Biochimica et Biophysica Acta, 1148 (1993) 223–233
© 1993 Elsevier Science Publishers B.V. All rights reserved 0005-2736/93/$06.00

BBAMEM 75947

The site of action of pantoprazole in the gastric H^+/K^+-ATPase

Jai Moo Shin [a], Marie Besancon [a], Alexander Simon [b] and George Sachs [a]

[a] *Wadsworth VAMC and UCLA, Los Angeles, CA (USA) and* [b] *BykGulden, Konstanz (Germany)*

(Received 4 September 1992)

Key words: ATPase, H^+/K^+-; Gastric acid secretion; Pantoprazole; Topology; Conformation

Pantoprazole is a pyridinyl-2-methylenesulfinyl-2-benzimidazole derivative. This compound inhibits the vesicular gastric H^+/K^+-ATPase (cytoplasmic side out) under acid transporting conditions by accumulating in the acid space generated by the pump. Pantoprazole is then converted in an acid-catalysed reaction to a cationic sulfenamide and reacts with cysteines available in or from the acidic extracytoplasmic space. This compound binds to the hog gastric H^+/K^+-ATPase with a stoichiometry of 3 nmol per mg protein, resulting in 94% inhibition of ATPase activity. Tryptic cleavage of the intact vesicles which had been reacted with [^{14}C]pantoprazole at a 1 to 4 trypsin to protein ratio removed most of the cytoplasmic domain leaving the pairs of membrane spanning segments and their connecting extracytoplasmic loops intact. The peptides remaining in the membrane were dissolved in SDS and available cysteine residues labelled with fluorescein-5-maleimide. The peptides were separated on Tricine gradient gels, transferred to PVDF membranes and identified by fluorescence and radioactivity. From N-terminal sequence, fluorescence and molecular mass, it is concluded that pantoprazole is able to label both Cys-813 and Cys-822. These cysteines are predicted to be located in the extracytoplasmic loop connecting membrane segments 5 and 6 and in membrane segment 6. The major cytoplasmic tryptic cleavage site at this location moved from position 776 in unmodified enzyme to positions 784 and 792 following pantoprazole labelling, showing that the configuration of this region changed with pantoprazole labelling. A similar result was obtained by reduction of the enzyme with dithiothreitol. Covalent binding of the cationic sulfenamide to this region of the enzyme is able to block the conformation necessary for phosphorylation of the enzyme by ATP, accounting for its inhibitory effect on acid secretion.

Introduction

The gastric H^+/K^+-ATPase is the pump responsible for H ion secretion by the gastric mucosa [1]. It is a member of the phosphorylating, ion transport ATPase family and exchanges cytosolic protons for extracytoplasmic K ion in an electroneutral exchange [2]. It is an α,β-heterodimer with considerable homology to the Na^+/K^+-ATPase [3,4].

This type of ATPase is thought to undergo conformational changes coupled to phosphorylation and dephosphorylation. These changes result in an alteration of affinity and sidedness of ion binding sites such that H^+ moves from the cytoplasmic domain across the membrane domain and is released into the extracytoplasmic space. K^+ on the other hand moves from the extracytoplasmic domain across the membrane domain and is released into the cytoplasmic space. Information about conformationally important regions in this type of pump has been obtained from effects and location of group selective reagents [5] and mutagenesis [6].

The number and location of transmembrane segments has been controversial for this and other EP type pumps. Strict biochemical evidence is available for 8 membrane segments [14] in the α subunit of the H^+/K^+-ATPase and evidence for an additional two segments at the C-terminal end has been obtained using in vitro translation methods (Bamberg, K. and Sachs, G., unpublished observations). A model generated from these data differs significantly in the C-terminal one-third from that based on hydropathy predictions [18]. The latter method predicts only 8 or 9 such segments, and in particular only a single segment in the place of the M5/M6 pair found by chemical methods. However, a 8 or 10 membrane segment model corresponds closely to the model predicted for the Ca^{2+}-ATPase of sarcoplasmic reticulum [21,22]. Of relevance here is the presence of the membrane inserted pair, M5/M6 between positions 792 and 844, defined

Correspondence to: G. Sachs, Wadsworth VAMC and UCLA Medical Center, Bldg. 113, rm. 324, Los Angeles, CA 90073, USA.

Abbreviations: PVDF, polyvinylidene difluoride; Tris, tris(hydroxymethyl)aminomethane; F-MI, fluorescein-5-maleimide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; NEM, *N*-ethylmaleimide.

by tryptic cleavage and site selective labelling. The numbering of amino acid residues is based on the known hog N-terminal and cDNA sequences.

The gastric ATPase, being the terminal step of acid secretion, is a target for anti-ulcer drugs that act as pump inhibitors [7]. The first of these introduced into general clinical use was omeprazole [7] and it has been followed by a similar drug, lansoprazole [8]. A third, pantoprazole, is under development [9].

All of these drugs are pyridinyl-2-methylenesulfinyl-2-benzimidazole derivatives variously substituted on the pyridine and benzimidazole rings. In common they have a protonatable pyridine N, which in the case of omeprazole has a pK_a of 4.0 and in the case of pantoprazole has a pK_a of 3.9. This allows accumulation in membrane enclosed acid spaces where the $pH_i < pK_a$. In the case of the secretory canaliculus of the parietal cell, where the pH_i is about 1.0, these inhibitors accumulate by about 1000-fold. In acidic spaces, protonation of the pyridine N is followed by a rearrangement to a cationic sulfenamide. This is illustrated in Fig. 1, for the case of pantoprazole, the compound investigated in this paper. The $t_{1/2}$ of the rearrangement is, at pH 1 about 5 min for pantoprazole as compared to 2 min for omeprazole.

The cationic sulfenamides are able to react with the thiol groups of cysteines in proteins, forming a disulfide, as also illustrated in Fig. 1. Since these sulfenamides are cations, they are relatively impermeable across biological membranes. Hence they form disulfides only with those cysteines present in or accessible from the extracytoplasmic space. They react weakly or not at all with the disulfide group of cystine. These compounds therefore act as probes for that group of cysteines accessible from the extracytoplasmic surface of the enzyme. The α subunit of hog enzyme has a total of 28 cysteines, whereas the β subunit has a total of 9 cysteines.

Omeprazole inhibits the H^+/K^+-ATPase under acid transporting conditions with a stoichiometry of about 2 mol omeprazole per mol phosphoenzyme [10,11]. The inhibition is reversed by reducing agents such as β-mercaptoethanol [12]. Inhibition of the enzyme is by formation of a covalent derivative and prevents phosphorylation of the enzyme by ATP [13]. In the case of omeprazole, it has been shown that inhibition is associated with labelling of cysteines at position 813 or 822 and at position 892.

These cysteines are located in or between membrane spanning segments M5 and M6 and between M7 and M8 [14]. Since the reaction between sulfenamides and thiols is rapid and diffusion limited, it was not possible to determine by inhibition kinetics which of the two regions of the enzyme reacting with omeprazole was crucial for inhibition of the pump.

