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Determination of the sidedness of the C-terminal region of the gastric H,K-ATPase α subunit

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It cannot be predicted from hydropathy analysis whether the C-terminal end of the α subunit of the gastric H,K-ATPase is cytoplasmic or extracytoplasmic. The sideness of the C-terminal amino acids was determined by taking advantage of the two C-terminal tyrosines in the primary sequence of the enzyme. Intact, cytoplasmic side out vesicles derived from hog gastric mucosa or detergent solubilized vesicles were iodinated by the lactoperoxidase method and then the C-terminal amino acids hydrolyzed by carboxypeptidase Y. The α and β subunits were separated by SDS gel electrophoresis. The level of iodination of the α subunit following solubilization was about three fold greater than when intact vesicles were iodinated, and the β subunit was iodinated only when solubilized enzyme was used. Carboxypeptidase Y removed 28 ± 4% of the radioactivity from the α subunit iodinated in intact vesicles. These data are consistent with a cytoplasmic location of the C-terminal amino acids of the α subunit and with a mostly extracytoplasmic location of the amino acids of the β subunit.

Introduction

The H,K-ATPase and the Na,K-ATPase are composed of a large α subunit of about 1000 amino acids and a smaller glycosylated β subunit of about 300 amino acids [1,2]. The location of the C-terminal region of these enzymes α subunits is not known. The location of the C-terminal region of the plasma membrane Ca-ATPase is almost certainly cytoplasmic since it contains the calmodulin binding region. Mutation and antibody sidedness studies on the Ca-ATPase of the sarcoplasmic reticulum have been interpreted as demonstrating 10 membrane spanning regions [3]. Hydropathy plots of the α subunits of the H,K-ATPase and the Na,K-ATPase have been interpreted as representing 6, 7, 8, 9 and 10 membrane spanning domains for the α subunit and one or four such domains for the β subunit [1,4]. Tryptic hydrolysis and sequencing of fragments have led to the conclusion that the β subunit has only one membrane spanning domain. Reaction with antibodies against the Na,K-ATPase [4] and determination of the site of pyridoxal 5-phosphate binding have been interpreted as showing that the C-terminal region is extracellular, which means that there must be an odd number of membrane spanning regions [5].

In the case of the gastric H,K-ATPase as well as the Na,K-ATPase, the α subunits terminate with two tyrosine residues. In the β subunit, only one tyrosine is predicted to be in the cytoplasmic N-terminal region, three in the membrane and the other 14 in the extracellular domain for a single membrane spanning model (Fig. 1b).

Gastric vesicles from hog mucosa are isolated as largely cytoplasmic side out particles. Electron microscopic analysis and WGA staining have shown that at least 94% of the vesicles are in the inside-out orientation [6], therefore the C-terminal tyrosines if located in the cytoplasmic domain are available for lactoperoxidase dependent iodination and subsequent carboxypeptidase Y cleavage from intact vesicles. On the assumption that mostly only the cytoplasmic tyrosines are likely to be iodinated under mild conditions, only 14 of the 29 tyrosines of the α subunit would be available for iodination in the eight membrane segment model presented in Fig. 1a. In fact the C-terminal region may be more accessible than residues buried in the secondary structure of the N-terminal and central cytoplasmic region (at least two of these tyrosines are in hydrophobic regions here). Based on these predictions minimally 15% (two tyrosines out of 14) of the label should be

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found at the C-terminal end of the molecule following controlled iodination of intact vesicles and perhaps as much as 25% given restriction of iodination of some tyrosines of the cytoplasmic domain of the α subunit. Only 5% of the label would be in the cytoplasmic domain of the β subunit, in a single membrane segment model.

In this paper we present data showing labelling of intact vesicles with peroxidase catalysed iodination of the α subunit, as detected following separation on SDS gels. About 30% of the label is removed by carboxypeptidase Y digestion. Significant labelling of the β subunit was obtained only after solubilization in $C_{12}E_8$. Hence the C-terminal end of the catalytic subunit of the H,K-ATPase is cytosolic and most of the β subunit is extracellular.

