Determination of the epitope for the inhibitory monoclonal antibody 5-B6 on the catalytic subunit of gastric Mg^{2+} -dependent H^+ -transporting and K^+ -stimulated ATPase

Tom J. F. VAN UEM, Herman G. P. SWARTS and Jan Joep H. H. M. DE PONT*

Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

The monoclonal antibody 5-B6, directed against the α -subunit of pig gastric H^+,K^+ -ATPase (Mg^{2+} -dependent H^+ -transporting and K^+ -stimulated ATPase), was shown to be a potent inhibitor of the K^+ -ATPase activity, thereby binding to the cytoplasmic side of the α -subunit of the enzyme [Van Uem, Peters & De Pont (1990) *Biochim. Biophys. Acta* 1023, 56–62]. In order to define the epitope for 5-B6 on pig gastric H^+,K^+ -ATPase more precisely, the α -subunit of the enzyme was subjected to limited proteolysis followed by chemical cleavage. Restricted proteolysis with papain followed by sequence analysis yielded an immunoreactive fragment of 27 kDa beginning at Ser³⁷⁹. This fragment was water-soluble and possessed the fluorescein isothiocyanate-reaction site. Limited tryptic digestion in the presence of K^+ gave rise to an immunoreactive 56 kDa fragment beginning at Ile⁴⁵⁶, thus restricting the location of the epitope from Ile⁴⁵⁶ to the C-terminal end of the 27 kDa fragment (around residue 620). Further degradation of the 27 kDa fragment by means of formic acid cleavage at Asp–Pro bonds resulted initially in the formation of two non-immunoreactive fragments of 17 kDa and 11 kDa, indicating that the epitope for 5-B6 has to be localized around the chemical cleavage sites Asp⁵⁰⁷ and/or Asp⁵¹⁰. Comparison of the primary structure of the α -subunits of gastric H^+,K^+ -ATPase and non-immunoreactive rat kidney Na^+,K^+ -ATPase shows almost no similarity for the sequence containing these formic acid cleavage sites (Thr⁵⁰⁴-Leu-Glu-Asp-Pro-Arg-Asp-Pro-Arg⁵¹²), whereas the adjacent sequences are nearly 100 % identical. These findings strongly suggest that the epitope for 5-B6 includes (part of) this sequence.

INTRODUCTION

Gastric H^+,K^+ -ATPase (Mg^{2+} -dependent H^+ -transporting and K^+ -stimulated ATPase) is an intrinsic membrane protein complex responsible for gastric acid secretion [1]. The enzyme belongs to the class of P-type ATPases [2,3] and catalyses *in vitro* an electroneutral exchange of 2 K^+ ions and 2 H^+ ions per molecule of ATP hydrolysed [4,5]. Recently it has been demonstrated that, in addition to the catalytic α -subunit of 114 kDa [6–8], the enzyme contains a glycosylated β -subunit with a core protein of 33 kDa [9–13].

The hydropathy profile of the α -subunit of H^+,K^+ -ATPase strongly suggests the presence of eight transmembrane segments with the N-terminus facing the cytoplasm [6]. Evidence for the actual folding of the α -subunit resembling the predicted one comes from studies on specific chemical modifications at the extracellular or cytoplasmic side. With the use of tightly sealed inside-out-oriented vesicles it was shown that Lys⁴⁹⁷ reacted with the impermeant reagent pyridoxal 5'-phosphate, indicating its location on a cytoplasmic domain of the enzyme [14]. Additional evidence that this residue faces the cytosol comes from the observation that ATP prevents its chemical modification by pyridoxal 5'-phosphate. ATP also prevents FITC (fluorescein isothiocyanate) labelling of Lys⁵¹⁸ [15], indicating the cytoplasmic orientation of this important recognition site on the α -subunit of P-type ATPases.

The monoclonal antibody 5-B6, directed against the α -subunit of pig gastric H^+,K^+ -ATPase, is a potent inhibitor of the K^+ -ATPase activity of the enzyme [16]. By using inside-out-oriented vesicles containing native pig gastric H^+,K^+ -ATPase we were able to show that 5-B6 recognizes the cytoplasmic side of the α -subunit [16]. In order to determine the epitope for 5-B6

(apparently probing a functional domain on the α -subunit), identification of immunoreactive and non-immunoreactive peptides, obtained by limited tryptic digestion [17] or papain digestion [18] of gastric H^+,K^+ -ATPase, was undertaken. This approach made it necessary to identify the specific cleavage sites on the α -subunit. The localization of the tryptic sites enabled us, moreover, to correlate the time course of formation of tryptic fragments of the α -subunit with the concomitant biphasic inactivation of the K^+ -ATPase activity of the enzyme as previously found by us [17]. Chemical fragmentation of a soluble fragment of the α -subunit, which was obtained by papain digestion [18], and an amino acid-by-amino acid comparison with the non-immunoreactive Na^+,K^+ -ATPase was carried out to characterize the epitope.