The variation in the ring substitutions of the pyridylmethylenesulfinylbenzimidazoles suggested the possibility that pantoprazole might show a different reaction pattern with the enzyme. For example, the half-life of the protonated drug is different between omeprazole and pantoprazole. Also the shape or bulk of the sulfenamide is different, perhaps allowing different sites to be accessed by the pantoprazole derivative as compared to the omeprazole derivative. Another possibility is that the stability of the disulfide formed might be different.

Accordingly in the work to be reported here, we investigated inhibition by pantoprazole of the hog gastric H^+/K^+-ATPase in intact, cytoplasmic side out vesicles, using [^{14}C]pantoprazole. 94% inhibition was

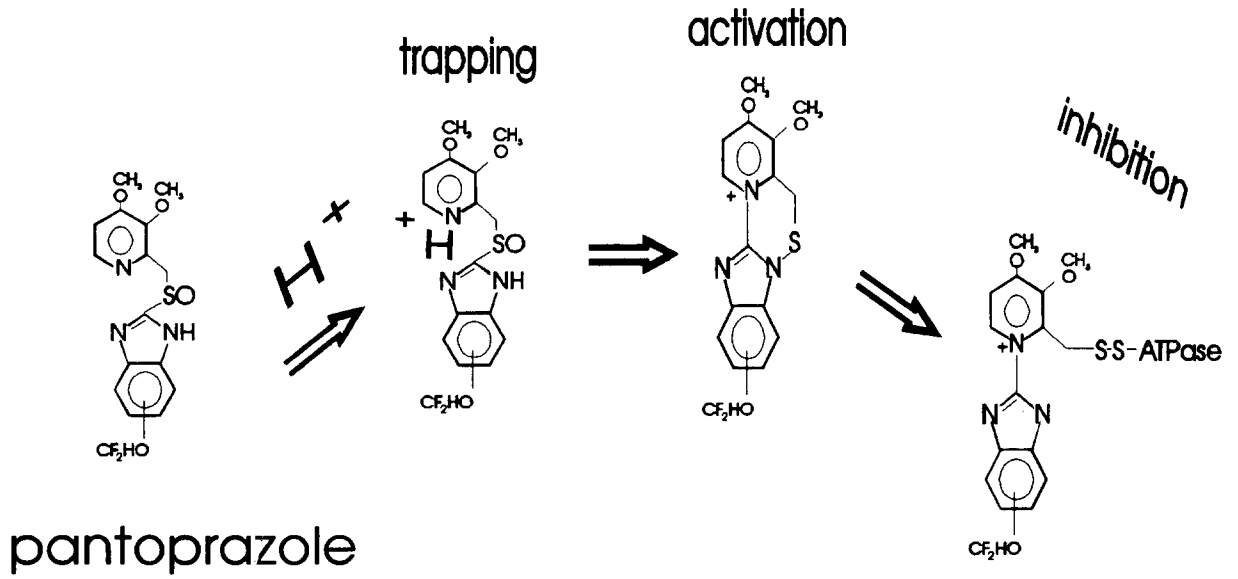

Fig. 1. The reaction pathway of pantoprazole, illustrating acid space trapping, acid-catalysed conversion and reaction with SH groups of the ATPase.

obtained at a stoichiometry of 3 nmol pantoprazole bound per mg protein. Tryptic hydrolysis at a trypsin to protein ratio of 1:4 was followed by SDS solubilisation of the washed membrane residue. After labelling with the cysteine reagent, fluorescein-5-maleimide, the peptide fragments retained in the membrane were separated on Tricine gradient gels and detected by fluorescence. N-terminal sequencing of the pantoprazole labelled peptides detected by autoradiography or counting showed that pantoprazole reacted with both Cys-813 and Cys-822, but not with Cys-892. It was also found that binding of pantoprazole changed the tryptic cleavage pattern in this region of the enzyme as did DTT reduction of the β subunit, moving the major cleavage site at the N-terminal end of the M5/M6 region from Arg-775 to Lys-791, whereas the C-terminal cleavage remained constant at Lys-835 or Arg-844.

A similar shift in tryptic cleavage pattern was observed after DTT reduction of the β subunit of the enzyme suggesting that the disulfides of the β chain affect conformation of the M5/M6 region of the enzyme.

Hence it is the binding of sulfenamides in the M5/M6 region of the H^+/K^+-ATPase and not in the M7/M8 region that is important for inhibition of the enzyme by this class of compound and binding of the sulfenamide alters the conformation of the enzyme, perhaps explaining the mechanism of inhibition.

Methods and Materials

ATPase preparation

The H^+/K^+-ATPase was derived from hog gastric mucosa by previously published methods, which involve differential and density gradient centrifugation [15]. The vesicles obtained have been shown to be $> 90\%$ cytoplasmic side out [15]. The ion impermeability of the vesicles was determined by the difference in K^+ stimulation of ATPase activity in the presence of KCl and in the presence of KCl and nigericin. The specific activity in the presence of nigericin was 120 μmol ATP hydrolysed per mg protein per h, and in the absence of nigericin 10 μmol mg^{-1} h^{-1}. Thus greater than 90% of the ATPase activity was dependent on the addition of the ionophore, nigericin. The large majority of the vesicles were therefore ion tight. No inhibition of ATPase activity by pantoprazole was observed in the absence of the formation of an acid gradient by the pump, namely in the presence of nigericin rather than valinomycin.

Labelling reaction

The ATPase vesicles at a concentration of 200 μg protein per ml were incubated in a flask containing 125 mM KCl, 3 mM Tris-HCl buffer (pH 7.0), 250 mM sucrose, valinomycin at 1 μg ml^{-1} and 20 μM [^{14}C]pantoprazole (specific activity 106 cpm $pmol^{-1}$). At this time a duplicate set of 10-μl aliquots was assayed for P_i, and another duplicate 10 μl set of aliquots were added to ATPase assay medium (see below) for measuring K^+-stimulated ATPase activity at zero time. The flask was warmed to 37°C and MgATP added at 5 mM, and the reaction followed for 30 min. At 5-min intervals quadruplicate 10-μl samples were removed. One pair was used for measurement of P_i that had been released in the primary incubation, the other pair added to ATPase assay medium for measurement of residual ATPase activity, obtained from the difference between P_i values in the initial and second incubation.

This activity was measured at each time point in a solution of 40 mM Tris-HCl (pH 7.0), 100 mM NH_4Cl, 2 mM MgATP and 250 mM sucrose at 37°C for 15 min. The NH_4^+ was present as a permeable surrogate for K^+, and to prevent continued acidification of the vesicle interior [10]. P_i was measured by the method of Yoda and Hokin [16] and protein by the Lowry method [17].