Methods

Labelling procedure. Purified hog gastric vesicles were prepared as described previously [6]. The vesicles (360 μg) were resuspended in 20 mM Tris buffer (pH 7.4) containing 8.9% sucrose, 100 μCi/ml $^{125}I_2$, 400 μg/ml lactoperoxidase and 3 μM NaI. The labelling reaction was initiated by the addition of 0.06% H_2O_2 at room temperature. After 10 min another pulse of 0.06% H_2O_2 was added. Following 10 min of incubation the labelling reaction was terminated by the addition of ice-cold 20 mM Tris buffer (pH 7.4) containing 8.9% sucrose. The vesicle suspension was centrifuged at 100000 $\times g$ in a Beckman Ti 65 rotor for 45 min at 4°C and the supernatant discarded. For the experiments with soluble enzyme the vesicles were treated with $C_{12}E_8$ (2:1, $C_{12}E_8$/enzyme) as previously described [7] and labelled as above.

Carboxypeptidase Y digestion. The pellet was resuspended in 50 mM Na citrate buffer (pH 7.0) containing 8.9% sucrose. Carboxypeptidase Y (4 μmol/μl) was added to the vesicle suspension and the digestion mixture was incubated at 37°C for 1 h. The digestion was terminated by the addition of electrophoresis sample buffer containing 0.3 M Tris, 10% SDS, 50% sucrose, 0.025% Bromophenol blue and 10% β-mercaptoethanol. The digested vesicle suspension was then subjected to analysis by PAGE on a tricine gradient gel (8%–15%). The gel was sliced at 1 mm intervals and the amount of radioactivity in each gel slice was determined by liquid scintillation counting in an LKB 1219 scintillation counter using BCS (Amersham) scintillation cocktail. The background radioactivity in the intact vesicles was 220 cpm, and in the solubilised experi-

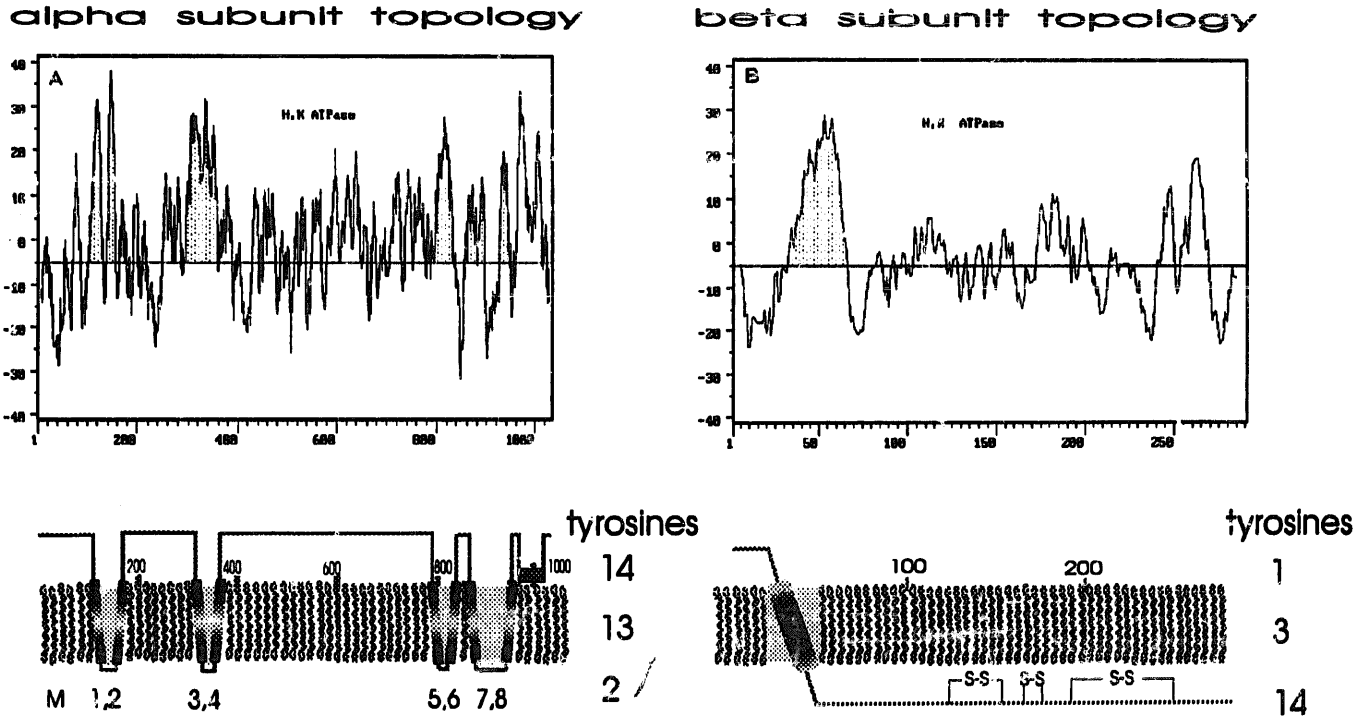

Fig. 1. Hydropathy plots and suggested topology maps of the α and β subunits of the H,K-ATPase. (A) Kyte-Doolittle hydropathy plot of the α subunit of the hog gastric H,K-ATPase. Membrane spanning regions are identified as shaded areas. Below is a topological model of the α subunit based on eight membrane spanning regions with the N- and C-terminals being on the cytoplasmic face. (B) Kyte-Doolittle hydropathy plot of the β-subunit of the hog gastric H,K-ATPase with the suggested membrane spanning region shaded. Below is a topological model for a single membrane spanning segment. A moving average of 11 amino acids was used to generate these plots.

