

# The functional roles of disulfide bonds in the $\beta$ -subunit of (Na,K)ATPase as studied by site-directed mutagenesis

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Received 21 June 1993; revised version received 7 February 1994

## Abstract

The  $\beta$ -subunit of *Torpedo californica* (Na,K)ATPase contains seven cysteine residues; one (Cys<sup>46</sup>) is in the single transmembrane segment and the other six (Cys<sup>127</sup>, Cys<sup>150</sup>, Cys<sup>160</sup>, Cys<sup>176</sup>, Cys<sup>215</sup> and Cys<sup>278</sup>) are in the extracellular domain and form three highly conserved disulfide bonds. A  $\beta$ -subunit mutant with replacement of Cys<sup>46</sup> by Ser could assemble with the  $\alpha$ -subunit, and the resulting  $\alpha\beta$ -complex was catalytically active. Mutants in which either the N-terminal side or both Cys residues of the Cys<sup>127</sup>–Cys<sup>150</sup> bond were replaced by Ser could also tightly assemble with the  $\alpha$ -subunit, but the resulting  $\alpha\beta$ -complex was catalytically inactive. On the other hand, disruption of either the Cys<sup>160</sup>–Cys<sup>176</sup> or Cys<sup>215</sup>–Cys<sup>278</sup> bond by substituting the N-terminal side only or both Cys residues with Ser led to a  $\beta$ -subunit that could not assemble with the  $\alpha$ -subunit. We conclude that the structure of the  $\beta$ -subunit around the Cys<sup>160</sup>–Cys<sup>176</sup> and Cys<sup>215</sup>–Cys<sup>278</sup> loops is indispensable for assembly with the  $\alpha$ -subunit, whereas the Cys<sup>127</sup>–Cys<sup>150</sup> loop is not essential for assembly but is required for enzyme activity.

**Key words:** (Na,K)ATPase;  $\beta$ -Subunit; Disulfide bond; Site-directed mutagenesis

## 1. Introduction

The ion-transporting (Na,K)ATPase of animal plasma membranes consists of  $\alpha$ - and  $\beta$ -subunits: all the known catalytic functions of the enzyme are associated with the  $\alpha$ -subunit [1]. Although the functional role of the  $\beta$ -subunit, which is glycosylated, is not yet fully understood, there is evidence that it is required for biogenesis of the enzyme [2,3]. The  $\beta$ -subunit stabilizes the nascent  $\alpha$ -subunit in the endoplasmic reticulum [4–6] and is also thought to play a role in targeting the  $\alpha\beta$ -complex to the plasma membrane [7–10].

The  $\beta$ -subunit spans the membrane once, leaving a short N-terminal segment in the cytoplasm and, therefore, the large C-terminal domain is exposed to the extracellular side [11]. This subunit (isoform  $\beta$ 1) contains seven conserved cysteine residues. One (Cys<sup>46</sup>) is located in the transmembrane segment and the other six, which are in the extracellular domain, form three highly conserved disulfide bonds, i.e. Cys<sup>127</sup>–Cys<sup>150</sup>, Cys<sup>160</sup>–Cys<sup>176</sup> and Cys<sup>215</sup>–Cys<sup>278</sup> [12,13] (numbering refers to *Torpedo californica* (Na,K)ATPase  $\beta$ -subunit), the reduction of which leads to the loss of the enzyme activity [14,15].

In this study, site-directed mutagenesis was used to disrupt each of the three disulfide bonds by substituting one or both cysteine residues with serine. It was thus found that the mutant  $\beta$ -subunit in which the Cys<sup>127</sup>–Cys<sup>150</sup> bond was disrupted could assemble with the  $\alpha$ -subunit, but the resulting  $\alpha\beta$ -complex showed little enzyme activity. On the other hand, disruption of either the Cys<sup>160</sup>–Cys<sup>176</sup> or Cys<sup>215</sup>–Cys<sup>278</sup> bond produced the  $\beta$ -subunit that lacks the ability to form a complex with the  $\alpha$ -subunit. It is concluded that the highly conserved disulfide bonds of the  $\beta$ -subunit are important for the correct assembly with the  $\alpha$ -subunit.