At the end of the 30-min incubation, the inhibition reaction was terminated by a 1:5 dilution in ice cold solution containing 250 mM sucrose, 15 mM KCl, 20 mM Tris-HCl (pH 7.0) and 1 mM NEM. The suspension was allowed to stand for 1 h and then the vesicles were washed three times by centrifugation at 100 000 $\times$ g for 60 min following resuspension by homogenisation for 1 min at 1500 rpm in a teflon/glass homogeniser in 250 mM sucrose containing 20 mM Tris-HCl (pH 7.0). The counts retained by membranes were determined on an aliquot of the washed vesicles, following TCA precipitation.

Tryptic digestion

The vesicles subjected to tryptic digestion were either untreated, inhibited by panatoprazole or had been treated with DTT. In the case of the pantoprazole-labelled gastric vesicles, a digestion protocol using 1/4 trypsin to protein was used, but in addition a digest in the presence of the enzyme ligands SCH28080 and ATP was carried out to define the general region of labelling since one of the bands containing label derived from two regions of the enzyme. The data presented reflect at least three experiments for each experimental protocol.

DTT reduction of disulfides present in the β subunit was carried out by incubation of the vesicles at 37°C for 30 min at pH 8.8 in the presence of 100 mM DTT, followed by washing by centrifugation at 100 000 $\times g$.

Labelling with fluorescein-5-maleimide was used to identify the membrane spanning segment pairs expected to be produced by the 1/4 digestion of the intact cytoplasmic side out vesicles, since each of the

predicted pairs has at least one cysteine present [14,18] and Coomassie staining is not efficient in terms of detecting low-molecular mass peptides.

(a) Digestion in the absence of ligands. The native, pantoprazole-labelled, or DTT-reduced membrane vesicles were digested with trypsin at an enzyme to protein ratio of 1/4, previously shown [14] to rapidly cleave almost all of the cytosolic sites. 100 μg of the intact vesicles were digested for 10 min under isotonic conditions at 37°C in 250 mM sucrose, 100 mM Tris/HCl buffer (pH 8.2) and the reaction stopped by the addition of a 15-fold excess of trypsin inhibitor on ice. The membranes were washed by centrifugation at $100000 \times g$ for 60 min. 68% of the counts remained with the pellet under these conditions. The pellet was suspended in 100 μl of a solution containing 0.5% SDS, 50 mM Tris-HCl (pH 6.1), and fluorescein-5-maleimide added at 50 μM final concentration to label the membrane retained cysteines. Incubation was continued for 30 min at room temperature, and the solution was then added to 20 μl of electrophoresis sample buffer.

The samples were electrophoresed on Tricine gradient gels and transferred to PVDF membranes as described below. The location of the F-MI labelled bands was defined by the fluorescence of the PVDF membrane and the counts located by slicing the membrane into 1 mm pieces and scintillation counting or by autoradiography as described.

(b) Digestion in the presence of ATP and SCH28080. In order to distinguish between labelling of the M1/M2 and the M5/M6 sectors that overlapped at the 6.5 kDa band, a limited digest was carried out in the presence of ATP and SCH28080, since it had been shown that under these conditions the α subunit of the H^+/K^+-ATPase is fragmented by trypsin into a 68 kDa fragment extending from Glu-47 to Ala-671 and a 33 kDa fragment running from Arg-670 to Arg-960 [14,19]. Accordingly, 300 μg of labelled vesicles were resuspended in 150 μl 100 mM Tris, pH 6.8 and 250 mM sucrose. 70 μM SCH28080, 3 mM EGTA, 0.6 mM Na_2ATP and 13 mM $MgCl_2$ were added giving a final volume of 165 μl. 10 μg trypsin was added and incubation was carried out at 37°C for 75 min. The reaction was stopped by an 18-fold excess of trypsin inhibitor and the suspension pelleted in the airfuge. The pellet was dissolved in 40 μl sample buffer and separated on Tricine gels as described below. The two major peptide fragments at 68 and 33 kDa were sequenced as before [14].

SDS gel separation

The peptides retained in the membrane were dissolved in 20% volume of sample buffer, (0.3 M Tris, 10% SDS, 50% sucrose and 0.025% Bromophenol blue) and the solution placed on top of a 10% (34:1 acrylamide/methylene bisacrylamide) to 21% (17:1 acrylamide/methylene bisacrylamide) 1.5 mm gradient slab gel using the Tricine buffer method of Schagger and Von Jagow [20]. The gel was run in the cold room (4°C) for 20–24 h at 48 mA constant current, along with a lane for prestained molecular mass (Bio-Rad, 106–18 kDa) standards and CNBr fragments of horse myoglobin (Sigma, 17–2.5 kDa). In every case a duplicate lane was run, to provide sufficient material for sequencing. Standard curves of ln(molecular mass) as a function of relative mobility were used to estimate the molecular mass of the peptide products of digestion. The accuracy of the molecular mass determination appeared to be within 10% based on predicted tryptic cleavage sites within the primary sequence of catalytic subunit of the enzyme.

The peptides were transferred electrophoretically to PVDF membranes (Millipore) for 18–24 h in the cold room (4°C) in a tank transfer apparatus at 150 mA constant current, in a transfer buffer of 0.15 M glycine, 0.02 M Tris and 20% methanol. A sandwich of three sheets of Whatman 3 mm filter paper was placed on either side of the gel which had a prewetted PVDF membrane on the anode side. After transfer, the blots were rinsed twice in distilled water and stained with Coomassie blue in 10% glacial acetic acid and 45% methanol. The fluorescent bands were visualised by a hand held UV lamp both on the PVDF membrane and on the gel before and after transfer.

Identification of radioactive bands

One third of one lane of the PVDF membrane was sliced into 1 mm sections, the pieces dissolved in dimethyl formamide and counted in liquid scintillant in a LKB scintillation counter. Alternatively the PVDF was placed on X-ray film and exposed for 14 days at −80°C. The position of the radioactive bands could then be compared with the position of the fluorescent bands. The bands identified as radioactive by either method were cut out from the PVDF and sequenced.

Sequencing

Both radioactive and non-radioactive bands were sequenced with a gas phase sequencer at the UCLA Protein Microsequencing facility using the Applied Biosystems 475A system composed of a 470A sequencer, a 120A phenylhydantoin analyser and a 900A data module. To identify the presence of a given sequence, each amino acid position was inspected for elevation in one particular cycle and decrease at the next cycle. The quantity of a given peptide was obtained by averaging the elevation above background for the amino acid in each cycle for sequencing cycles 2 through 10. For each peptide it was possible to follow the sequence for 10 amino acids or more, allowing unambiguous assignment of sequence.

Stoichiometry of peptide fragment labelling

The counts in the 1 mm PVDF slice allowed calculation of the amount of pantoprazole bound as compared to the amount of sequence found at the same position in the analyser. Typically about half of the applied counts were recovered on the PVDF membrane following electrotransfer, and as noted above, 1/3 of the counts were lost during the digestion procedure prior to electrophoresis.