The results of our experiments show that the epitope includes (part of) the amino acid sequence from residue 504 to 512. This finding is fully consistent with the proposed folding of the α -subunit, with a large cytoplasmic domain from amino acid residue 359 to 796 [6], and suggests that the recently found carbohydrate moiety at Asn⁴⁹³ [9] faces the cytosol.

EXPERIMENTAL

Preparation of gastric H^+,K^+ -ATPase

Membranes enriched in H^+,K^+ -ATPase were isolated from the gastric mucosa of freshly slaughtered pigs as described previously by us [16]. Protein was determined by the Lowry method with BSA as standard.

Labelling of H^+,K^+ -ATPase with FITC

FITC-labelling of gastric H^+,K^+ -ATPase was carried out as described by Jackson *et al.* [19]. Briefly, gastric H^+,K^+ -ATPase

Abbreviations used: H^+,K^+ -ATPase (EC 3.6.1.36), Mg^{2+} -dependent H^+ -transporting and K^+ -stimulated ATPase; FITC, fluorescein isothiocyanate; Caps, 3-(cyclohexylamino)-1-propanesulphonic acid.

* To whom correspondence should be addressed.

was incubated at a protein concentration of 3 mg/ml in a reaction medium containing 100 mM-Tris/HCl buffer, pH 9.2, 2 mM-EDTA and 5 μ M-FITC (dimethylformamide solution). After incubation for 30 min at 22 °C in the dark, a 10-fold excess of ice-cold 20 mM-Tris/HCl buffer, pH 7.2, containing 4 mM-lysine hydrochloride was added. The modified H⁺,K⁺-ATPase was spun down (100 000 *g* for 1 h), washed with the same buffer and resuspended to 5 mg/ml in 20 mM-Tris/HCl buffer, pH 7.2, in which 0.25 M-sucrose was included.

Tryptic digestion of H⁺,K⁺-ATPase

Treatment of H⁺,K⁺-ATPase with trypsin was carried out as described by Helmich-De Jong *et al.* [17]. Peptide fragments characteristic for the E₁ conformation (67 and 35 kDa) were obtained by tryptic digestion of 1.25 mg/ml H⁺,K⁺-ATPase for 30 min at 37 °C in a medium containing 50 mM-imidazole/HCl buffer, pH 7.0, 5 mM-Na₂ATP, 100 mM-choline chloride, 2 mM-EDTA and 100 μ g of trypsin/ml. Digestion under similar conditions, but in the presence of 100 mM-KCl and 30 μ g of trypsin/ml gave peptide fragments (56 and 42 kDa) characteristic for the E₂ conformation of the enzyme. Tryptic digestion was stopped by precipitation with 5 % (w/v) trichloroacetic acid. The pellets were washed with 30 % (w/v) sucrose and resuspended in the sample buffer for SDS/PAGE described by Laemmli [20].

Papain digestion of H⁺,K⁺-ATPase

Limited papain digestion of native and FITC-labelled H⁺,K⁺-ATPase was carried out by the procedure of Saccomani & Mukidjam [18]. Before use, papain (6 units/ml) was activated for 20 min at 22 °C in a solution containing 20 mM-cysteine hydrochloride and 2 mM-Tris/EDTA, pH 7.0. The solution was kept under a stream of N₂. After activation, papain (0.6 unit/ml) was incubated at 37 °C with native or FITC-labelled enzyme (1.0 mg/ml). The digestion was stopped after 30 min by adding iodoacetic acid (20-fold molar excess over cysteine hydrochloride). The digested sample was centrifuged (100 000 *g* for 1 h) and the supernatant, containing a soluble FITC-labelled 27 kDa peptide of the catalytic subunit, was dialysed against deionized water and freeze-dried. Finally, this peptide fragment was resuspended in 20 mM-Tris/HCl buffer, pH 7.2, and concentrated to a protein concentration of 10 mg/ml with Centricon 30 microconcentrators (Amicon).

Cleavage of the 27 kDa fragment with formic acid

Selective chemical cleavage of the 27 kDa fragment, obtained by papain digestion of FITC-labelled H⁺,K⁺-ATPase, at Asp-Pro bonds was achieved by treatment of the peptide fragment with 70 % (v/v) formic acid for 48 h at 37 °C [21]. After incubation, samples were freeze-dried and resuspended in sample buffer for Tricine gels [22].

Gel electrophoresis and Western blotting

The SDS/polyacrylamide slab gels utilized were prepared and used according to the procedure of Laemmli [20] and were either 10 % or 8–15 % gradient gels. To separate the peptide fragments after chemical cleavage of the 27 kDa peptide, Tricine gels [22] of 16.5 % with 3 % cross-linker were used. After electrophoresis, the gels were soaked for 5 min in transfer buffer [10 mM-Caps/NaOH buffer, pH 11.0, containing 10 % (v/v) methanol] and subsequently electroblotted for 1 h at 50 V to poly(vinylidene difluoride) membranes (Immobilon Transfer, Millipore) [23]. After electroblotting, the Immobilon membranes were rinsed twice with deionized water. Subsequent blocking of non-reactive sites, incubation with monoclonal antibody 5-B6 and secondary antibodies and staining were carried out as described before [16].