ment, 490 cpm. This was subtracted from the radioactivity in each peak prior to calculation of the effects of carboxypeptidase Y digestion.

Materials. Trizma base, sucrose, sodium citrate, citric acid and sodium iodide were from Sigma. Lactoperoxidase and carboxypeptidase Y (sequencing grade) were obtained from Boehringer Mannheim. All chemicals used for electrophoresis were obtained from Bio-Rad.

Results

Lactoperoxidase dependent iodination of hog gastric vesicles followed by carboxypeptidase Y digestion and subsequent Tricine gel electrophoretic analysis showed that there was no apparent change in the molecular weight of the α subunit as shown in the Coomassie stained gel (Fig. 2A, lanes 1 and 3). Slicing the gel into 1 mm pieces and measuring the radioactivity in each slice showed a peak of radioactivity associated with the α subunit and a minor peak associated with lactoperoxidase. A typical experiment is shown in Fig. 3a. In the area between the two peaks, where the β subunit resides, the measured amount of radioactivity was not above background suggesting that the β subunit was weakly labelled if at all. The absence of labelling of the β subunit is not unexpected as the proposed secondary structure of the β subunit would predict that only one tyrosine would be accessible for labelling in intact vesicles.

There was a $28 \pm 4\%$ ($n = 4$) loss of radioactivity from the α subunit following 1 h of carboxypeptidase Y digestion of the intact vesicles (Fig. 3a) as compared to non-digested labelled material. The loss of counts from the α subunit is in approximate agreement with the model that predicts that of the 29 tyrosines in the α subunit, only 10 are probably iodinated in intact inside-out vesicles and of those 10, the two C-terminal tyrosines and possibly a third, are cleaved by carboxypeptidase Y digestion. This also implies that of the 14 cytoplasmically located tyrosines, four are largely unavailable for iodination by this procedure.

The self iodination of lactoperoxidase in the case of the intact vesicles was significant (Fig. 3a). The counts incorporated are about two thirds of those incorporated into the α subunit. Treatment with carboxypeptidase Y had no effect on the lactoperoxidase self labelling. The position of lactoperoxidase is indicated in Fig. 2.

Carboxypeptidase Y digestion of the solubilized enzyme also resulted in no detectable change in the molecular weight of the enzyme (Fig. 2B, lanes 1 and 2). The level of iodination of the solubilized enzyme was about three fold that of the intact enzyme, as shown in Fig. 3b. This would be anticipated from increased exposure of tyrosines following detergent