Materials

The materials used were of the highest grade purity available. Trypsin type XIII was obtained from Sigma, PVDF membranes from Millipore. [^{14}C]Pantoprazole was a gift from BykGulden, Konstanz, Germany. Fluorescein-5-maleimide was obtained from Molecular Probes.

Results

(a) Inhibition and labelling

The enzyme at the 30 min time point was inhibited by about 94%. This degree of inhibition is consistent with the intactness of the vesicle preparation, which was 90%. Only those vesicles capable of acidification in the presence of KCl, valinomycin and ATP would be able to accumulate and convert pantoprazole to its active form. In the absence of acid transport, pantoprazole did not inhibit the enzyme at neutral pH, nor was there labelling of the protein.

(b) Tryptic digestion pattern

Digestion in the presence of SCH28080 and ATP

As before, this protocol fragmented the enzyme into two major fragments of 68 kDa and 33 kDa [14]. 90% of the pantoprazole label was present in the 33 kDa fragment, which had as its N-terminal sequence, ACV, beginning at position 671. From the molecular mass this fragment would be expected to terminate at Arg-960. The 68 kDa peptide had as its N-terminal sequence EME beginning at position 47 and probably extending to position 670. Virtual absence of label from this peptide excludes cysteines between or in M1 through M4 as reacting with pantoprazole.

Unmodified enzyme

The pattern of Coomassie staining and fluorescence due to incorporation of F-MI following tryptic digestion of unmodified enzyme is illustrated in Fig. 2. There are three bands of low-molecular mass readily

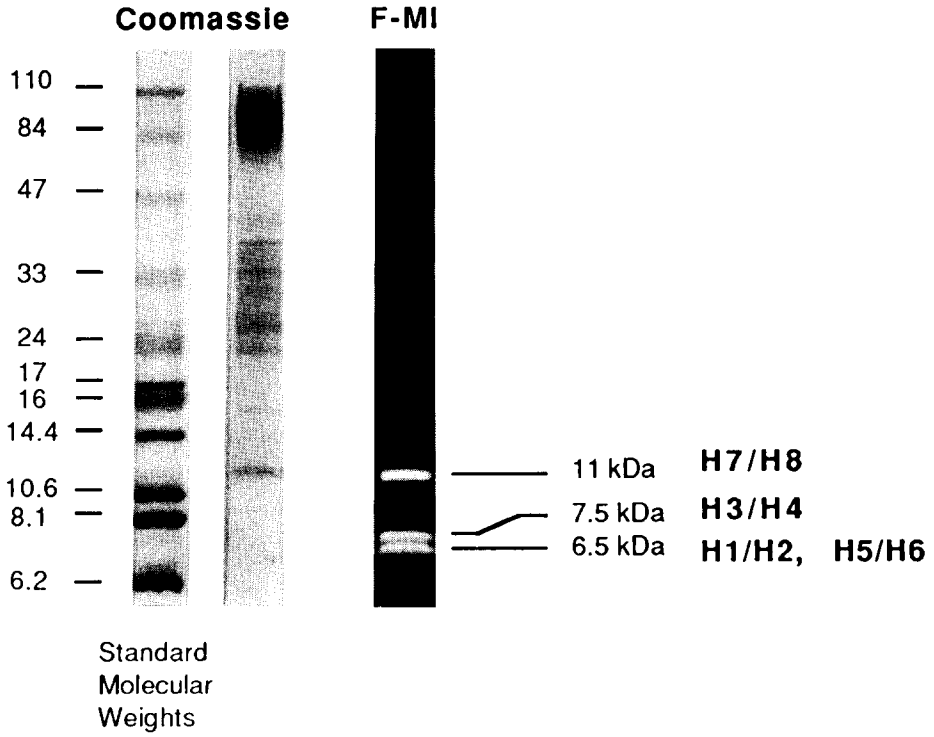

Fig. 2. The electrophoretic pattern of a tryptic digest of unmodified H^+/K^+-ATPase. Trypsinolysis (at a trypsin to protein ratio of 1:4) was carried out as described in Methods. The 11 kDa band shows one single peptide fragment with N-terminal sequence, L^{853}VNE and the 7.5 kDa band provides one peptide fragment beginning at T^{291}PIA. The 6.5 kDa band provides two peptides. One is a peptide beginning at Q^{104}LAG and the other is a peptide beginning at L^{776}IFD. This pattern is typical of at least four experiments. The designation H represents a hydrophobic sequence capable of spanning the bilayer.

identified by fluorescence, at about 11, 7.5 and 6.5 kDa. The upper one is also seen by Coomassie staining, but the lower two are not visible.

Sequence of these bands shows that the upper band begins at Leu-853, the 7.5 kDa band begins at Thr-291, and the 6.5 kDa band has two sequences beginning at Gln-104 and at Leu-776.

The N-terminal sequence, molecular mass of the bands and the placement of arginines or lysines in the primary sequence of the enzyme allowed deduction as to both the N-terminal and C-terminal amino acids of each band.

The 11 kDa band would be predicted to extend from Leu-853 to Arg-946. This corresponds to a pair of membrane spanning segments and an extracytoplasmic loop joining these segments. These would correspond to M7 and M8 and the intervening loop.

The 7.5 kDa band would correspond to a sequence extending from Thr-291 to Lys-358. This would correspond to membrane spanning segments M3 and M4 and the extracytoplasmic loop joining these segments. Both of these bands had unique sequences.

In contrast the 6.5 kDa band had two sequences. The first would probably extend from position Gln-104 to Lys-162. This would include M1 and M2 and the intervening extracytoplasmic loop. The second would extend from Leu-776 to Lys-835 and includes M5 and M6 and the intervening loop.

TABLE I

The peptide bands labelled by pantoprazole on SDS-PAGE

Molecular mass	N-terminal sequences	yield (pmol) [a]	
		peptide	pantoprazole
6.5 kDa	Q^{104}LAGG...	4	–
	S^{784}IAYT...	2	2.8
5.9 kDa	S^{784}IAYT...	2.8	3.0
5.0 kDa	N^{792}IPEL...	4.1	4.0

[a] Yields were obtained by averaging pmoles of elevation above background for the amino acid in each cycle for sequencing cycles 2 through 10.

Pantoprazole-labelled enzyme

The results of digestion of the pantoprazole-labelled enzyme are presented in Fig. 3. Shown are the Coomassie pattern, the count profile from the sliced PVDF membrane and the autoradiogram. The bands detected by autoradiography were sequenced and the sequences and yields of peptide are also presented in the Table I.