N-Terminal sequence analysis

The N-terminal sequences of proteolytic fragments of H⁺,K⁺-ATPase were determined by automated Edman degradation in an Applied Biosystems 475A gas-phase Sequencer. Therefore proteolytic fragments of the enzyme were separated on a Laemmli gel that had been aged for 24 h and pre-run with sample buffer in the presence of 0.001 % (v/v) thioglycollic acid in the upper running buffer. Electroblotting to an Immobilon membrane was as above, except for addition of 0.001 % (v/v) thioglycollic acid in the transfer buffer. The Immobilon membrane was washed in deionized water, stained with 0.1 % (w/v) Coomassie Blue R-250 in 50 % (v/v) methanol and then destained in 50 % (v/v) methanol/10 % (v/v) acetic acid. The membrane was finally rinsed with deionized water, air-dried, and the peptide bands (40 pmol for each peptide) of interest were cut out for N-terminal sequence analysis.

RESULTS

Specificity of monoclonal antibody 5-B6

The monoclonal antibody 5-B6, which we described before [16], is an antibody that specifically inhibits the K⁺-ATPase activity of gastric H⁺,K⁺-ATPase membrane fragments. Since the antibody also inhibits the enzyme in inside-out-oriented membrane vesicle preparations the antigenic determinant must be at the cytoplasmic side.

Fig. 1 demonstrates the specificity of antibody 5-B6 for the α -subunit of gastric H⁺,K⁺-ATPase. The Immobilon membranes presented contain purified membrane samples from pig H⁺,K⁺-ATPase (lane 1), rat H⁺,K⁺-ATPase (lane 2) and rabbit kidney Na⁺,K⁺-ATPase (lane 3). The blot on the left was stained with Coomassie Blue. The blot on the right was stained with monoclonal antibody 5-B6. It is evident that antibody 5-B6 reacts equally well with the 95 kDa α -subunit of both pig and rat gastric H⁺,K⁺-ATPase, but not with the 95 kDa α -subunit of Na⁺,K⁺-ATPase. The same results were obtained in an enzyme-linked immunosorbent assay (work not shown).

Limited trypsin and papain digestion

The first step in the determination of the 5-B6 epitope on the catalytic subunit of gastric H⁺,K⁺-ATPase was made by carrying out limited tryptic and papain digestions of gastric H⁺,K⁺-ATPase, as described by Helmich-De Jong *et al.* [17] and Saccomani & Mukidjam [18]. These experiments were carried out in order to determine which of the obtained peptide fragments contains the binding site for antibody 5-B6.

We have previously shown that the products formed upon tryptic digestion of gastric H⁺,K⁺-ATPase depend on the conformational state of the enzyme [17]. Thus in the presence of ATP two fragments are produced with apparent molecular masses of 35 and 67 kDa, whereas in the presence of K⁺ the α -subunit is split into fragments of 42 and 56 kDa. The phosphorylation site, Asp³⁸⁶, is present in both the 42 kDa and the 67 kDa fragment. The presence of Asp³⁸⁶ in the 42 kDa peptide means that this fragment must be the N-terminal cleavage product of the α -subunit. On the other hand, the 67 kDa fragment can originate from the C-terminal as well as from the N-terminal part of the α -subunit, since in both cases it will overlap Asp³⁸⁶. However, on the basis of structural analogy with the α (1)-subunit of Na⁺,K⁺-ATPase [24] we then concluded that the 67 kDa tryptic-digest fragment of H⁺,K⁺-ATPase was most probably on the C-terminal part, with Arg²⁷⁹ as the putative cleavage site. Although two of six amino acid assignments were uncertain (represented in Table 1 by X), we unequivocally confirm in the present study the C-terminal origin of the 56 kDa product by

Fig. 1. Western-blot staining of gastric H⁺,K⁺-ATPases by monoclonal antibody 5-B6

Pig H⁺,K⁺-ATPase (lane 1, 3 μ g), rat H⁺,K⁺-ATPase (lane 2, 3 μ g) and rabbit kidney Na⁺,K⁺-ATPase (lane 3, 5 μ g) were run on a 10% Laemmli gel and then transferred to Immobilon membranes. The blot on the left (a), containing also molecular-mass marker proteins of 94, 67, 43, 30 and 20.1 kDa, was stained with Coomassie Blue. The blot on the right (b) was stained with antibody 5-B6.

Table 1. *N*-Terminal sequences of proteolytic-digest fragments from pig gastric H⁺,K⁺-ATPase

The sequences were determined by automated Edman degradation and aligned to the pig H⁺,K⁺-ATPase amino acid sequence [7]. X represents uncertain amino acid assignments where less than 10 pmol of amino acid phenylthiohydantoin derivative was detected.

