ELSEVIER

Biochimica et Biophysica Acta 1236 (1995) 105–113

BBA Biochimica et Biophysica Acta

Structural interactions between α- and β-subunits of the gastric H,K-ATPase

K. Tyagarajan [a], D.C. Chow [a], A. Smolka [b], J.G. Forte [a,*]

[a] *Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA*
[b] *Department of Medicine, University of South Carolina, Charleston, SC 29425, USA*

Received 25 October 1994; revised 2 January 1995; accepted 2 February 1995

Abstract

Structural and functional interactions between α- and β-subunits of the H,K-ATPase were explored. The sensitivity to trypsinolysis of α-subunit was monitored by SDS-PAGE in control H,K-ATPase-enriched microsomes and in microsomes in which disulfide bonds of the β-subunit were reduced using 2-mercaptoethanol (2-ME). Reduction of β-subunit disulfide bonds increased the susceptibility of the α-subunit to tryptic digestion. Kinetics of trypsinolysis were also carried out in the presence of ligands known to bind with H,K-ATPase and favor a particular conformer state in the native enzyme. The time-course for release of tryptic peptides was monitored in protein stained gels and Western blots probed with monoclonal antibody α-H,K,12.18. In control preparations, where β-subunit disulfides remained intact, trypsinolysis in the presence of ATP or K^+ produced distinctive patterns of tryptic fragments, each characteristic of the conformational states induced by the respective ligand. For 2-ME-treated microsomes the altered α-subunit was unable to undergo ligand-induced conformational changes. The increased susceptibility of the α-subunit to trypsinization, the change in accessibility of tryptic cleavage sites and the inability of the α-subunit to undergo ligand-induced conformational changes after reduction of the β-subunit disulfides suggest that the interactions between α- and β-subunits are important for the conformational stability of the functional holoenzyme. A model localizing the most susceptible tryptic cleavage sites in control and 2-ME-reduced states is presented.

Keywords: ATPase; Acid secretion; Disulfide bond; Tryptic sensitivity; Conformational stability

1. Introduction

The H,K-ATPase is the gastric proton pump which secretes acid into the gastric lumen by catalyzing the electroneutral exchange of H^+ for K^+ [1]. The H,K-ATPase belongs to the family of P-type ATPases and shares a high degree of homology with the Na,K-ATPase in terms of sequence, function and structure [2]. The gastric enzyme functions as a heterodimer and consists of two subunits – the catalytic α-subunit, a protein of about 96 kDa which contains the sites of nucleotide binding and phosphorylation, and a glycoprotein β-subunit whose precise function is not clearly known and which runs as a broad band of 60–80 kDa in SDS-PAGE gels and as a sharp 34 kDa band in its deglycosylated form. Both subunits, from several species, have been cloned and analyzed [3–5]. Topology studies have demonstrated that the α-subunit spans the membrane 8–10 times and is oriented with most of its mass, including the nucleotide binding site, exposed to the cytoplasm [2]. On the other hand, the best estimate of β-subunit topology suggests a single transmembrane segment, a short N-terminal cytoplasmic piece and a large extracellular C-terminal domain containing six highly conserved cysteine residues and over 70% of the peptide mass [4,6]. By analogy with the Na,K-ATPase it has been proposed that the extracellular cysteine residues in the β-subunit of H,K-ATPase exist as oxidized disulfide bonds in a sequential pattern [7].

Several experiments have demonstrated close associations between the α- and β-subunits of H,K-ATPase and the importance of these associations. The heterodimeric α–β interactions are stable to non-ionic detergents [8], and the importance of the β-subunit for functional H,K-ATPase activity has been inferred from studies with β-specific antibodies [9]. Furthermore, recent studies using a baculovirus expression system have demonstrated that both α-

* Corresponding author. E-mail: john_forte@maillink.berkeley.edu. Fax: +1 (510) 6436791.

SSDI 0005-2736(95)00044-5

and β-subunits are required for functional H,K-ATPase activity [10]. Close association between α-and β-subunits has been shown to be also important in the stable expression of the α-subunit of Na,K-ATPase in *Xenopus* oocytes [11]. The β-subunit of H,K-ATPase can serve as a substitute in supporting some of the functional activities of Na,K-ATPase when expressed together with the α-subunit of Na,K-ATPase in both *Xenopus* [12] and yeast expression systems [13]. Treatment of either Na,K-ATPase [14,15] or H,K-ATPase [7] with 2-mercaptoethanol (2-ME) inactivates the respective enzymatic activity, and the inactivation has been directly correlated with the reduction of disulfide bonds within the β-subunit. Furthermore, the stability of β-subunit disulfide bonds and H,K-ATPase activity was preserved in the presence of K^+ to promote the E_2 conformer state. Recent work has also demonstrated the importance of β-subunit disulfides in the occlusion of K^+ in the catalytic cycle of the Na,K-ATPase [16].

The present work was initiated to determine whether the β-subunit of H,K-ATPase plays a role in the structural stabilization of the α-subunit. H,K-ATPase-enriched gastric microsomes (which are oriented cytoplasmic side out) were subjected to 2-ME followed by controlled trypsinolysis to study how changes in the extracellular domain, caused by reduction of the disulfide bridges in the β-subunit, would affect enzyme conformation as judged by the accessibility of the cytoplasmic side of the α-subunit to trypsin. We offer evidence that reduction of the β-subunit disulfides results in a destabilization of the α-subunit which now becomes trypsinized very rapidly. Furthermore, the altered α-subunit is unable to undergo ligand-induced conformational changes thereby indicating the importance of the β-subunit for the conformational stability of the functional holoenzyme.

2. Materials and methods

2.1. Materials

Trypsin, 2-mercaptoethanol (2-ME), *N*-ethylmaleimide, sodium dodecylsulfate (SDS) and tributylphosphine were purchased from Sigma. Pefabloc was purchased from Boehringer-Mannheim. Fluorescein-5-maleimide was from Molecular Probes. All other chemicals were of reagent grade.