There were three radioactive bands at the lower end of the gel. The highest molecular mass radioactive

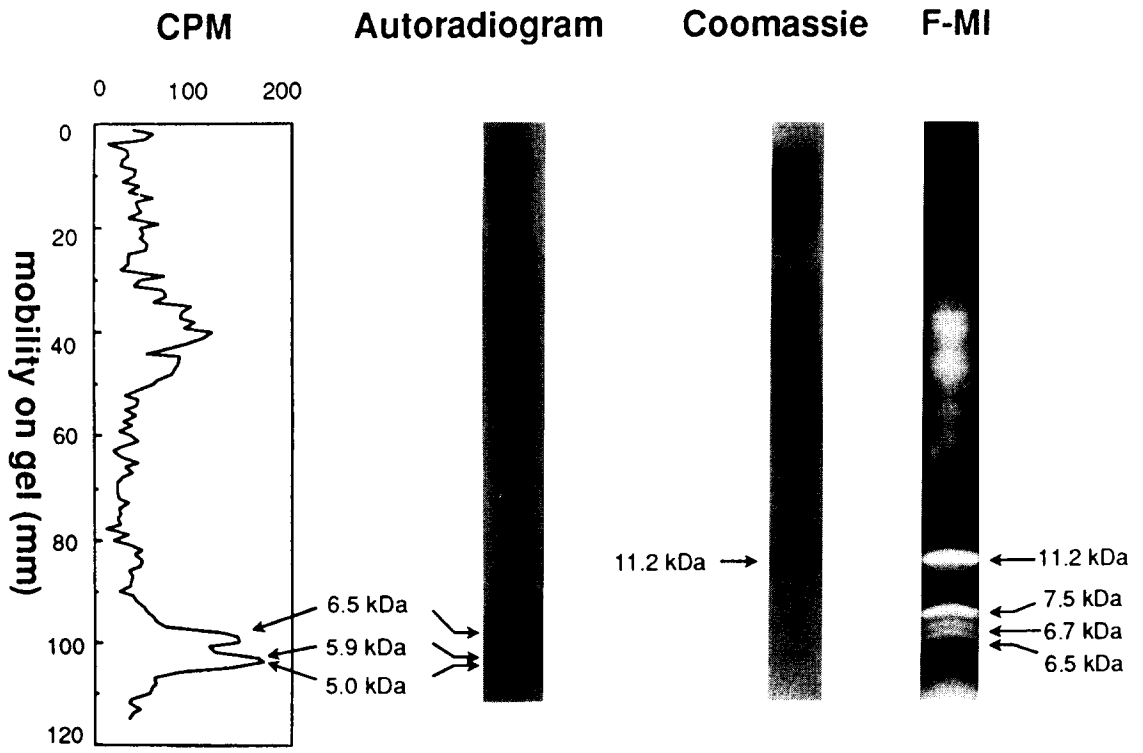

Fig. 3. The electrophoretic pattern of a tryptic digest of pantoprazole-labelled H^+/K^+-ATPase. Trypsinolysis was carried out as described in the methods section. The 11 kDa band represents the M7/M8 segment beginning at Leu-853 and the 7.5 kDa band represents M3/M4 segment beginning at Thr-291. Both of these were not labelled by pantoprazole. The 6.5, 5.9, and 5.0 kDa bands beginning at Ser-784, Ser-784 and Asn-792, respectively, showed pantoprazole labelling, and these data along with the yields of peptide and label are summarized in Table I. This is a pattern typical of at least three experiments.

band at 6.5 kDa represented the mixture of two peptides. One began at Ser-784 and would be predicted to end at Arg-844. The other began at Gln-104 and ended at Lys-162, containing the M1 and M2 membrane spanning segments.

Digestion of the labelled enzyme in the presence of SCH28080 and ATP produced two major peptide fragments at 68 kDa and 33 kDa as previously shown for unmodified or omeprazole-labelled enzyme [14] and the 33 kDa fragment had 90% of the retained counts and should represent a sequence from position 671 to 960. Hence the M1/M2 region was not significantly labelled by pantoprazole, leaving only the M5/M6 region as a candidate for labelling at this molecular mass in the 1/4 digest conditions.

The second radioactive band found at 5.9 kDa began at Ser-784 and would be predicted to end at Lys-835. No other peptide was detected at this molecular mass. The third band at 5.0 kDa, which had the most radioactivity, began at Asn-792 and ended at Lys-835.

All these bands correspond to the region of the enzyme that contains M5 and M6 and the intervening loop. It can be seen that if the sequences found here from the M5/M6 region are compared to the sequences found from the unmodified enzyme, the two major sequences detected are the one beginning at position Asn-792 and the other beginning at position Ser-784 and the least prominent is the sequence beginning at Leu-776. This latter sequence is the major sequence found after trypsinolysis of the unmodified enzyme.

The labelling with F-MI is also of interest. The labelling with F-MI appears to be just slightly below the radioactive regions on the autoradiogram at 6.5 kDa, as if the retention of pantoprazole labelling alters the mobility of the peptide. The bottom band at 5.0 kDa is seen to have no F-MI labelling at all, but to contain the major amount of radioactivity.

The only two cysteines in this region of the primary sequence of the labelled enzyme are Cys-813 and Cys-822. These are therefore the cysteines that can be modified by pantoprazole or by F-MI.

DTT-reduced enzyme

The pattern of trypsin digestion of enzyme, which had been reduced by DTT prior to trypsinolysis and F-MI labelling, is presented in Fig. 4. As shown in Fig. 4, the trypsin digestion of the reduced enzyme provided six fluorescent bands of 11.2, 7.5, 6.7, 6.5, 6.0, and 5.0 kDa. Among these six bands, sequences representing M5/M6 were found in the four bands of 6.7,