Fragment	Residue	<i>N</i> -Terminal sequence
67 kDa	48	XMEINDXQLSV EMEINDHQLSV
56 kDa	456	XVIXDA IVIGDA
27 kDa	379	XTXVIXXD STSVICSD

means of automated Edman degradation of the *N*-terminal part of this fragment and comparison with the known sequence of pig gastric H⁺,K⁺-ATPase. The 56 kDa fragment starts at Ile⁴⁵⁶, indicating that in the presence of K⁺ trypsin preferentially cleaves the α -subunit of H⁺,K⁺-ATPase at Arg⁴⁵⁵ (tryptic-cleavage site T1 of Na⁺,K⁺-ATPase). Interestingly, the *N*-terminal sequence of the 67 kDa fragment aligns with an *N*-terminal sequence of the α -subunit of H⁺,K⁺-ATPase starting at Glu⁴⁸. Our results demonstrate that in the presence of ATP the α -subunit of H⁺,K⁺-ATPase is preferentially split at Lys⁴⁷ (tryptic-cleavage site T2 on Na⁺,K⁺-ATPase) and not at Arg²⁷⁹ (tryptic-cleavage site T3 on Na⁺,K⁺-ATPase). Fig. 2 summarizes these findings in a linear polypeptide map of the catalytic subunit of H⁺,K⁺-ATPase. This map shows besides the tryptic-cleavage sites also the phosphorylation site and the FITC-reaction site. In this map the position of the third tryptic-cleavage site, which gives rise to the 35 kDa fragment, is presumed to be at Lys⁶⁶⁸. This would be the site trypsin preferentially attacks, since there are two basic amino acid residues (Arg⁶⁶⁷ and Lys⁶⁶⁸) next to each other. Indications for such a preference are given by the finding that the tryptic-cleavage sites T1 and T2 of Na⁺,K⁺-ATPase also have such a pair of basic amino acid residues [24]. The Na⁺,K⁺-ATPase however, has only one basic amino acid residue in the comparable sequence near to residue 650, which would explain why this enzyme has no restricted tryptic-cleavage site in this region.

Prolonged papain digestion of gastric H⁺,K⁺-ATPase produces a soluble 28 kDa peptide fragment that has both the

Fig. 2. Tryptic- and papain-digestion sites on the catalytic subunit of H⁺,K⁺-ATPase

The Figure shows a linear polypeptide map of the catalytic subunit of the pig enzyme. Besides Asp³⁸⁶, which is phosphorylated during catalysis (P), and Lys⁵¹⁸, which can be modified by FITC, the specific tryptic-digestion sites in either the presence of ATP or in the presence of K⁺ as well as the digestion sites of papain are shown. The indicated molecular masses of the fragments are the apparent molecular masses observed on SDS/PAGE gels.

Fig. 3. Western-blot staining of proteolytic-digest fragments of gastric H⁺,K⁺-ATPases by monoclonal antibody 5-B6

Pig gastric H⁺,K⁺-ATPase (lane 1) was digested with trypsin in the presence of K⁺ (lane 2) or ATP (lane 3) and with papain (lane 4) as described in the Experimental section. Samples were run on a 10% Laemmli gel and transferred to Immobilon membranes. The blot on the left (a) was stained with Coomassie Blue, and molecular-mass markers are 94, 67, 43, 30 and 20.1 kDa. The blot on the right (b) was stained with monoclonal antibody 5-B6.

phosphorylation and the FITC-reaction site [18]. Because of these characteristic sites, and because of its solubility, the fragment is believed to originate from the putative large cytoplasmic domain between the fourth and the fifth transmembrane segments of the catalytic subunit. Since the exact cleavage sites for this papain digestion were also not known, the *N*-terminal sequence of this soluble peptide fragment (27 kDa in our experiments) was determined. The *N*-terminal sequence is presented in Table 1. Although four of eight amino acid residues could not be assigned, the *N*-terminal sequence could be aligned with certainty beginning at Ser³⁷⁹. The apparent molecular mass indicates that the fragment extends to about residue 620. This correlates nicely with the findings obtained by Saccomani & Mukidjam [18], who observed that the fragment contains both the phosphorylation site Asp³⁸⁶ and the FITC-reaction site Lys⁵¹⁸. The location of the 27 kDa papain-digest fragment is also shown in the linear polypeptide map of Fig. 2.

The binding of monoclonal antibody 5-B6 to the specific proteolytic-cleavage peptide fragments of the α -subunit of H⁺,K⁺-ATPase is shown in Fig. 3. In this experiment pig gastric H⁺,K⁺-ATPase (lane 1) was digested with trypsin in the presence of K⁺

Fig. 4. Formic acid cleavage of the FITC-labelled 27 kDa fragment

The purified and FITC-labelled 27 kDa fragment obtained after papain digestion of pig H^+,K^+ -ATPase (lane 1) was treated with formic acid for 48 h (lane 2) as described in the Experimental section. The samples were separated on a Tricine gel and electroblotted on to an Immobilon membrane. Panel (a) shows a Coomassie Blue stain of the Tricine gel. The lane on the far left shows molecular-mass markers of 94, 67, 43, 30, 20.1 and 14.4 kDa. Panel (b) shows the FITC labelling of the fragments. Panel (c) shows an immunostaining of the fragments with monoclonal antibody 5-B6.