2.2. Isolation of H,K-ATPase-enriched microsomes (tubulovesicles)

The H,K-ATPase-containing gastric microsomes were isolated from rabbit stomach as previously described [17]. Crude microsomes were harvested from homogenized mucosa of unstimulated rabbit stomach (H_2 receptor-blocked) as the membrane pellet sedimenting between 10 min at $13\,000 \times g$ and 1 h at $100\,000 \times g$. The pellet was resuspended in 10% sucrose, brought to 40% sucrose in ~ 8 ml volume, and overlaid with successive layers of 35% sucrose (15 ml) and 10% sucrose (12 ml) in a 37 ml tube. All sucrose media were made in 5 mM tris(hydroxymethyl)aminomethane (Tris), and 0.2 mM EDTA, pH 7.4. After centrifugation at $80\,000 \times g$ for 4 h, the purified gastric microsomes were collected from the interface between 10% and 35% sucrose and stored at 4° C until use. H,K-ATPase was assayed on the diluted samples as potassium-stimulated ouabain-insensitive *p*-nitrophenylphosphatase activity (K^+-*p*NPPase) as previously described [7]. Specific activities for various preparations were in the range of 800–1400 nmol/mg per min.

2.3. Treatment of gastric microsomes with 2-ME

Gastric microsomes (~ 4–5 mg/ml) were incubated for 25 min at 44° C with 0.4 M 2-ME. The microsomes were then diluted 15-fold at 0° C to prevent any further reduction [9]. Two sets of controls were routinely carried out: one control used 0.4 M ethanol as an organic solvent control, and the other control was a zero time of 2-ME treatment (i.e., sample diluted immediately after adding 2-ME). An additional experimental test, treated with 25 mM KCl during the 2-ME reduction process, was also performed and described in Results. Before exposing the microsomal samples to tryptic digestion, ethanol or 2-ME were added to the respective test and control tubes so that both samples were chemically identical during trypsinization.

2.4. Disulfide labeling of H,K-ATPase subunits

We previously showed that inhibition of H,K-ATPase by 2-ME was correlated with the reduction of disulfide bonds within the β-subunit, but not the α-subunit [7]. Here, we employed an alternative protocol, using tributylphosphine to reduce the oxidized cystine, to further confirm that the reduction of H,K-ATPase disulfide bonds is restricted to the β-subunit. The advantage of using tributylphosphine is in its power to reduce disulfide bonds completely without interfering with the subsequent labeling reaction. H,K-ATPase-enriched microsomes (~ 1 mg/ml) were treated with 0.4 M 2-ME at 44° C as in the protocol described above. At selected intervals, 1 ml of sample was diluted 20-fold in ice-cold suspending medium, 25 mM *N*-ethylmaleimide was added to quench any remaining 2-ME, and the sample was centrifuged at $100\,000 \times g$ for 60 min. The membrane pellet was solubilized in 2% SDS, 10 mM Tris, pH 7.4. 10 mM *N*-ethylmaleimide was then added and incubated at 55° C for 20 min in order to alkylate all available SH groups. Proteins were precipitated with 70% methanol at −20° C. The protein was then solubilized in 2% SDS, 20 mM phosphate, pH 7.1, containing 1 mM fluorescein-5-maleimide, and tributylphosphine was added to a final concentration of 1 mM. The

mixture was incubated at room temperature for 10 min, then warmed to 55° C for 20 min. The proteins were again precipitated with 70% methanol, separated by SDS-PAGE and the fluorescence was recorded on film as previously described [7]. In cases where we wished to label total cysteine SH, the microsomes were not treated with 2-ME or alkylated with *N*-ethylmaleimide, but only the last two steps were followed. That is, proteins were precipitated with methanol, solubilized in 2% SDS, 20 mM phosphate, pH 7.1, and treated with 1 mM tributylphosphine to reduce all disulfide bonds and 1 mM fluorescein-maleimide in order to label total cysteine sulfhydryl.

2.5. Tryptic digestion

Treatment with trypsin was carried out at 37° C in 25 mM imidazole-HCl (pH 7.5) at a microsomal protein concentration of 0.25–0.35 mg/ml. In some experiments either 10 mM KCl or 2 mM ATP were included as modifying ligands during trypsinization. The ratio of trypsin to membrane protein was in the range of 1:100–1:500, as stated for particular experiments. Digestion was initiated by the addition of trypsin. Aliquots were taken at selected times and quenched with trypsin inhibitor, Pefabloc, such that the final concentration of inhibitor was 1 mM. The zero time point in all cases has trypsin added to inhibitor followed by the addition of microsomes.

2.6. Immunoblots

Proteins were separated by SDS-PAGE using 10 or 12% polyacrylamide gels. For immunoprobing, proteins were transferred to nitrocellulose, and blocked for 1 h at room temperature in 5% non-fat milk. The monoclonal antibody anti-α-H.K.12.18 was used to probe for the 96 kDa α-subunit and tryptic fragments containing the epitope [20]. Nitrocellulose blots were incubated at room temperature for 1 hr with the primary antibody in 150 mM NaCl and 5 mM phosphate (pH 7.4) containing 0.5% bovine serum albumin (w/v), and then washed three times for 10 min each in 150 mM NaCl, 5 mM phosphate (pH 7.4). The blots were incubated for 1 h with goat anti-mouse secondary antibody conjugated with alkaline phosphatase, washed as above and developed with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and *p*-nitroblue tetrazolium color reagents.