## 2. Materials and methods

### 2.1. Materials

Rabbit antisera raised against the  $\alpha$  and  $\beta$ -subunits purified from the electric organ of *T. californica* (Na,K)ATPase were described previously [2]. Enzymes used for site-directed mutagenesis and in vitro transcription were purchased from TaKaRa, Nippon Gene and Toyobo. Other chemicals and biochemicals used were obtained from Nacalai Tesque Inc. and were of reagent or higher grade.

### 2.2. Site-directed mutagenesis

Template plasmids for mutation were constructed from pSPT $\beta$  containing the entire coding region of the  $\beta$ -subunit of *T. californica* ((Na,K)ATPase [16], as shown in Fig. 1. The oligonucleotide primers used for mutagenesis were synthesized in an Applied Biosystems Model 380A DNA synthesizer. Site-directed mutagenesis was performed according to the kit supplier's instructions (Amersham, RPN.1523). For double mutants, mutagenesis was repeated twice. The gene fragments carrying the mutations were cut from pUC119 and used to replace the corresponding segment of the  $\beta$ -subunit cDNA that had been inserted into pSP65.

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### 2.3. Expression in *Xenopus* oocytes

The plasmid carrying the wild-type or mutated cDNA for the  $\alpha$ - or  $\beta$ -subunit was linearized and transcribed in vitro with the aid of SP6 RNA polymerase. The mRNA thus synthesized was injected into *Xenopus laevis* oocytes as described previously [6]. The concentration of mRNA was 0.5  $\mu\text{g}/\mu\text{l}$  for each mRNA, and 20 nl, on average, was injected per oocyte. The oocytes were incubated at 19°C for 3 days in modified Barth's medium containing 100  $\mu\text{g}/\text{ml}$  each of ampicillin, streptomycin and cefmonexime, and 15  $\mu\text{g}/\text{ml}$  of nystatin. The incubation medium also contained L-[U-<sup>14</sup>C]leucine (312 mCi/mmol, 0.15 mCi/ml) to label the translation products.

### 2.4. Immunoprecipitation of translation products

Eighteen labeled oocytes were homogenized. The homogenate was brought to 1% with respect to Triton X-100 and centrifuged at 160,000  $\times g$  for 30 min. Anti- $\alpha$ -subunit or anti- $\beta$ -subunit antiserum or both were added to the supernatant and the mixture was incubated at 4°C for 8 h. Then protein A-Sepharose CL-4B beads were added to adsorb the immunoprecipitate. The beads carrying the immunoprecipitate were collected, washed, and analyzed by SDS-PAGE (0.1% SDS, 10% polyacrylamide gel).

### 2.5. Other methods

For ATPase assay, about 400 oocytes were homogenized, microsomes were isolated from the homogenate and treated with 1 M NaSCN, as described before [6]. ATPase activity was measured in a reaction mixture (0.2 ml) containing 50 mM imidazole/HCl buffer (pH 7.5), 140 mM NaCl, 14 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM ATP and microsomes (20–80  $\mu\text{g}$  protein) in the presence and absence of 1 mM ouabain. (Na,K)ATPase activity was obtained by subtracting the activity measured in the presence of ouabain from that measured in its absence. Limited trypsinolysis was performed as described previously [6].