Fig. 5. Formic acid-cleavage sites on the 27 kDa fragment

The Figure shows a linear polypeptide map of the catalytic subunit of pig H^+,K^+ -ATPase with the papain-digestion sites and the formic acid-cleavage sites. The indicated molecular masses of the fragments are apparent molecular masses on Tricine gel.

(lane 2) or ATP (lane 3), and with papain (lane 4) as described in the Experimental section. Samples of the native enzyme, the trypsin-treated enzyme and the purified soluble 27 kDa peptide obtained after papain digestion were separated on SDS/PAGE gels and electroblotted on to Immobilon membranes. The blot on the left, which also contained the low-molecular-mass markers, was stained with Coomassie Blue. The blot on the right was incubated with monoclonal antibody 5-B6. The antibody recognized the undigested 95 kDa α -subunit as well as the C-terminal 56 kDa fragment, the N-terminal 67 kDa fragment and the soluble 27 kDa fragment. Taking into account the location of the 56 kDa, 67 kDa and 27 kDa fragments on the linear polypeptide map of Fig. 2, this implies that the sequence beginning at Ile⁴⁵⁶ and ending near to amino acid residue 620 contains the commonly shared antigenic determinant of these fragments.

Cleavage of the 27 kDa fragment with formic acid

A more exact localization of the epitope on the sequence Ile⁴⁵⁶ to about amino acid residue 620 was carried out by further fragmentation of the purified 27 kDa fragment by specific chemical cleavage of Asp-Pro bonds with formic acid as described in the Experimental section. The 27 kDa fragment contains three of these bonds, at position Asp⁵⁰⁷, Asp⁵¹⁰ and Asp⁶⁰³. Complete fragmentation of the purified 27 kDa fragment with formic acid will therefore lead to four peptide fragments. On Tricine gels, however, the small 0.3 kDa fragment that results from cleavage at Asp⁵⁰⁷ and Asp⁵¹⁰ cannot be detected. Identification of the

fragments obtained is facilitated by using FITC labelling of Lys⁵¹⁸. The results of chemical cleavage with formic acid of the FITC-labelled and purified 27 kDa fragment and staining with monoclonal antibody 5-B6 are shown in Fig. 4. In this experiment the FITC-labelled 27 kDa fragment (lane 1) was treated with formic acid for 48 h and the fragments obtained (lane 2) were separated on a Tricine gel. The Coomassie Blue-stained gel (Fig. 4a) shows that the chemical cleavage resulted in complete disappearance of the 27 kDa fragment and in the appearance of a 17 kDa, an 11 kDa and a weakly stained 9 kDa fragment. Treatment of the 27 kDa fragment with formic acid for different times demonstrated an initial cleavage of the fragment into a 17 kDa and an 11 kDa fragment, whereafter the 11 kDa fragment was degraded to a 9 kDa fragment (results not shown). This phenomenon and the fact that only the 11 kDa and the 9 kDa fragments are FITC-labelled (Fig. 4b) demonstrate an initial cleavage of the 27 kDa FITC-labelled fragment at Asp⁵⁰⁷ and/or Asp⁵¹⁰, which results in an N-terminal 17 kDa fragment and an 11 kDa FITC-labelled C-terminal fragment. Subsequent cleavage at Asp⁶⁰³ results in degradation of the 11 kDa FITC-labelled fragment to a 9 kDa FITC-labelled fragment. Although the calculated molecular mass of 14773 Da of the N-terminal fragment (residue 379 to 507 or 510) is smaller than the apparent mass of 17 kDa determined by Tricine-gel electrophoresis, the other calculated molecular masses of 11 kDa (residue 507 or 510 to about 620) and 9 kDa (residue 507 or 510 to 603) are in good agreement with the observed apparent masses (12220 and 10487 Da respectively). A summary of this cleavage by formic acid of the FITC-labelled 27 kDa fragment is shown in a linear polypeptide map in Fig. 5.

When an electrophoretic blot of this Tricine gel was stained with monoclonal antibody 5-B6, only the 27 kDa FITC-labelled fragment was recognized (Fig. 4c). Chemical modification of the epitope by formic acid leading to a loss of immunoreactivity is not very likely, since in the case of shorter treatment of the 27 kDa fragment with formic acid the remaining 27 kDa fragment is still recognized by the monoclonal antibody (results not shown). The fact that there is no staining with the 17 kDa or 11 kDa fragment suggests that the epitope includes the amino acid sequence that is initially split by formic acid: Asp⁵⁰⁷-Pro⁵⁰⁸ and/or Asp⁵¹⁰-Pro⁵¹¹. Cleavage in this region apparently leads to destruction of the epitope and thus to loss of immunoreactivity. It cannot, however, be excluded that suitable folding of another site is inhibited when the two formic acid-derived fragments are separated.