3. Results

We had previously shown that the H,K-ATPase was irreversibly inhibited by 2-ME, and that the inhibition was correlated with the reduction of disulfide bonds within the β-subunit, but not within the α-subunit [7]. Here we used an alternative protocol, as described in Materials and methods, to more efficiently localize and assay the reduction of

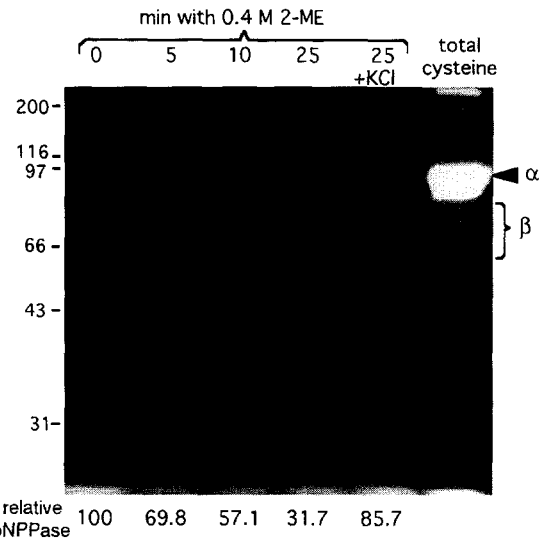

Fig. 1. Progressive reduction of β-subunit disulfide bonds as a function of time of treatment with 2-ME. H,K-ATPase-enriched microsomes (~ 1 mg/ml) were treated with 0.4 M 2-ME at 44° C for 0, 5, 10 and 25 min, as indicated. In one case the 25 min incubation with 2-ME included 25 mM KCl. An aliquot of each sample was taken for measurement of *p*NPPase activity; values are shown relative to the 0 time control. In the remaining sample, membrane proteins were precipitated, solubilized in 2% SDS and treated with 10 mM *N*-ethylmaleimide in order to block all available SH groups, as described in Materials and methods. Proteins were again precipitated and solubilized in 2% SDS containing 1 mM tributylphosphine to reduce all remaining SS bonds and 1 mM fluorescein-maleimide to label the resulting SH groups. In the case where total cysteine SH was labeled (last lane), the samples were not treated with 2-ME or alkylated with *N*-ethylmaleimide, but they were solubilized and subjected to total reduction by tributylphosphine and total SH labelling by fluorescein-maleimide. All samples were subjected to SDS-PAGE and photographed under ultraviolet light.

disulfide bonds within subunits of the H,K-ATPase under the same conditions that were employed throughout this work. A typical assay is exemplified in Fig. 1, along with relative *p*NPPase activity for the same samples. The first four lanes represent fluorescent gel images of a time-course of disulfide bond reduction in the β-subunit following treatment with 0.4 M 2-ME at 44° C. Fluorescence (index of disulfide bonds) of the 60–80 kDa β-subunit was most intense at 0 time of treatment (lane 1) and progressively diminished up to 25 min of treatment with 2-ME (lane 4), although these reducing conditions were not sufficient to eliminate all β-subunit disulfides. The inclusion of 25 mM KCl in the incubation, along with 0.4 M 2-ME at 44° C for 25 min (lane 5), prevented the reduction of β-subunit disulfides as well as the loss of enzyme activity. For all of these assays (lanes 1–5), the β-subunit was the only peptide with significant disulfide bond content; the α-subunit showed no labeling. On the other hand, when total cysteine SH content was assayed (lane 6), a predictably large signal was observed in the α-subunit. Thus, consistent with our previous observations [7], treatment with 2-ME caused exclusive reduction of disulfide bonds in the β-subunit, which was correlated with a decrease in enzy-

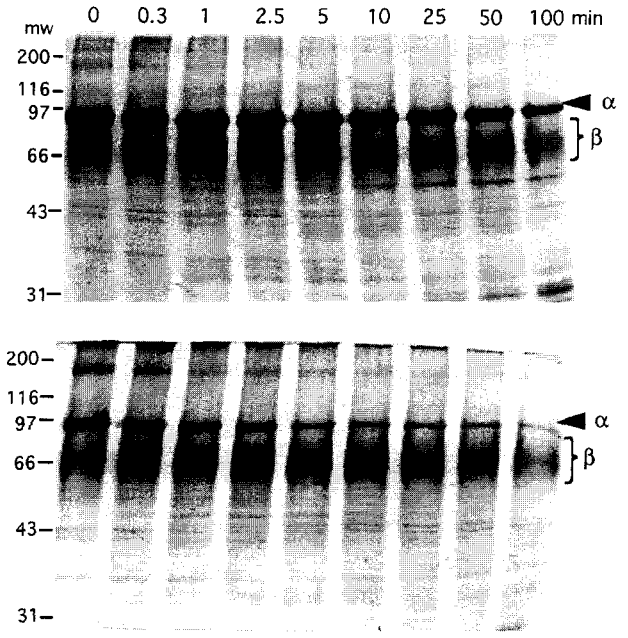

Fig. 2. Time-course of trypsinization for control (upper panel) and 2-ME treated microsomes (lower panel). Gastric microsomes were treated with 0.4 M ethanol (control) or 2-ME, as described in Materials and methods, diluted and then trypsinized at 37° C using a trypsin/substrate ratio of 1:500. Aliquots were taken at the indicated time points, subjected to SDS-PAGE, and the proteins stained with Coomassie blue. Each lane was loaded with 25 μg of protein.

matic activity, and these effects were attenuated by the presence of K^+.

