, as for the Na,K-ATPase (Kirley, 1989), are disulfide linked. Hydrolysis of the enzyme in intact vesicles produces no visible cleavage of the β subunit, in contrast to the α subunit. When lyophilized vesicles are cleaved and PAGE carried out in the absence of reducing reagents, there is also remarkably little evidence of cleavage. However, in the presence of reducing agents, a peptide is produced in high yield, along with disappearance of the β subunit, which has a sequence beginning AQP, at position 231 of the β subunit (Hall *et al.*, 1990). On either side of this position there are cysteines at position 201 and position 262, which must be disulfide linked to give these data. Accordingly, the second region of hydrophobicity cannot be membrane spanning, and it is likely therefore that the C terminal segment of the β subunit is extracytoplasmic, with the proviso that β sheets are not present.

STRUCTURAL ASPECTS OF TRANSPORT

For all the members of this class of ATPase, the coupling of scalar ATP hydrolysis to vectorial transport is thought to depend on conformational changes. These pumps are thus chemomechanical devices designed to use changes in protein conformation to alter sidedness and affinity of ion-binding sites with respect to the membrane. The ultimate goal of all research into mechanisms of transport by these pumps is to be able to define the regions of the enzyme that comprise the ion sites and the pumping sectors. The eventual solution will be obtained using crystals of adequate resolution of the enzyme in at least two conformations. Much effort is being expended in that direction, but it is not clear when the breakthroughs will occur.

Techniques such as those discussed above can be used, however, to give partial information on this topic. As an example, the use of K-competitive, photoaffinity-modified reagents in the H,K-ATPase has shown that segments M1/M2 and their intervening loop are the site of interaction of this class of reagent.

The fluorescent K-competitive arylquinoline, MDPQ, shows conformational changes with binding and phosphorylation (Rabon *et al.*, 1991b). In the latter instance, the probe appears to move into a more hydrophobic environment. Kinetic analysis has shown that this reagent type stabilizes an E_2P form of

enzyme. Hence the binding site can be thought of as moving further into the membrane domain with the generation of the E_2P form. FITC labels the enzyme at lys^{516}. With the addition of Na as a proton surrogate, i.e., with the formation of the E_1 form, the quantum yield from FITC increases, as if the probe is becoming more hydrophobic. With the addition of K, forming the E_2 form, the FITC loses fluorescence, as if moving into a more hydrophilic environment (Rabon *et al.*, 1991c). Lys^{516} is thought to be present in the cytoplasmic loop between M4 and M5. Accordingly, the M1/M2 region moves reciprocally to the M4/M5 region as the enzyme adopts E_1 and then E_2 conformations.

These data show also that conformational changes are transmitted from inside to outside the enzyme, presumably across the membrane sector of the protein. Additional evidence for this is that the two cysteines that react with omeprazole are present in the extracellular domain, and binding of the sulfenamide prevents ATPase activity and phosphorylation which occurs at asp^{385} (Linberg *et al.*, 1986).

It would seem that motion of the membrane sector vertical to the plane of the membrane, or relative motion of membrane sectors vertically with respect to each other, would require the breaking and making of too many bonds. However, if the membrane segments are tilted with respect to each other, it is imaginable that changes of tilt in response to a change of position of a region of the cytoplasmic sector could alter sidedness of the ion-binding sites, and their affinity. Establishment of this hypothesis is a major goal of research on the gastric H,K-ATPase.

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