Fine specificity mapping of the epitope

Since antibody 5-B6 showed no cross-reactivity with rabbit kidney Na^+,K^+ -ATPase, the determination of the epitope could be obtained by an amino acid-by-amino acid comparison in the region of Asp⁵⁰⁷ and Asp⁵¹⁰ of H^+,K^+ -ATPases and Na^+,K^+ -ATPase. The aligned sequences of pig H^+,K^+ -ATPase, rat H^+,K^+ -ATPase and rat $\alpha 1$ Na^+,K^+ -ATPase (this species is closest to rabbit on the evolutionary scale) are presented in Fig. 6. The pig sequence begins at Asn⁴⁹³, the residue that is most likely glycosylated [9,25], and ends at Lys⁵¹⁸, the residue that reacts with FITC [15]. Besides this site of covalent modification, Fig. 6 also shows the location of the lysine residue that is specifically modified with pyridoxal 5'-phosphate [14]. The analogous lysine residue of lamb kidney Na^+,K^+ -ATPase has recently also been demonstrated to be reactive towards pyridoxal 5'-phosphate [26]. Examination of these aligned sequences revealed one stretch of residues, amino acids 504-512 of the pig sequence, which is identical in the rat enzyme, but which is nearly completely different in Na^+,K^+ -ATPase. Since the adjacent sequences show nearly 100% identity, it is very likely that the binding site for

		PLP		Formic acid		FITC
Pig H ⁺ ,K ⁺ -ATPase	493	N S T N K F Q L S I H T L E D P R D P R H V L V M K				
Rat H ⁺ ,K ⁺ -ATPase	492	N S T N K F g L S I H T L E D P R D P R H I L V M K				
Rat Na ⁺ ,K ⁺ -ATPase	478	N S T N K y Q L S I H k n p n a s e P k H I L V M K				

Fig. 6. Sequence comparison between the catalytic subunits of pig and rat H⁺,K⁺-ATPases and rat α 1 Na⁺,K⁺-ATPase

Data are from Maeda *et al.* [7], Shull & Lingrel [6] and Shull *et al.* [27]. Residues that are not identical are indicated by lower-case letters. The pyridoxal 5'-phosphate (PLP)- and FITC-reaction sites and the formic acid-cleavage sites are also shown.

antibody 5-B6 includes (part of) this essential sequence from residue 504 to 512.

DISCUSSION

Epitope of antibody 5-B6 on the catalytic subunit

The first step in the determination of the antigenic binding site on the α -subunit of gastric H⁺,K⁺-ATPase was carried out by identifying immunoreactive proteolytic peptide fragments of the α -subunit of the enzyme. This approach made identification of the restricted proteolytic sites on H⁺,K⁺-ATPase necessary. The tryptic-cleavage sites that have been identified were for the condition where ATP was present Lys⁴⁷ and probably Lys⁶⁸⁸, and for the condition where K⁺ was present Arg⁴⁵⁵. The identification of Ser³⁷⁹ as the *N*-terminus of the 27 kDa soluble fragment obtained by papain digestion showed that this fragment is almost identical with a soluble proteolytic-digest fragment of the catalytic subunit that was recently isolated by Tai *et al.* [25]. They carried out a limited tryptic digestion of FITC-labelled H⁺,K⁺-ATPase in the presence of an amphiphilic detergent. The soluble 27 kDa peptide fragment obtained this way showed not only the FITC-binding site but also concanavalin A-binding activity. Its *N*-terminal sequence began at Asn³⁶⁹ of the rat H⁺,K⁺-ATPase. Although the *N*-terminal sequence of the 27 kDa fragment obtained in the present study is different, both sequence determinations demonstrate that a large part of the putative cytoplasmic domain, which is proposed to start at Lys³⁵⁹ and to end at Glu⁷⁹⁶ [6], indeed has a cytoplasmic disposition.

The binding of antibody 5-B6 to proteolytic-digest fragments of 67 kDa, 56 kDa and 27 kDa made it possible to limit the epitope to a sequence beginning at Ile⁴⁵⁶ and ending near to residue 620. Further chemical cleavage of the 27 kDa fragment with formic acid then showed that there was loss of immunoreactivity upon the primary cleavage of Asp-Pro bonds at residue 507 and/or 510. An amino acid-by-amino acid comparison between the region of initial formic acid cleavage in H⁺,K⁺-ATPase and the similar region in non-immunoreactive Na⁺,K⁺-ATPase showed large differences, in contrast with the adjacent regions of both enzymes. The sequence comparison revealed a stretch of nine amino acid residues beginning at Thr⁵⁰⁴ and ending at Arg⁵¹² that is, except for Pro⁵¹¹, completely different in rat kidney Na⁺,K⁺-ATPase. Because of its hydrophilicity (Thr-Leu-Glu-Asp-Pro-Arg-Asp-Pro-Arg) it is assumable that this sequence has the potency to function as a strong antigenic determinant. These data, taken together, seem to justify the conclusion that the epitope of antibody 5-B6 comprises the complete or part of the sequence from residue 504 to 512.