To test whether reductive alterations in the extracellular domain of the β-subunit influence the structural integrity of the α-subunit, H,K-ATPase-enriched microsomes were treated with 0.4 M 2-ME (or 0.4 M ethanol as a control) at 44° C for 25 min, the reaction was quenched by dilution, and the microsomes were subjected to trypsinization as described in Materials and methods. The time-course of trypsinization at 37° C was monitored over a broad range of trypsin concentrations and analyzed. Fig. 2 shows the time-course of trypsinization for control and 2-ME-treated microsomes at a trypsin to substrate ratio of 1:500 (w/w), and with no additional ligands in the digestion medium. The Coomassie blue stained gel for the control microsomes (upper panel) shows that the 96 kDa α-band was degraded at a gradual rate, with a decrease becoming prominent at about 5 min. On the other hand the 96 kDa α-subunit band was rapidly trypsinized for the 2-ME-treated microsomes (lower panel), such that more than 50% disappeared in less than 2 min. The broad 60–80 kDa β-subunit band was also decreased slightly with time in trypsin, but unlike the α-subunit there were no apparent differences between 2-ME-treated and control microsomes. The same effect of rapid trypsinization of the α-subunit for 2-ME-treated microsomes versus control microsomes was observed over a range of trypsin to substrate concentrations. At higher trypsin concentrations, the α-subunit of the 2-ME-treated microsomes was degraded very rapidly, making it difficult to analyze the rate of degradation. Hence subsequent analyses were performed at the lower trypsin to substrate ratios in the range of 1:300–1:500 using more discrete time points. It was also noted that the α-subunit in 2-ME-treated microsomes showed increased aggregation as evidenced by the bands at 200 kDa and at the interface between the stacking and resolving gel as compared to control. The reduction of the β-subunit disulfides which results in a loosened α-conformation might also produce a more sticky form for enhanced interaction of the α-subunit with its neighbors. The time-course of trypsinization for five preparations was quantitated by measuring the relative volume density of the Coomassie blue stained 96 kDa α-band. These data, plotted in Fig. 3, clearly show that the α-subunit of the 2-ME-treated microsomes is degraded at a faster rate than the control microsomes. Thus 2-ME-treatment which results in the reduction of β-subunit disulfide bonds and inhibition of enzyme activity also increased the susceptibility of the α-subunit to trypsinization.

To verify that increased susceptibility to trypsin of α-subunit in 2-ME-treated is due to reduction of β-subunit disulfides, a test was performed which included 25 mM KCl during the 2-ME-treatment process. It has been demonstrated that K^+ and its congeners protect against the 2-ME-induced inactivation of the H,K-ATPase and that the protective effect of K^+ is accompanied by a preservation of β-subunit disulfides [7]. Measurements of enzyme activity for samples prior to trypsinization revealed that the

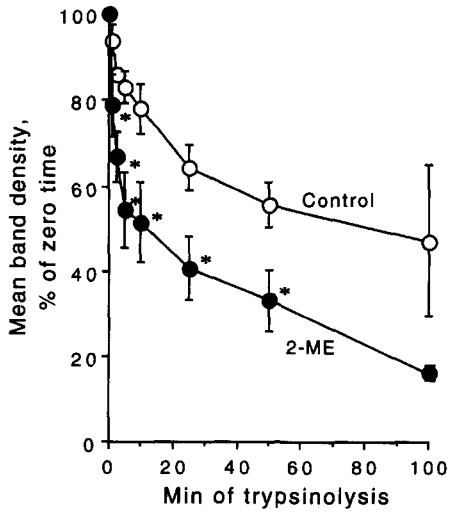

Fig. 3. Reduction of β-subunit disulfide bonds makes the α-subunit more susceptible to trypsinolysis. Preparations of gastric microsomes were treated with ethanol (control) or 2-ME, subjected to trypsinolysis (1:500) just as described in Fig. 2. The 96 kDa α-subunit on Coomassie blue stained gels was assayed by densitometry, and the band density, relative to the measured value at zero time for each preparation, was plotted as a function of time. For all time points $n = 4$, except for 0 and 25 min where $n = 5$, and for 100 min where $n = 3$. The asterisk (*) indicates a significance of $P < 0.05$.

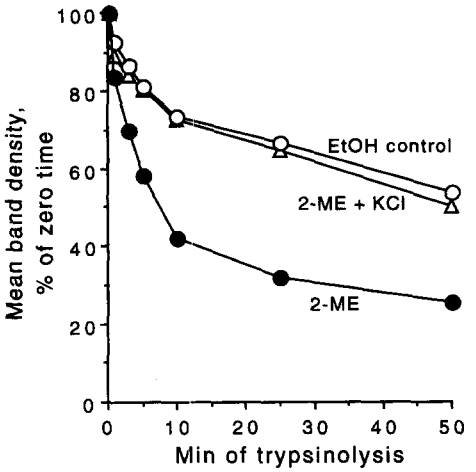

Fig. 4. A comparison of α-subunit trypsinolysis for microsomes treated with 2-ME in the presence and absence of 25 mM KCl. Gastric microsomes were treated for 25 min at 44° C with 0.4 M 2-ME (closed circles) or 0.4 M 2-ME plus 25 mM KCl (open triangles); a control set of microsomes were treated with 0.4 M ethanol (open circles). The preparations were then diluted and trypsinized, as described in Materials and methods, and aliquots taken for SDS-PAGE. The gels were stained with Coomassie blue and the 96 kDa α-band quantitated by densitometry.

presence of 25 mM KCl in the preincubation mixture attenuated the inhibition by 2-ME, such that the enzyme retained almost all of its activity, as compared to the ethanol-treated control. Patterns of trypsinization were compared for 2-ME-treated microsomes with and without KCl protection, as well as for control microsomes treated with ethanol. Results in Fig. 4 reveal that the α-subunit of 2-ME-treated microsomes was highly susceptible to trypsin, whereas, when 25 mM KCl was present during the 2-ME treatment tryptic digestion of the α-subunit was very much attenuated and similar to the non-reduced, ethanol-treated, control. Thus, the data support the postulate that increased rate of trypsinization of the α-subunit is a direct consequence of disulfide bond reduction in the β-subunit.

Previous studies have shown that specific products formed on tryptic digestion of gastric H,K-ATPase depend on the conformational state of the enzyme [18,19]. In the presence of K^+, for example, different sites on the protein are exposed than in the presence of ATP and each of the two conformations, termed K^+ conformation and ATP conformation, respectively, can be defined by their characteristic patterns of digestion. To study the conformational responsiveness of the α-subunit in control and 2-ME-treated microsomes, the patterns of tryptic digestion were followed in the presence of ATP, which is known to favor the E_1 conformation of the enzyme, or in the presence of K^+, which is known to induce the E_2 conformer.