## 3. Results

As mentioned earlier, Cys<sup>46</sup> of isoform  $\beta$ 1 of the  $\beta$ -subunit of *T. californica* (Na,K)ATPase is the only cysteine residue that is not disulfide bonded and is located in the transmembrane segment. We first examined the effect of substitution of Cys<sup>46</sup> with serine ( $\beta$ C46S) on its ability to assemble with the  $\alpha$ -subunit. To this end, *Xenopus* oocytes were injected with both  $\beta$ C46S mRNA $\beta$  and wild-type mRNA $\alpha$  (mRNA $\alpha$  and mRNA $\beta$  refer to mRNAs for the  $\alpha$ - and  $\beta$ -subunits, respectively), incubated at 19°C for 3 days, and assayed for translation products by immunoprecipitation followed by SDS-PAGE and fluorography. As shown in Fig. 2, the mutant  $\beta$ -subunit (both fully glycosylated  $\beta$ m and core glycosylated  $\beta$ c) and the wild-type  $\alpha$ -subunit were synthesized in the oocytes (lanes 1–3) in nearly the same fashion as upon injection of wild-type mRNA $\beta$  and wild-type mRNA $\alpha$  (lanes 4–6). In confirmation of our previous finding [6], much larger amounts of the  $\alpha$ -subunit were detected in oocytes injected with both mRNA $\alpha$  and  $\beta$ C46S mRNA $\beta$  than in those injected with mRNA $\alpha$  alone (data not shown, but see Fig. 4, lane 10), indicating that this mutant  $\beta$ -subunit could stabilize the  $\alpha$ -subunit. Anti- $\beta$ -subunit antiserum could precipitate not only the  $\beta$ -subunit but also the  $\alpha$ -subunit, though only slightly (lanes 3 and 6). This poor precipitation of the  $\alpha$ -subunit is thought to be due to the dissociation of the  $\alpha\beta$ -complex by the 1% Triton X-100 used for immunoprecipitation.

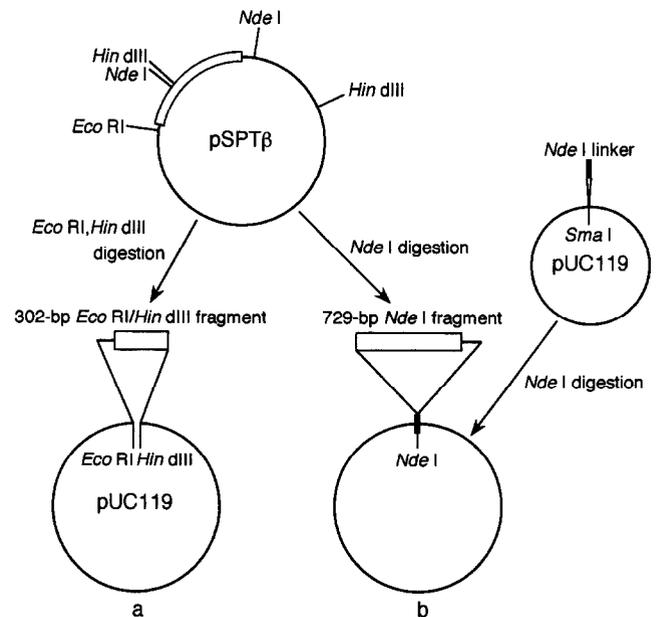


Fig. 1. Construction of template plasmids for mutagenesis. Open and closed boxes indicate the coding sequence for the  $\beta$ -subunit of *T. californica* (Na,K)ATPase and *Nde*I linker, respectively. Plasmid a was used for construction of  $\beta$ C46S. For all the other mutations, plasmid b was used.

These results suggest that the  $\beta$ C46S mutant subunit could assemble with the  $\alpha$ -subunit and thereby stabilize it. As shown in Table 1, ouabain-sensitive ATPase activity of microsomes from the oocytes injected with both wild-type mRNA $\alpha$  and  $\beta$ C46S mRNA $\beta$  was comparable with that obtained after injection of both wild-type mRNA $\alpha$  and wild-type mRNA $\beta$ . These activities were significantly higher than the value determined for the uninjected control. We conclude that the  $\beta$ C46S mutant has no functional defect. This is in agreement with the finding that the  $\beta$ -subunit isoforms,  $\beta$ 2 and  $\beta$ 3, that lack