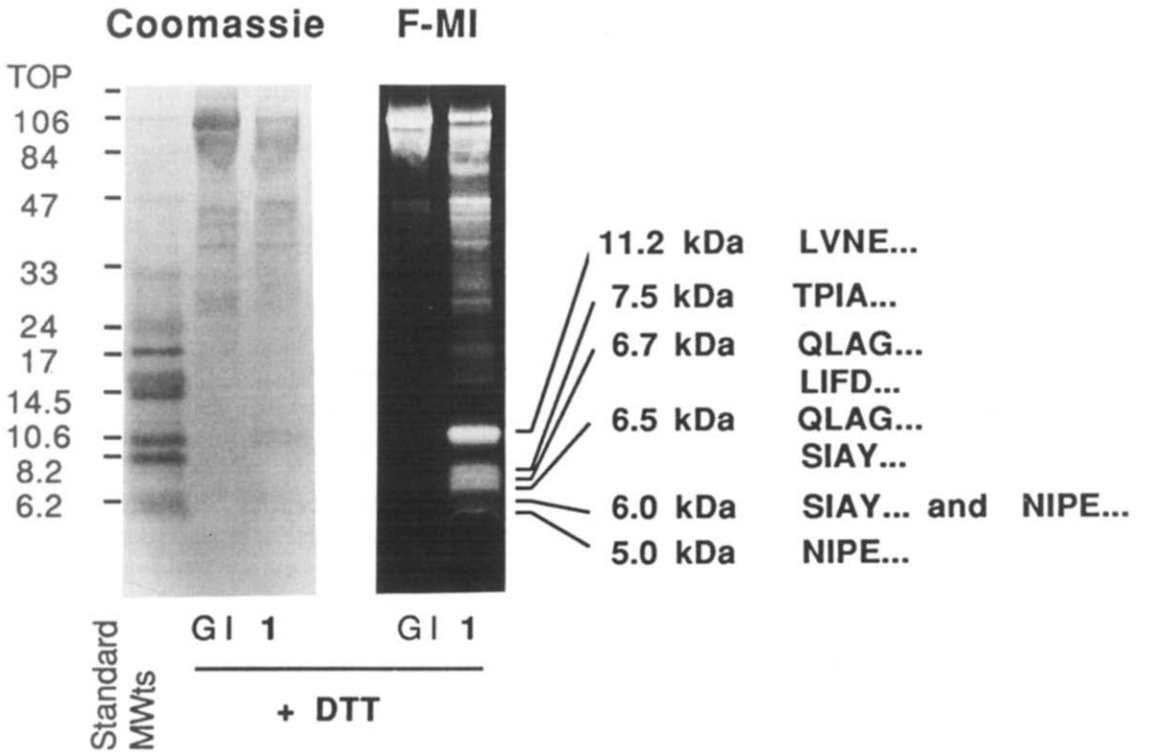

Fig. 4. The electrophoretic pattern of tryptic digest of DTT-treated H^+/K^+-ATPase. Reduction of the enzyme and trypsin digestion were carried out as described in Materials and Methods. The lane labelled G1 represents untreated enzyme, 1 trypsin-digested enzyme. The M5/M6 segment was split into several peptide fragments with different N-terminal sequences such as L^{776}IFD, S^{784}IAY, and N^{792}IPE, while the segments derived from M1/M2, M3/M4, and M7/M8 showed single N-terminal sequences identical with those of unmodified enzyme.

6.5, 6.0, and 5.0 kDa. These bands represented Leu-776 to Lys-835 (6.7 kDa), Ser-784 to Arg-844 (6.5 kDa), Ser-784 to Lys-835 (6.0 kDa), Asn-792 to Arg-844 (6.0 kDa) and Asn-792 to Lys-835 (5.0 kDa).

Like the pantoprazole-modified enzyme, Lys-783 and Lys-791 appeared to become trypsin-sensitive, in addition to Leu-776 when the enzyme was reduced by DTT, while trypsin seems not to access Asn-792 in unmodified enzyme. It can be seen that the F-MI fluorescence corresponds to the location of the bands seen with the pantoprazole-labelled enzyme rather than to the bands seen with unmodified enzyme.

It should also be noted that the β subunit shows much increased labelling with F-MI after reduction indicative of the presence of one or more disulfide bridges.

Stoichiometry of labelling

The overall stoichiometry of labelling by pantoprazole was 3 nmol per mg protein. Mercaptoethanol addition to the labelled membrane suspension prior to centrifugation and TCA precipitation removed all the pantoprazole counts from the protein, showing that only cysteines had reacted with the sulfenamide.

The stoichiometry of pantoprazole binding is shown in Table I. It was observed that about 50% of the applied counts was recovered on the PVDF. A total yield of peptide that was radioactive was 9 pmol as compared to 10 pmol of pantoprazole, making the stoichiometry close to unity. However, correcting for loss of counts during preparation, digestion and separation would bring the overall stoichiometry to greater than 2. In terms of the 5 kDa band, this stoichiometry would indicate that both cysteines are labelled, explaining the absence of F-MI labelling at this molecular mass.

Discussion

Topology

The digestion of intact vesicles by trypsin under the conditions chosen here apparently can cleave the cytoplasmic domain virtually completely. The only small peptides that were retained in the digested and washed vesicles correspond to sequences containing hydrophobic stretches of the α subunit of the protein. These would be predicted to be membrane embedded or membrane spanning. From the molecular mass, the sequences are composed of a pair of membrane embedded sequences connected by an extracytoplasmic loop and cleaved at the N-terminal and C-terminal end of the pair close to the membrane at lysine or arginine residues.

The sequences determined correspond to M7/M8, M3/M4 and M1/M2,M5/M6 at molecular masses of 11, 7.5 and 6.5 kDa, respectively. These sequence pairs were found to be intact, without cleavage having occurred at the potential tryptic sites between M3/M4 and M7/M8. Moreover, there was no cleavage detected of the β subunit, which is thought to have most of the protein present in the extracytoplasmic domain. Thus penetration of trypsin to the interior of the vesicles appears negligible under the conditions of these experiments, as deduced previously [14].

The tryptic digestion pattern is therefore dependent on the membrane topology of the H^+/K^+-ATPase and therefore on cytoplasmic exposure of trypsin sensitive bonds. The pattern provides explicit evidence for at least 8 membrane spanning segments, corresponding to sequences M1 through M8. This pattern and location of the segments corresponds exactly to the first 8 membrane sequences postulated to be present in the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum vesicles [21,22]. M1, M2, M3, M4 and M5 are predicted by hydropathy, M6 is not. M7 and M8 are also predicted by hydropathy analysis. Notable is the absence of F-MI labelled peptides that would be predicted to be membrane spanning subsequent to Arg-946 in the primary sequence of the enzyme. In the H9 segment, between positions Ile-963 and Met-984, there are four cysteines in the primary sequence. The H9 segment however, when used in an in vitro translation system, is able to span the microsomal membrane (unpublished observations). Perhaps the cysteines of H9 are either inaccessible to F-MI or present as disulfides. Another possible explanation for the absence of this segment and the following H10 segment from the membrane-retained peptide fragments is that this region of the enzyme is not membrane spanning in spite of the hydropathy. Perhaps it is a protein region which would be membrane embedded, rather than membrane spanning.

Sites of pantoprazole labelling

The sequences that contained radioactivity were all related to membrane spanning segments M5 and M6, and the intervening loop. The only two cysteines present in this sequence are located at positions 813 and 822.

The stoichiometry found previously for omeprazole in the presence of NEM was 2.2 nmol per mg protein [10,11,14]. For both compounds, the ratio of incorporation to phosphoenzyme is therefore 2 or greater, arguing for reaction with more than 1 cysteine per mol enzyme. In the case of omeprazole, Cys-892 reacts, but in the case of pantoprazole there is virtually no reaction with this cysteine. The losses during digestion, electrophoresis and transfer would allow the calculation that more than one pantoprazole binds in the M5/M6 region, namely that both Cys-813 and Cys-822 react. This conclusion is consistent with the absence of

F-MI labelling of the most radioactive, 5.0 kDa band, which would have both cysteines modified and therefore unavailable for maleimide modification.