When control microsomes were trypsinized in the presence of 2 mM ATP, the Coomassie blue stained gels showed a gradual degradation of the 96 kDa α-subunit band with the appearance of characteristic intermediates at 67 kDa and 38 kDa (Fig. 5A, upper panel). The prominent 67 kDa intermediate first appeared at about 10 min and remained over the time-course of the experiment. Fainter bands at 38 kDa and 55 kDa also appeared at 10 min and remained over the experimental time-course. When the 2-ME-treated microsomes were trypsinized in the presence of ATP, the 96 kDa α-subunit rapidly disappeared (Fig. 5A, lower panel), similar to the rapid loss of α-subunit in the case with no additional ligands (cf. Figs. 2 and 5). The pattern of tryptic fragments for the 2-ME-treated microsomes did not reveal the presence of sharp and well defined intermediates seen in the control microsomes.

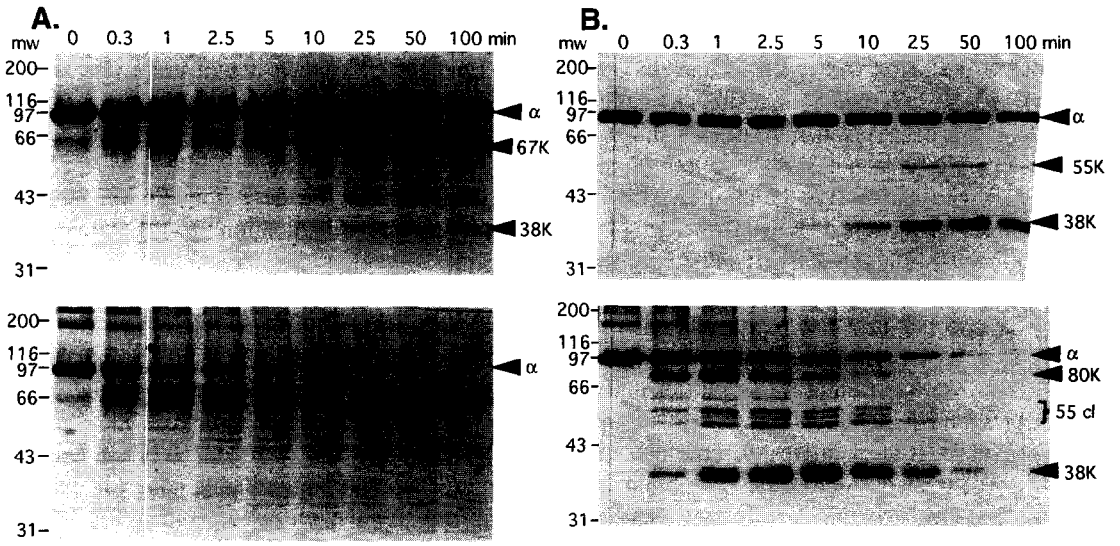

Fig. 5. The time-course of trypsinolysis in the presence of ATP for control (upper panels) and 2-ME treated microsomes (lower panels). Gastric microsomes were treated with ethanol (control) or 2-ME as described in Materials and methods. The preparations were then diluted and trypsinized in the presence of 2 mM ATP at a trypsin-to-substrate ratio of 1:500 at 37° C. Aliquots were taken at the various indicated times and the samples subjected to SDS-PAGE. (A) Gels were loaded with 12 μg of protein per lane and stained with Coomassie blue. (B) Western blots probed with anti-α-H,K12.18. Gels were loaded with 7 μg per lane then blotted to nitrocellulose. Molecular mass standards (kDa) are shown on the left.

Several faint bands could be seen in the region of 35–80 kDa which seemed to fade away and disappear after 10 min. The Coomassie stained tryptic pattern of the 2-ME treated microsomes in the presence of ATP also appeared similar to the tryptic pattern with no additional ligands.

In order to visualize the intermediate-pattern more clearly, immunoblots were performed using anti-α-H.K.12.18 which binds in the region of amino acids 888–906 of the α-subunit [20]. For control microsomes, blots of the trypsinization pattern in the presence of ATP showed immunoreactivity with two tryptic products: a dominant 38 kDa band, which starts to appear at about 10 min and reaches a maximum at about 50 min; and a faint 55 kDa band (Fig. 5B, upper panel). This is in agreement with published data demonstrating that, in the presence of ATP, tryptic cleavage of the α-subunit gives rise to a N-terminal 67 kDa band and a carboxy-terminal 38 kDa band, respectively [19]. On the other hand, for the 2-ME-treated microsomes tryptic mapping in the presence of ATP revealed that the α-subunit is degraded more rapidly, and proceeds though additional intermediates as compared to control (Fig. 5B, lower panel). A dominant band at 80 kDa was seen as early as 20 s and almost disappeared by 5 min; a fainter triplet cluster in the region of 45–55 kDa was seen at about 1 min and disappeared by 10 min; and a dominant doublet appeared at 38–40 kDa, first appearing at about 20 s, reaching a maximum at about 5 min and disappearing by 50 min. These data suggest that reduction of extracytoplasmic disulfide bonds in the β-subunit results in an altered α-conformation which then exposes additional tryptic sites on the cytoplasmic side giving rise to additional intermediates.

To further test the sensitivity of 2-ME-treated H,K-ATPase to ligand-induced conformational changes, trypsinization of the microsomes was performed in the presence of KCl. Control microsomes treated with trypsin in the presence of 10 mM KCl showed the characteristic pattern of 55 kDa and 42 kDa bands on Coomassie blue stained gels, while the 2-ME-treated microsomes did not show these distinct bands (data not shown). Blots of the tryptic pattern probed with anti-α-H,K 12.18 showed that, for control microsomes in presence of KCl, the 55 kDa band is the predominant immunoreactive band while the 38 kDa band was very faint (Fig. 6, upper panel). Immunoblots of the tryptic pattern of 2-ME-treated microsomes in presence of KCl revealed additional immunoreactive fragments at 80 kDa, a fainter triplet cluster in the region of 45–55 kDa and a dominant doublet at 38–40 kDa (Fig. 6, lower panel). It is of interest to note that the immunoblots for 2-ME-treated microsomes exhibited an almost identical pattern of intermediates whether trypsinization was carried out in presence KCl or ATP, or when no ligands were present (cf. lower panels of Figs. 5 and 6). This contrasted with control microsomes, where trypsinization in presence of ATP revealed a 38 kDa predominant intermediate and trypsinization in the presence of KCl showed the predominant 55 kDa intermediate, demonstrating the more typical response to ligands. The digestion pattern for 2-ME treated microsomes was the same and did not change in the presence or absence of different ligands. Thus it appears that the α-subunit of H,K-ATPase is unable to undergo ligand-induced conformational changes when the β-subunit disulfides are reduced.