This sequence is located at the cytoplasmic side of the catalytic subunit, as has been demonstrated by the inhibitory action of antibody 5-B6 on the K⁺-ATPase activity of inside-out-oriented H⁺,K⁺-ATPase vesicles [16]. Therefore the topological disposition of this sequence brings new light on the discussion about the

sidedness of the *N*-linked carbohydrate moiety at Asn⁴⁹³, which shows concanavalin A-binding activity [9,25]. Hall *et al.* [9] determined by concanavalin A/colloidal-gold binding to post-embedded inside-out-oriented H⁺,K⁺-ATPase vesicles that 72 % of the reactivity towards concanavalin A was on the interior of the vesicle membranes. It was therefore concluded that Asn⁴⁹³ has an extracellular location. As there was a discrepancy in the topological disposition of Asn⁴⁹³ and Lys⁴⁹⁷, the residue that binds pyridoxal 5'-phosphate and that is protected by ATP [14], it was suggested that there is a membrane-spanning segment in the putative cytoplasmic domain that enables extracellular glycosylation to occur only four amino acid residues away from Lys⁴⁹⁷. However, a membrane-spanning segment, which will have an α -helix structure, should be at least about 20 amino acid residues long. The fact that the sequence from residue 504 to residue 512 faces the cytoplasmic side therefore suggests that the carbohydrate moiety at Asn⁴⁹³ is located at the cytoplasmic side. This topological disposition would be in much better agreement with the cytoplasmic disposition of Lys⁴⁹⁷ and Lys⁵¹⁸, chemical modification of which with pyridoxal 5'-phosphate and FITC respectively in both cases is prevented by ATP. Moreover, a cytoplasmic assignment of this glycosylated amino acid residue, which would be contradictory to the current models of glycosylation of membrane-bound proteins, has recently also been demonstrated for the catalytic subunit of Na⁺,K⁺-ATPase [28].

Tryptic cleavage of H⁺,K⁺-ATPase

Digestion of gastric H⁺,K⁺-ATPase by trypsin is known to occur at restricted sites [17]. The more precise identification of specific tryptic-cleavage sites, described in the present study, makes it possible to correlate the time courses of the biphasic inactivation of the K⁺-ATPase activity, which is observed during tryptic digestion in the presence of both K⁺ and ATP [17], with the appearance of well-defined peptide fragments on SDS/PAGE gels. During the initial rapid phase of tryptic inactivation of the K⁺-ATPase activity, neither the K⁺-stimulated *p*-nitrophenyl phosphatase activity nor the phosphorylation capacity of the enzyme was inactivated. It was therefore concluded that it is the interconversion from E₁P into E₂P that is interrupted during this rapid phase [17].

In the presence of ATP, which induces the E₁ conformation, tryptic cleavage occurs at Lys⁴⁷ and probably at Lys⁶⁸⁸. The latter residue is part of a highly conserved sequence in P-type ATPases [29] that forms part of the ATP-binding domain [30]. Although the second tryptic-cleavage site at Lys⁶⁸⁸ is not definitely determined, there are two arguments for this site, as follows. (1) Tryptic cleavage at this site will result in the observed fragments with apparent masses of 35 and 67 kDa, the latter containing the aspartic acid residue that is phosphorylated by ATP [17]. (2) High ATP concentrations protect against tryptic inactivation [17]. Such a shielding effect of nucleotides has also been demonstrated for the chymotryptic-cleavage site and the tryptic-cleavage site T3 on Na⁺,K⁺-ATPase [31]. Tryptic cleavage at

these two tryptic-cleavage sites in the E_1 conformation of the enzyme explains the observation that the K^+ -ATPase activity is inhibited in a biphasical manner [17]. Since the time course of formation of the 35 kDa and 67 kDa fragment corresponds to the rate of the slow phase of inactivation of the K^+ -ATPase activity [17], it can be concluded that tryptic cleavage at Lys⁶⁶⁸ is responsible for the slow phase of inactivation of the K^+ -ATPase activity, i.e. inactivation of phosphorylation capacity and K^+ -stimulated *p*-nitrophenyl phosphatase activity.