Table 1  
(Na,K)ATPase activity of *Xenopus* oocytes injected with wild-type or mutant mRNA $\beta$  together with wild-type mRNA $\alpha$

mRNA $\beta$ injected	Ouabain-sensitive ATPase activity ( $\mu\text{mol P}_i/\text{mg protein/h}$ )		
	Exp. 1	Exp. 2	Exp. 3
Wild-type	4.2 (2.1)	3.3 (1.0)	2.5 (0.7)
$\beta$ C46S	4.9 (2.8)	4.0 (1.7)	3.2 (1.4)
$\beta$ C127S	2.4 (0.3)	2.3 (0)	1.6 (–0.2)
$\beta$ C127/150S	2.6 (0.5)	2.4 (0.1)	1.8 (0)
Uninjected	2.1 (–)	2.3 (–)	1.8 (–)

*Xenopus* oocytes were injected with the indicated mRNA $\beta$  (10 ng/oocyte) together with wild-type mRNA $\alpha$  (10 ng/oocyte) and incubated at 19°C for 3 days. Microsomes were isolated from the oocytes, treated with 1 M NaSCN, and assayed for (Na,K)ATPase (ouabain-sensitive ATPase) activity as described previously [6]. The figures in parentheses are the activities after subtraction of the activity of microsomes from uninjected oocytes.

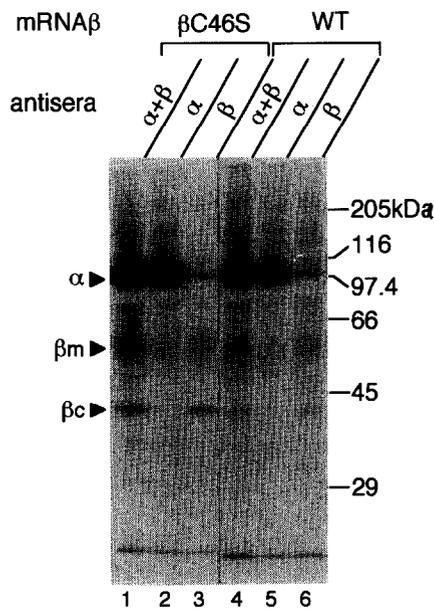


Fig. 2. Functional expression of  $\beta$ C46S mutant.  $\beta$ C46S mRNA $\beta$  (10 ng/oocyte) (lanes 1–3) or wild-type mRNA $\beta$  (10 ng/oocyte) (lane 4–6) of *T. californica* (Na,K)ATPase was injected into *Xenopus* oocytes together with wild-type mRNA $\alpha$  (10 ng/oocyte). After incubation at 19°C for 3 days in the presence of [ $^{14}$ C]leucine, the oocytes were homogenized in the presence of 1% Triton X-100. The labeled translation products were immunoprecipitated with anti- $\alpha$ -subunit antiserum (lanes 2 and 5), anti- $\beta$ -subunit antiserum (lanes 3 and 6), or a mixture of both antisera (lanes 1 and 4). The immunoprecipitate was subjected to SDS-PAGE and the subsequent fluorography.  $\beta$ m and  $\beta$ c indicate fully glycosylated and core glycosylated  $\beta$ -subunit, respectively.

the corresponding cysteine residue in the transmembrane segment, can assemble with the  $\alpha$ -subunit to form the fully active  $\alpha\beta$ -complex [17,18].