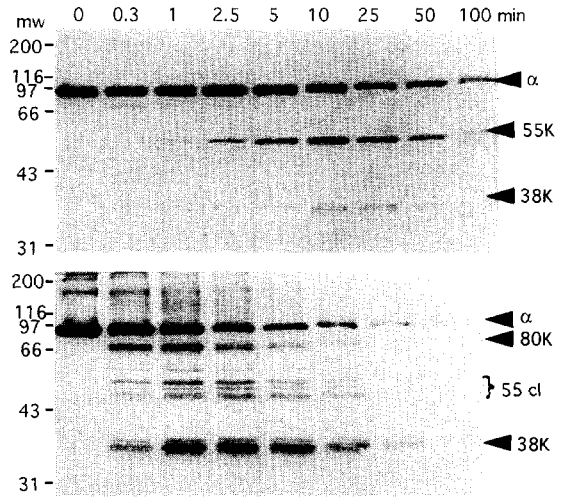

Fig. 6. The time-course of trypsinolysis in the presence of KCl for control (upper panel) and 2-ME treated microsomes (lower panel). Trypsinization of control and 2-ME-treated microsomes was performed at 37° C in the presence of 10 mM KCl at a trypsin to substrate ratio of 1:500. Aliquots were taken at the indicated times and subjected to SDS-PAGE and Western blotting. Nitrocellulose blots were probed with anti-α-H,K-12.18. Each lane was loaded with 7 μg of protein.

4. Discussion

The present studies demonstrate that the α-subunit of H,K-ATPase becomes increasingly susceptible to trypsinization on reduction of disulfide bonds within the β-subunit. Control studies indicated that the increased tryptic sensitivity of the α-subunit was not directly due to the conditions of incubation or to the effect of 2-ME as an organic solvent; rather, tryptic sensitivity was correlated with the reduction of disulfide bonds in the β-subunit. Any temporary instability or unfolding of the enzyme held at 44° C for 25–30 min is reversible, and does not directly account for the permanent alterations of enzyme activity or susceptibility to trypsin. However, cleavage of β-subunit disulfide bonds for both H,K-ATPase and Na,K-ATPase requires rather strong reducing conditions that are certainly facilitated by the elevated temperature used here [7,14]. We can also rule out the direct effects of 2-ME as solvent as the primary cause for the irreversible inactivation of H,K-ATPase. We have measured the oil/water partition coefficient of 2-ME to be 0.76 (personal observation), between the values of 0.48 and 0.98 reported for ethanol

and 2-propanol, respectively [21], and neither of these latter solvents inhibits H,K-ATPase activity when employed at the conditions used here for 2-ME [22]. However, reversible destabilization of 2-ME as it partitions into the membrane may serve to facilitate the reduction of disulfide bonds as enzyme structure is permanently altered. Such a multiple participation of bonding forces to stabilize/destabilize protein structure is the hallmark of the phenomenon of cooperativity [23]. At this time we can not specify the degree to which the thermal and solvent effects contribute to holoenzyme inactivation. Under the conditions used here treatment with 2-ME is essential for the correlative reduction of disulfide bonds in the β-subunit, thus presenting a system for to study the corresponding changes in α-subunit conformation.

The increased proteolytic susceptibility of the α-subunit is accompanied by the presence of new and additional intermediate peptides which were seen on immunoblots with the α-subunit antibody, H,K-12.18. This implies that reduction of β-subunit disulfides results in a loosening of α-conformation, exposing additional tryptic sites on the cytoplasmic side of the gastric vesicles. Tryptic cleavage of the control microsomes in presence of ATP as a ligand has been previously identified to occur at Lys-47 to give the 67 kDa peptide that contains the phosphorylation site and is non-reactive to carboxy-terminal antibodies [24,25]. The second cleavage in presence of ATP occurs preferentially at Lys-670 to give the 38 kDa peptide that has been sequenced to contain the region from Ala-672 of the α-subunit (Adam Smolka, unpublished observation). The control α-subunit in the KCl conformation has been shown to undergo preferential cleavage at amino acids 47 and 455 to give the 42 kDa N-terminal peptide containing the phosphorylation site and the 55 kDa peptide that has been sequenced to contain the region from Ile-456 onward of the α-subunit and is reactive to C-terminal antibodies [24,25]. Some amount of cleavage also occurs at Lys-670 to give the 37 kDa peptide but the predominant product under our conditions was the 55 kDa peptide. Thus, as schematically represented in Fig. 7 the control α-subunit in the KCl conformation shows preferential cleavage at amino acids 47 and 455, while in the ATP bound conformation preferential cleavage occurs at amino acid 47 and 670. Tryptic cleavage of the α-subunit when the β-subunit was reduced exposes three regions of highly sensitive sites. These sites, schematically represented in Fig. 7, are (1) an N-terminal cleavage, which gives rise to the immunoreactive 80 kDa tryptic fragment, (2) sites in the neighborhood of Lys-455, which give rise to the immunoreactive 50–55 kDa cluster, and (3) sites in the neighborhood of amino acid Lys-670, which give rise to the two immunoreactive bands in the region of 35–38 kDa. All of these sites are in the cytoplasmically exposed regions of the α-subunit and appear to be all in the large cytoplasmically