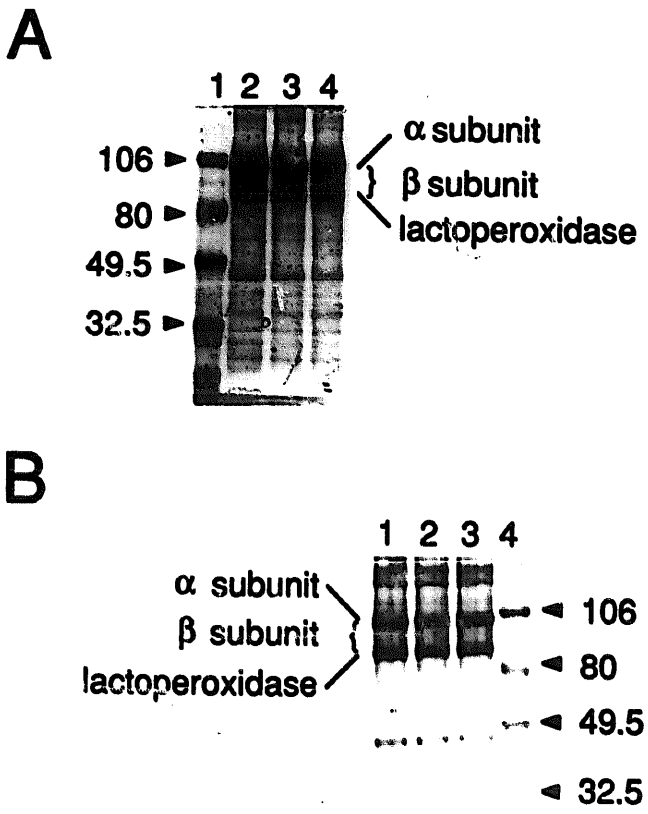

Fig. 2. Polyacrylamide gel electrophoresis of intact and $C_{12}E_8$ solubilized H,K-ATPase enriched gastric vesicles with and without carboxypeptidase Y digestion. (A) Intact gastric vesicles subjected to PAGE on a tricine gradient gel (lane 1, molecular mass standards in kDa; lane 2, gastric vesicles following lactoperoxidase driven iodination; lane 3, gastric vesicles following 60 min of incubation at 37 °C in the presence of carboxypeptidase Y; lane 4, gastric vesicles following 60 min of incubation at 37 °C in the absence of carboxypeptidase Y. (B) $C_{12}E_8$ solubilized gastric vesicles subjected to PAGE on a tricine gradient gel (lane 1, $C_{12}E_8$ solubilized gastric vesicles following lactoperoxidase driven iodination; lane 2, $C_{12}E_8$ solubilized gastric vesicles following 60 min of incubation at 37 °C in the presence of carboxypeptidase Y; lane 3, $C_{12}E_8$ solubilized gastric vesicles following 60 min of incubation at 37 °C in the absence of carboxypeptidase Y; lane 4, molecular weight standards in kDa. The position of the lactoperoxidase band is indicated by the arrows.

treatment. Further, there were counts now associated with the region containing the β subunit, again predicted if the extracytoplasmic tyrosines were now available for iodination. In contrast there was no change in the level of lactoperoxidase labelling, which represented about one fifth of the counts incorporated into the α subunit. As before, treatment with carboxypeptidase Y digestion had no effect on the label found over the lactoperoxidase peak. The percentage of counts released from the α subunit upon carboxypeptidase Y digestion was 20%. This lower release of counts is probably due to the fact that after solubilization and iodination, the C-terminal tyrosines represent a smaller

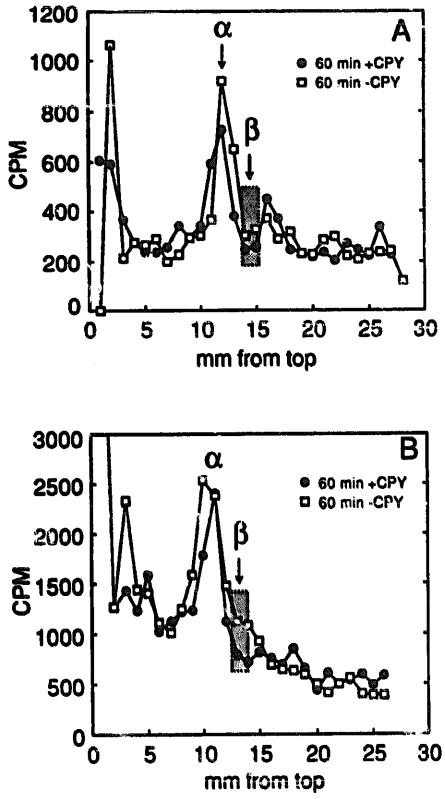

Fig. 3. Radioactive profile of intact and solubilized H,K-ATPase enriched gastric vesicles labelled with $^{125}I_2$. The position of the α and β subunits are indicated by the arrows. (A) Representative graph of the amount of radioactivity in 1 mm slices from a tricine gradient gel of intact gastric vesicles labelled with $^{125}I_2$ incubated for 60 min without and with carboxypeptidase Y. (B) Representative graph of the amount of radioactivity in 1 mm slices from a tricine gradient gel of $C_{12}E_8$ solubilized gastric vesicles labelled with $^{125}I_2$ again incubated for 60 min without and with carboxypeptidase Y.

percentage of the iodinated tyrosines in the enzyme. However, if all the tyrosines were equally iodinated, the two C-terminal tyrosines would represent only 6% of the radioactivity. Hence even after solubilization, there appears to be preferential iodination of the C-terminal residues, and probably they become more accessible after detergent treatment compared to intact, detergent free vesicles.