Since the sum of the masses of the 35 kDa and 67 kDa fragments virtually equals the apparent mass of 95 kDa of the undigested α -subunit, the initial rapid phase of inactivation of the K^+ -ATPase activity should be caused by splitting off a small fragment from the α -subunit. Although this initial tryptic cleavage can not be seen on the SDS/PAGE gels because of poor resolution [17], the presence of this site is confirmed in the present study by the finding that the *N*-terminus of the 67 kDa fragment begins at Glu⁴⁸. Therefore the rapid phase of inactivation of the K^+ -ATPase activity, caused by interrupting the interconversion from E_1P into E_2P [17], very probably can be ascribed to cleavage at Lys⁴⁷. This would be in agreement with Na^+, K^+ -ATPase, where cleavage of the tryptic-cleavage site T2 at the *N*-terminus of the catalytic subunit, corresponding to the Lys⁴⁷ of pig H^+, K^+ -ATPase, also affects the conformational transition from E_1P to E_2P as well as the transition from E_1 to E_2 [24].

We thank Dr. W. W. De Jong for his valuable suggestion to apply the formic acid cleavage method and Dr. P. H. G. M. Willems for his help in the preparation of this manuscript.

REFERENCES

- Rabon, E. C. & Reuben, M. A. (1990) *Annu. Rev. Physiol.* **52**, 321–344
- Pedersen, P. L. & Carafoli, E. (1987) *Trends Biochem. Sci.* **12**, 146–150
- Pedersen, P. L. & Carafoli, E. (1987) *Trends Biochem. Sci.* **12**, 186–189
- Sachs, G., Chang, H. H., Rabon, E., Schackmann, R., Lewin, M. & Saccomani, G. (1976) *J. Biol. Chem.* **251**, 7690–7698
- Skrabanja, A. T. P., De Pont, J. J. H. H. M. & Bonting, S. L. (1984) *Biochim. Biophys. Acta* **774**, 91–95
- Shull, G. E. & Lingrel, J. B. (1986) *J. Biol. Chem.* **261**, 16788–16791
- Maeda, M., Ishizaki, J. & Futai, M. (1988) *Biochem. Biophys. Res. Commun.* **157**, 203–209
- Maeda, M., Oshiman, K., Tamura, S. & Futai, M. (1990) *J. Biol. Chem.* **265**, 9027–9032
- Hall, K., Perez, G., Anderson, D., Gutierrez, C., Munson, K., Hersey, S. J., Kaplan, J. H. & Sachs, G. (1990) *Biochemistry* **29**, 701–706
- Okamoto, C. T., Karpilow, J. M., Smolka, A. & Forte, J. G. (1990) *Biochim. Biophys. Acta* **1037**, 360–372
- Shull, G. E. (1990) *J. Biol. Chem.* **265**, 12123–12126
- Toh, B.-H., Gleeson, P. A., Simpson, R. J., Moritz, R. L., Callaghan, J. M., Goldkorn, I., Jones, C. M., Martinelli, T. M., Mu, F.-T., Humphris, D. C., Pettitt, J. M., Mori, Y., Masuda, T., Sobieszczuk, P., Weinstock, J., Mantamadiotis, T. & Baldwin, G. S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6418–6422
- Reuben, M. A., Lasater, L. S. & Sachs, G. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6767–6771
- Tamura, S., Tagaya, M., Maeda, M. & Futai, M. (1989) *J. Biol. Chem.* **264**, 8580–8584
- Farley, R. A. & Faller, L. D. (1985) *J. Biol. Chem.* **260**, 3899–3901
- Van Uem, T. J. F., Peters, W. H. M. & De Pont, J. J. H. H. M. (1990) *Biochim. Biophys. Acta* **1023**, 56–62
- Helmich-De Jong, M. L., Van Ermat-De Vries, S. E. & De Pont, J. J. H. H. M. (1987) *Biochim. Biophys. Acta* **905**, 358–370
- Saccomani, G. & Mukidjam, E. (1987) *Biochim. Biophys. Acta* **912**, 63–73
- Jackson, R. J., Mendlein, J. & Sachs, G. (1983) *Biochim. Biophys. Acta* **731**, 9–15
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Jauregui-Adell, J. & Marti, J. (1975) *Anal. Biochem.* **69**, 468–473
- Schägger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
- Jørgensen, P. L. & Collins, J. H. (1986) *Biochim. Biophys. Acta* **860**, 570–576
- Tai, M. M., Im, W. B., Davis, J. P., Blakeman, D. P., Zürcher-Neely, H. A. & Heinrikson, R. L. (1989) *Biochemistry* **28**, 3183–3187
- Hinz, H. R. & Kirley, T. L. (1990) *J. Biol. Chem.* **265**, 10260–10265
- Shull, G. E., Greeb, J. & Lingrel, J. B. (1986) *Biochemistry* **25**, 8125–8132
- Pedemonte, C. H., Sachs, G. & Kaplan, J. H. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9789–9793
- Green, N. M. & MacLennan, D. H. (1989) *Biochem. Soc. Trans.* **17**, 819–822
- Ohta, T., Nagano, K. & Yoshida, M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2071–2075
- Jørgensen, P. L. & Petersen, J. (1985) *Biochim. Biophys. Acta* **821**, 319–333

Received 5 March 1991/1 May 1991; accepted 31 May 1991