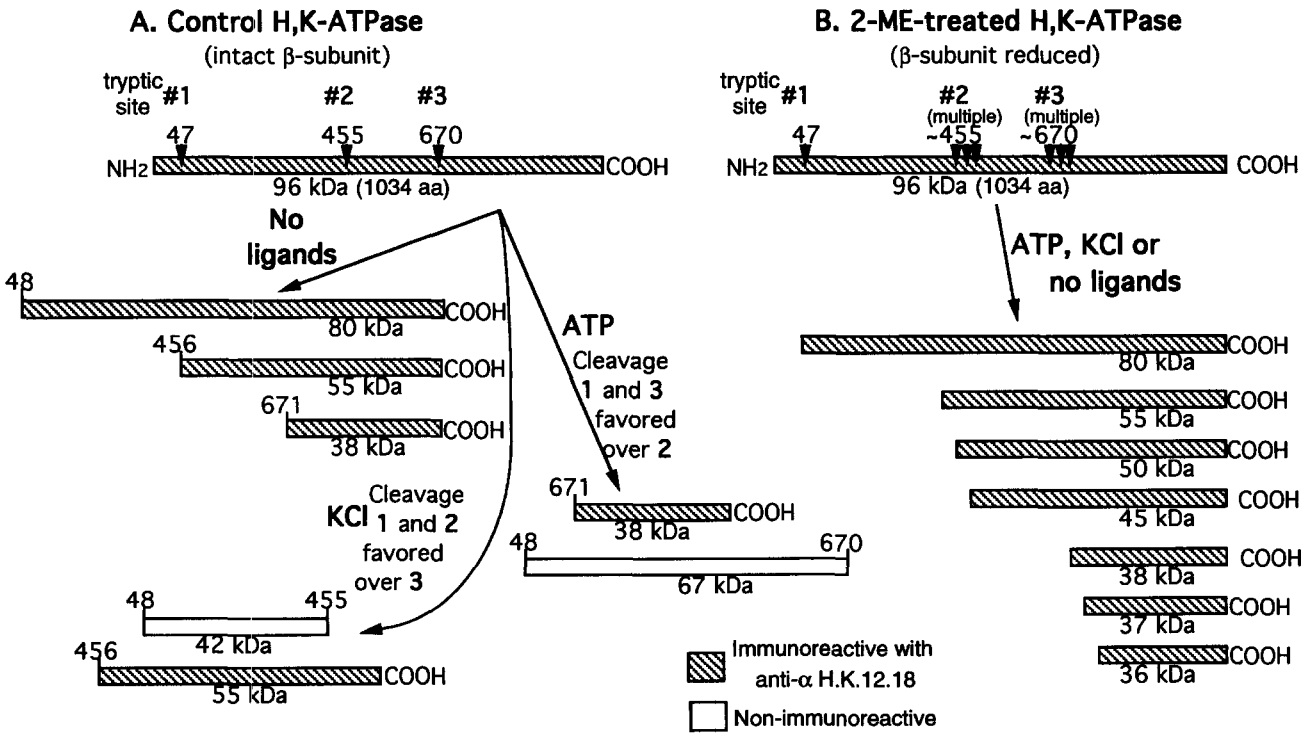

Fig. 7. Suggested tryptic digestion sites on the α-subunit of H,K-ATPase for control and 2-ME treated microsomes: the figure shows a linear polypeptide map of the catalytic subunit of rabbit H,K-ATPase. For the control microsomes tryptic cleavage sites in the presence of ligands are indicated on basis of immunoblots with anti-α-H,K 12.18 and previously published data. For the 2-ME treated microsomes speculated tryptic cleavage sites are indicated on basis of immunoblots with anti-α-H,K 12.18. The indicated molecular masses of the fragments are the apparent molecular masses observed on SDS-PAGE gels.

exposed loop of the α-subunit. Reduction of β-subunit disulfides has thus caused increased exposure of additional tryptic sites in the neighborhood of the already susceptible cleavage sites, in the cytoplasmic region of the H,K-ATPase. Thus, a change in β-conformation is communicated to its tightly associated α-conformation.

It is also interesting to note that on reduction of disulfides in the β-subunit, the α-subunit loses its ability to respond to conformational changes induced by ligands. This situation is highly reminiscent of the high sensitivity to trypsin and the lack of ligand response of the α-subunit of Na,K-ATPase immediately after synthesis, but which gains tryptic resistance and the capacity to undergo cation-dependent conformational changes on maturation and association with its β-subunit [26]. The β-subunit of both H,K-ATPase and Na,K-ATPase must therefore play a very important role in sustaining the right functional conformation in the α-subunit and the holoenzyme.

The finding that reduction of β-subunit disulfides destabilizes the α-subunit which can no longer assume ligand-specific conformation is particularly interesting from a topological viewpoint. As pointed out earlier the vesicles used in these studies are cytoplasmic side out and the conformational transitions in the α-subunit are both detected and most pronounced on the cytoplasmic side of the secretory membrane. The α-subunit has very little exposure on the lumenal side while the bulk of the β-subunit including the β-subunit disulfides are on the lumenal side of the membrane [6,4]. Thus the β-subunit initiated signals for α-subunit destabilization are transmitted across the membrane.

If we consider a 8-transmembrane domain model of the α-subunit, there are four small helical loops which can potentially interact with the lumenally exposed β-subunit disulfides. The disposition of one or more of these loops may be dictated by α–β interactions which in turn places conformational constraints on the large hydrophilic α-subunit catalytic domain in the cytoplasm. With the reduction of β-subunit disulfides, these α–β interactions are loosened, the hairpin helices are cast adrift and the α-subunit relaxes into non-functional, trypsin-accessible flaccidity possibly even losing its ligand binding capacity. Recent studies have suggested that it is the M7/loop/M8 sector which are the transmembrane segments in the carboxy terminal one-third of the α-subunit that are closely associated to the β-subunit [27].

It is also possible that the interactions between the β-subunit and α-subunit that influence the conformational response are mainly cytoplasmic. Evidence supportive of this speculation is the inhibition of ATPase activity by antibodies against the N-terminal domains of the β-subunit [9]. In such a model, the reduction in β-subunit disulfides induces rearrangement of extracellular β-subunit domains which are communicated to the N-terminus of β-subunit and the subsequent loosened cytoplasmic interactions between the α–β subunits are the ones that cause the loss of conformational response. The exact mechanism by which changes in the extracellular domain of the β-subunit are communicated and influence the conformational response of the α-subunit are not clear, but our data clearly point to the fact that for the H,K-ATPase the α–β-subunit interactions play an important role in the conformational response of the α-subunit.