Although the major peptide found in unmodified enzyme began at Leu-776, there were additional tryptic cleavage sites revealed in the pantoprazole modified enzyme. The N-terminal amino acids of the labelled sequences at 6.5, 5.9 and 5 kDa moved progressively from position Leu-776 to position Ser-784 and position Asn-792. The C-terminal end of these sequences would be predicted to be at Lys-835 or Arg-844 depending on the molecular mass found. The minimal length of the M5/M6 loop and the extracytoplasmic hairpin is defined by starting at the sequence NIP beginning at position 792 and ending with the lysine at position 835.

This implies that the binding of pantoprazole modifies the conformation of the enzyme, and positions Lys-783 and Lys-791 become trypsin accessible as a function of labelling either Cys-813 and/or Cys-822. The change in trypsin accessibility may relate to conformational changes that determine inhibition by the binding of the sulfenamide.

Effect of DTT reduction prior to digestion

Reduction of the enzyme by DTT prior to digestion produced a pattern similar to that observed with pantoprazole-labelled enzyme. The β subunit is known to have six cysteines present in the extracytoplasmic domain, linked by disulfide bridges [23,24]. Without DTT reduction, F-MI virtually does not label the β subunit, but does so after reduction (Fig. 4). Breaking the disulfide bonds of the β subunit therefore alters trypsin access to the N-terminal region of the fifth membrane spanning segment.

It has been reported that the gastric H^+/K^+-ATPase activity is inhibited by reduction of disulfide bonds in the β subunit [24]. It was concluded that the loss of the H^+/K^+-ATPase activity is correlated with a reduction of disulfide groups in the β subunit, while there was no significant change in the α subunit. Our present results suggest that reduction of the gastric vesicles may change the conformation of α subunit with the reduction of β subunit at least on the cytoplasmic side of the M5 transmembrane segment.

Fig. 5 shows the amino acids predicted to span the membrane in M5 and M6 and the very short hairpin turn between them. The M6 segment is relatively hydrophilic, as is the M6 segment of the Ca^{2+}-ATPase [21,22]. Mutation of the carboxylic acid residues in this segment of the Ca^{2+}-ATPase has been shown to have significant effects on function, arguing for a transport role for these amino acids in this segment of the enzyme in the Ca^{2+} pump and probably therefore in the H^+/K^+-ATPase [6]. The carboxylic acids are highlighted in this diagram, showing that there are three of these in addition to three serines, making this sector relatively hydrophilic. It may also be noted that there are four prolines in the M5 sector, which would distort the helical pattern.

In this work we have not fragmented this segment further, leaving both cysteines as potential targets for pantoprazole. However, since the sequence beginning at NIP had most of the radioactivity and was not labelled by F-MI, it is possible to conclude that in this band, both cysteines were modified preventing reaction with F-MI. Hence both Cys-813 and Cys-822 are accessible to the cationic sulfenamide formed by acid activation of pantoprazole. The model of Fig. 5 illustrates Cys-813 as being present in the hairpin and Cys-822 as being in membrane spanning segment 6. This prediction is based on tryptic cleavage at Lys-791 and Arg-835 and the minimum number of amino acids necessary to span the hydrophobic phase of the bilayer. The location of Cys-822 is almost two turns of an α helix into

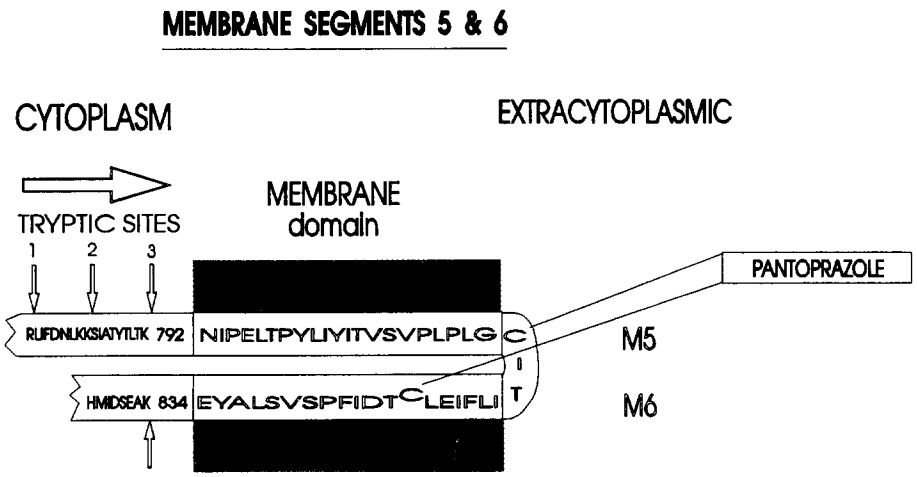

Fig. 5. The amino acid arrangement of the M5/M6 region showing the pattern of tryptic cleavage sites that modify as a function of either pantoprazole binding or β subunit reduction by DTT (arrows). Also indicated by the stars are the carboxylic acids in this segment. Note the four prolines in M5, and the additional serines in this region that make M6 relatively hydrophilic.

the hydrophobic phase of the membrane. Given that a bulky cation can penetrate to this location, a vestibule bounded by this segment of the pump may be present. Distortion of the α helical pattern by the prolines in M5 may contribute to this vestibule. It may also be noted that the cysteine at position 822 is between the two carboxylic acids thought to be in the transport pathway of the pump, and it is binding to this cysteine that changes the tryptic digestion pattern.

The putative membrane spanning segments defined biochemically are shown in Fig. 6. In addition we have illustrated the possible presence of an M9 and M10 segment, based on hydropathy and in vitro translation. We have no explanation for the lack of biochemical evidence for these segments. The labelling sites for pantoprazole are also indicated on the diagram, as are the beginning and end of the segments defined by tryptic hydrolysis for the first eight membrane spanning pairs. On the diagram, we have also shown the location of aspartic and glutamic acid and glutamine, the most hydrophilic of the amino acids in the membrane spanning segments. This illustrates the preponderance of these amino acids in M5/M6 and M7/M8, arguing that these regions participate in ion transport. It may be noted that the K competitive reagent, SCH28080, binds in the extracellular loop between M1 and M2. If K competition implies competition for the K site, the ion transport pathway also includes this region of the enzyme.