Discussion

The analysis of the secondary structure of the EP type transport ATPases has relied heavily on hydropathy plots. The hydropathy profile of the H,K-ATPase raises problems as to the interpretation of the number of membrane spanning segments. Fig. 1a shows a Kyte-Doolittle plot, using a moving average of 11 amino acids which has been shown to be the best for this scale of hydrophobicity [8]. Other plots predicting membrane spanning segments all show similar profiles but can differ in detail.

The Kyte-Doolittle plot, predicts the first third of the protein to contain two pairs of membrane spanning segments as outlined. The sector between M4 and M5 is considered as cytoplasmic, based on phosphorylation at Asp^{385}, pyridoxal phosphate Schiff base formation at Lys^{492} and FITC labelling at Lys^{516}. However, there are significant regions of hydrophobicity before M5 predicted to start at about Leu^{796}. The hydropathy analysis subsequent to M5 predicts the presence of 3 to 5 membrane spanning segments using the Kyte-Doolittle algorithm. In the case of the Ca-ATPase, five membrane spanning segments following M5 have been postulated [3,9] to give a total of 10 segments for this enzyme. It is clear that experimental evidence has to be obtained for the membrane segments in the H,K-ATPase.

With an even number of membrane segments, the C-terminal tyrosines would be cytoplasmic, and accessible not only to limited iodination but also to carboxypeptidase Y cleavage after labelling in the inside out preparation as obtained from hog gastric mucosa.

The data presented here show labelling of the α but not the β subunit in intact vesicles. Labelling of the β subunit is found only after $C_{12}E_8$ solubilization, and the labelling of the α subunit is increased 3-fold, demonstrating the intactness of the vesicles during the iodination procedure.

The loss of about 1/3 of the label in the α subunit following carboxypeptidase Y digestion of intact labelled vesicles, and a lesser loss from vesicles that were labelled after detergent treatment again supports a cytoplasmic location of the C-terminal tyrosines. Hence the α subunit has an even number of membrane spanning segments.

Experimental evidence has been obtained that tryptic cleavage of intact, inside out gastric vesicles produces four pairs of membrane spanning segments, between positions 104 and 162 (M1/M2), 291 and 358 (M3/M4), 776 and 835 (M5/M6) and 853 and 946 (M7/M8) [10]. Moreover, labelling with the extracytoplasmic reagents, MeDAZIP$^+$ [11], and omeprazole has provided additional evidence for the presence of M1 and M2 as well as M5 and M6, and M7 and M8, respectively [10]. These segments correspond to the first four pairs of membrane segments postulated for the Ca-ATPases. No chemical evidence has been obtained for the last pair of membrane segments suggested for the Ca-ATPase, corresponding to H9 and H10.

From the hydrophobicity analysis shown at the top of Fig. 1a there is sufficient hydrophobicity for the protein to have one or two membrane segments in this region. Since the C-terminal by the data presented here is cytoplasmic, this region of the enzyme either remains cytoplasmic or there is an additional pair of segments in this region, not detected by our biochemi-

cal methods. The model shown at the bottom of Fig. 1a illustrates the presence of eight membrane segments, with a hydrophobic stretch in the C-terminal sector, of unspecified location, perhaps membrane embedded. In this model, of the 29 tyrosines present, 14 are calculated to be cytoplasmic, 13 in the membrane and two extracytoplasmic. Since 30% rather than 15% of the radioactivity was released by carboxypeptidase digestion, it seems that about half of the cytoplasmic tyrosine residues are not accessible under the conditions of iodination in the intact enzyme. This then leads to a 3-fold increase in labelling of the α subunit with detergent solubilization, rather than a doubling.

The structure of the β subunit is usually thought of as having a single membrane spanning segment, basd on hydrophobicity, but in the case of the β subunit of the Na,K-ATPase, there has recently been speculation that there may be more such segments [12]. Since virtually no iodination was found in intact vesicles, it is clear from these data that the majority of the tyrosines in this protein are inaccessible to restricted iodination with the membrane intact. These data therefore lend support to a single membrane spanning segment for this subunit, as suggested by hydropathy plots, as in Fig. 1b. In the model shown in the figure, of the 18 tyrosines, one is cytoplasmic, three are in the membrane and 14 extracytoplasmic.

The finding that the C-terminal end of the α subunit of the H,K-ATPase is cytoplasmic is consistent with a similar membrane topology as compared to the Ca-ATPases. The strong homology between the H,K-ATPase and the Na,K-ATPases argues for a similar location of the C-terminal amino acids of the Na,K-ATPases. In fact, antibody epitope mapping has shown a cytoplasmic location for both the N- and C-terminal regions of the Na,K-ATPase [13], contradicting previous conclusions [4,5].

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