The present data are in agreement with much of the literature on the importance of subunit interactions in ion-transport ATPases. A recent report on H,K-ATPase expression in the baculovirus system demonstrated that the β-subunit is essential for functional expression, indicating the importance of α–β interaction for enzymatic function [10]. Experiments on Na,K-ATPase in yeast and *Xenopus* oocyte expression systems have demonstrated that maturation of functional pumps depends on the concomitant expression of both α- and β-subunits [28]. For *Xenopus* oocytes expressing an excess of Na,K-ATPase α over β, the α-subunit was highly sensitive to trypsin and became trypsin-resistant on injection of cRNA for the β-subunit, demonstrating that assembly of the β-subunit has a stabilizing effect on the α-subunit [26]. Thus, subunit interaction and assembly is a prerequisite for the nominal catalytic subunit to acquire a correct, stable, membrane conformation.

If the role of the β-subunit is to structurally assist the α-subunit in the right functional conformation then that would explain why the β-subunit of H,K-ATPase can substitute for the β-subunit of Na,K-ATPase [12,13]. It would also explain the loss of H,K-ATPase activity on reduction of β-subunit disulfide bonds, although a direct involvement of β-subunit in the catalytic cycle can not be ruled out. Recent experiments on Na,K-ATPase demonstrate the importance of β-subunit disulfide bonds in K^+ occlusion [16].

In summary, the results presented here indicate that the α- and β-subunits of gastric H,K-ATPase are closely and tightly associated. The data further demonstrate that the α–β interactions are important for holoenzyme conformation and structural and functional integrity of the H,K-ATPase.

References

[1] Forte, J.G. and Soll, A. (1989) in Handbook of Physiology: The Gastrointestinal System (Forte, J.G. and Schultz, S., eds.), Vol. III, Chapter 11, pp. 207–228, American Physiological Society, Bethesda.
[2] Sachs, G., Besancon, M., Shin, J.M., Mercier, F., Munson, K. and Hersey, S. (1992) J. Bioenerg. Biomembr. 24, 301–308.
[3] Chow, D.C. and Forte, J.G. (1995) J. Exp. Biol. 198, 1–17.
[4] Canfield, V.A., Okamato, C.T., Chow, D.C., Dorfman, J., Gros, P., Forte, J.G. and Levenson, R. (1990) J. Biol. Chem. 265, 19878–19884.
[5] Reuben, M.A., Lasater, L.S. and Sachs, G. (1990) Proc. Natl. Acad. Sci. USA 87, 6767–6781.
[6] Shull, G.E. (1990) J. Biol. Chem. 265, 12123–12126.

[7] Chow, D.C., Browning, C.M. and Forte, J.G. (1992) Am. J. Physiol. 263, C39–46.
[8] Okamoto, C.T., Karpilow, J.M., Smolka, A. and Forte, J.G. (1990) Biochim. Biophys. Acta 1037, 360–372.
[9] Chow, D.C. and Forte, J.G. (1993) Am. J. Physiol. 265, C1562–1570.
[10] Klaassen, C.H., Van Uem, T.J., De Moel, M.P., De Caluwe, G.L., Swarts, H.G. and De Pont, J.J. (1993) FEBS Lett. 329, 277–282.
[11] Geering, K., Theulaz, I., Verrey, F., Hauptle, M.T. and Rossier, B.C. (1989) Am. J. Physiol. 257, C851–858.
[12] Horisberger, J.D., Jaunin, P., Reuben, M.A., Lasater, L.S., Chow, D.C., Forte, J.G., Sachs, G., Rossier, B.C. and Geering, K. (1990) J. Biol. Chem. 266, 19131–19134.
[13] Eakle, K.A., Kim, K.S., Kabalin, M.A. and Farley, R.A. (1992) Proc. Natl. Acad. Sci. USA 89, 2834–2838.
[14] Kawamura, N. and Nagano, K. (1984) Biochim. Biophys. Acta 774, 188–192.
[15] Kirley, T.L. (1990) J. Biol. Chem. 265, 4227–4232.
[16] Lutsenko, S. and Kaplan, J.H. (1993) Biochemistry 32, 6737–6743.
[17] Reenstra, W.W. and Forte, J.G. (1990) Methods Enzymol. 192, 151–165.
[18] Saccomani, G., Dailey, D.W. and Sachs, G. (1979) J. Biol. Chem. 254, 2821–2827.
[19] Helmich-De Jong, M.L., Van Emst-De Vries, S.E. and De Pont, J.J. (1987) Biochim. Biophys. Acta 905, 358–370.
[20] Smolka, A., Alverson, L., Fritz, R., Swiger, K. and Swiger, R. (1991) Biochem. Biophys. Res. Commun. 180, 1356–1364.
[21] Leo, A., Hansch, C. and Elkins, D. (1971) Chem. Rev. 71, 525–613.
[22] Forte, J. and Chow, D.C. (1993) The Stomach (Domschke, W. and Konturek, S.J., eds.), pp. 22-37, Springer, Berlin.
[23] Creighton, T.E. (1993) Proteins – Structure and Molecular Properties, 2nd Edn., W.H. Freeman, New York.
[24] Van Uem, T.J., Swarts, H.G. and De Pont, J.J. (1991) Biochem. J. 280, 243–248.
[25] Asano, S., Arakawa, S., Hirasawa, M., Sakai, H., Ohta, M., Ohta, K. and Takeguchi, N. (1994) Biochem. J. 299, 59–64.
[26] Geering, K., Kraehenbuhl, J.P. and Rossier, B.C. (1987) J. Cell Biol. 105, 2613–2619.
[27] Shin, J.M. and Sachs, G. (1994) J. Biol. Chem, 269, 8642–8646.
[28] Ackerman, U. and Geering, K. (1990) FEBS Lett. 269, 105–108.