Since these cysteines are the only sites which retain the labelled pantoprazole, binding of the sulfenamide to one or both of these sites is responsible for the

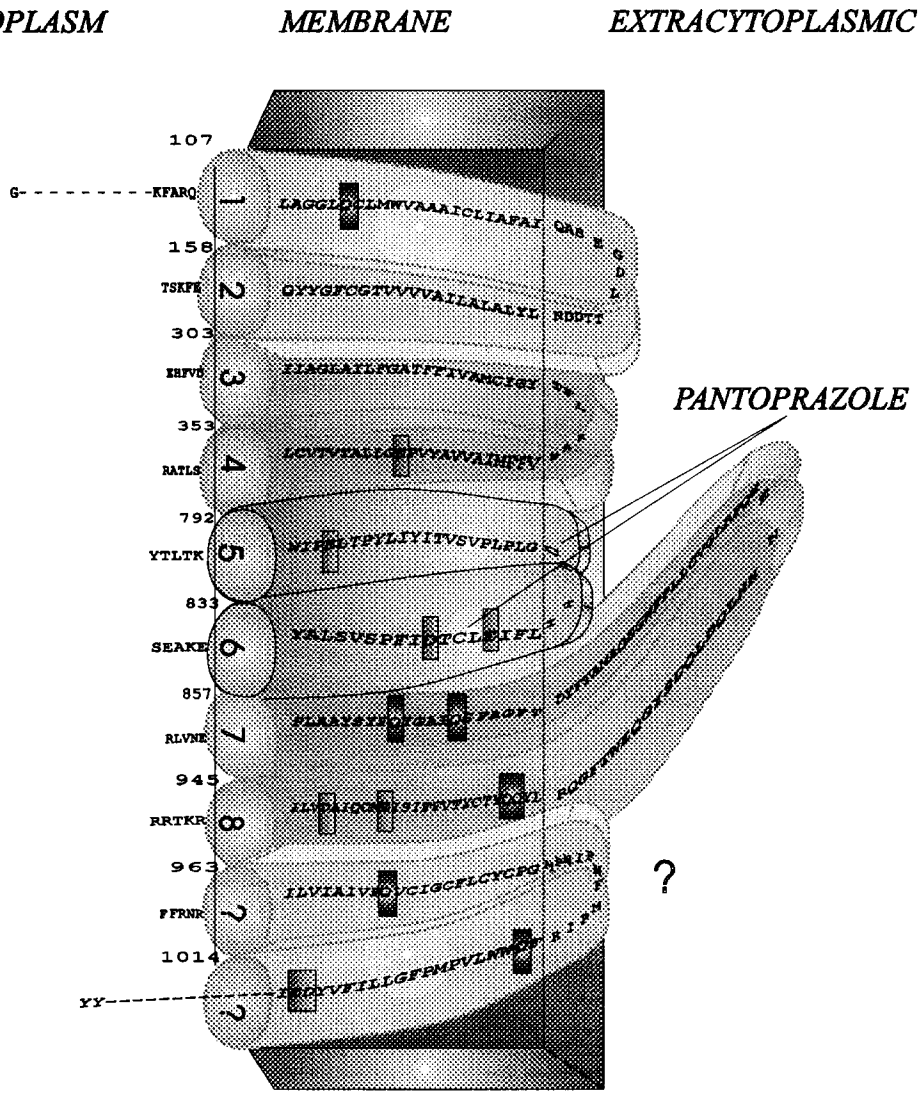

Fig. 6. A working model of the arrangement of the membrane segments of the catalytic subunit of the H^+/K^+-ATPase. 10 membrane segments are shown, with a question mark over the M9 and M10 segments since these are inferred, rather than proven by the techniques described here. Also illustrated is the location of hydrophilic caboxylic amino acids as well as those of glutamine present in the membrane spanning segments. The site of pantoprazole binding is shown, as well as the putative beginning and end of the membrane spanning segment pairs demonstrated by sequencing and molecular mass.

enzyme inhibition by pantoprazole. Omeprazole was found to bind to this region and also to Cys-892 [14]. The omeprazole data did not allow us to determine which of these cysteines was responsible for inhibition of the H^+/K^+-ATPase by this class of compound. Since pantoprazole did not label Cys-892, evidently it is derivatisation of the cysteines in the M5/M6 region of the enzyme that inhibits ATPase activity and is responsible for inhibition of acid secretion in vitro and in vivo by pantoprazole and also by omeprazole.

Acknowledgements

This study was supported by NIH grant Nos. DK40615, 41301 and USVA SMI Research Funds. Sequencing was supported by NIH BRS grant No. 1S10RR0554-01 to UCLA and was performed by Dr. Audree Fowler.

References

1 Rabon, E. and Reuben, M. (1990) Ann. Rev. Physiol. 52, 321–344.
2 Sachs, G., Chang, H.H., Rabon, E., Schackman, R., Lewin, M. and Saccomani, G. (1976) J. Biol. Chem. 251, 7690–7698.
3 Glynn, I.M. and Karlish, S.J.D. (1990) Annu. Rev. Biochem. 59, 171–205.
4 Hall, K., Perez, G., Sachs, G. and Rabon, E. (1991) Biochim. Biophys. Acta 1077, 173–179.
5 Pedemonte, C.H. and Kaplan, J.H. (1990) Am. J. Physiol. 258, C1–23.
6 Clarke, D.M., Loo, T.W. and MacLennan, D.H. (1990) J. Biol. Chem. 265, 6262–6267.
7 Sachs, G., Carlsson, E., Lindberg, P. and Wallmark, B. (1988) Annu. Rev. Pharm. Toxicol. 28, 269–284.
8 Nagaya, H., Satoh, H., Kubo, K. and Maki, Y. (1989) J. Pharm. Expt. Ther. 248, 799–805.
9 Simon, W.A., Keeling, D.J., Laing, S.M., Fallowfield, C. and Taylor, A.G. (1990) Biochem. Pharmacol. 39, 1799–1806.
10 Lorentzon, P., Jackson, R., Wallmark, B. and Sachs, G. (1987) Biochim. Biophys. Acta 897, 41–51.
11 Keeling, D.J., Fallowfield, C. and Underwood, A.H. (1987) Biochem. Pharmacol. 36, 339–344.
12 Fryklund, J., Gedda, K. and Wallmark, B. (1988) Biochem. Pharmacol. 37, 2543–2550.
13 Lindberg, P., Nordberg, P., Brandstrom, A. and Wallmark, B. (1986) J. Med. Chem. 29, 1327–1329.
14 Besancon, M., Shin, J.M., Mercier F., Munson, K., Miller, M., Hersey, S. and Sachs, G. (1993) Biochemistry, in press.
15 Hall, K., Perez, G., Anderson, D., Guttierez, C., Munson, K., Hersey, S.J., Kaplan, J.H. and Sachs, G. (1990) Biochemistry 29, 701–706.
16 Yoda, A. and Hokin, L.E. (1970) Biochem. Biophys. Res. Commun. 40, 880–88619.
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
18 Shull, G. and Lingrel, J.B. (1986) J. Biol. Chem. 261, 16788–16791.
19 Munson, K., Guttierez, C., Balaji, V.N., Ramnayaran, K. and Sachs, G. (1991) J. Biol. Chem. 266, 18976–8988.
20 Schagger, H. and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
21 Matthews, I., Sharma, R.P., Lee, A.G. and East, J.M. (1990) J. Biol. Chem. 265, 18737–18740.
22 MacLennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Nature 316, 696–724.
23 Reuben, M.A., Lasater, L.S. and Sachs, G. (1990) Proc. Natl. Acad. Sci. USA 87, 6767–6771.
24 Chow, D.C., Browning, C.M. and Forte, J.G. (1992) Am. J. Physiol. 263, C39–C46.