To study the roles of the three extracellular disulfide bonds of the  $\beta$ -subunit, each of these bonds was disrupted by substituting the N-terminal side only or both cysteine residues with serine. Thus we constructed the following six mutants:  $\beta$ C127S,  $\beta$ C127/150S,  $\beta$ 160S,  $\beta$ 160/178S,  $\beta$ C215S and  $\beta$ C215/276S. As shown in Fig. 3, in oocytes injected with  $\beta$ C127S or  $\beta$ C127/150S mRNA $\beta$  together with wild-type mRNA $\alpha$ , anti- $\beta$ -subunit antiserum precipitated the  $\alpha$ -subunit though only slightly (for the same reason as mentioned above) (lanes 3 and 6). The amounts of the  $\alpha$ -subunit precipitated with anti- $\alpha$ -subunit antiserum (lanes 2 and 5) were larger and nearly the same as detected in the oocytes co-injected with wild-type mRNA $\beta$  (lane 20). It is, therefore, evident that the  $\beta$ C127S and  $\beta$ C127S/150S subunits, like the wild-type  $\beta$ -subunit, could assemble with and stabilize the  $\alpha$ -subunit. However, microsomes from the oocytes injected with  $\beta$ C127S or  $\beta$ C127/150S mRNA $\beta$ , together with wild-type mRNA $\alpha$ , showed practically no ouabain-sensitive ATPase activity (Table 1). In contrast to  $\beta$ C127S and  $\beta$ C127/150S, the products of the other four mutant RNAs ( $\beta$ C160,  $\beta$ C160/176S,  $\beta$ C215 and  $\beta$ C215/278S) could neither assemble with nor stabilize the  $\alpha$ -

subunit (lanes 7–18). The faint  $\alpha$ -subunit bands detected in the oocytes injected with these four mutant mRNA $\beta$ s together with mRNA $\alpha$  (lanes 7, 8, 10, 11, 13, 14, 16 and 17) were due to the  $\alpha$ -subunit that had been extensively degraded because of the lack of stabilization by the  $\beta$ -subunit. The oocytes injected with mRNA $\alpha$  alone also showed a similar, faint band of the  $\alpha$ -subunit (see Fig. 4, lane 10). No ouabain-sensitive ATPase activity could be detected in microsomes from the oocytes injected with the mutant mRNA $\beta$  together with wild-type mRNA $\alpha$  (data not shown).

As shown in Fig. 4, the  $\alpha$ -subunit in the oocytes injected with wild-type mRNA $\alpha$  plus  $\beta$ C160S mRNA was completely digested by trypsinolysis even at a protein:trypsin ratio (w/w) of 100:1 (lanes 7–9). The  $\alpha$ -subunit in the oocytes injected with mRNA $\alpha$  plus  $\beta$ C127S or  $\beta$ C127/150S mRNA $\beta$ , on the other hand, was resistant to trypsin (lanes 1–6). It is thus evident that the assembly-competent  $\beta$ -subunit can confer trypsin-resistance on the  $\alpha$ -subunit.

#### 4. Discussion

The results described above indicate that the intactness of the structure of the C-terminal portion of the  $\beta$ -subunit, including the Cys $^{160}$ –Cys $^{176}$  and Cys $^{215}$ –Cys $^{278}$  loops, is essential for the subunit to assemble with and stabilize the  $\alpha$ -subunit, whereas disruption of the Cys $^{127}$ –Cys $^{150}$  bond does not affect the ability of the  $\beta$ -subunit to assemble with and stabilize the  $\alpha$ -subunit.

Although the reason for the importance of the Cys $^{160}$ –Cys $^{176}$  and Cys $^{215}$ –Cys $^{278}$  loops, for the assembly is not yet known, it is to be noted that the sequences of these loops in isoform  $\beta$ 1 of the  $\beta$ -subunit are significantly conserved among *Torpedo* [16], human [19], sheep [20], dog [21], pig [22], rat [23], chicken [24] and *Xenopus* [25]. It can be seen in Fig. 5 that the sequences of the Cys $^{160}$ –Cys $^{176}$  and Cys $^{215}$ –Cys $^{278}$  loops are 47% and 51% identical, respectively, among these species (the overall identity of isoform  $\beta$ 1 sequences is at most 40%). Moreover, 6 residues in the Cys $^{160}$ –Cys $^{176}$  loop (Ser $^{161}$ , Gly $^{162}$ , Asp $^{165}$ , Gly $^{169}$ , Gly $^{173}$  and Pro $^{175}$ ) and 11 residues in the Cys $^{215}$ –Cys $^{278}$  loop (Gly $^{239}$ , Leu $^{242}$ , Tyr $^{247}$ , Tyr $^{244}$ , Pro $^{246}$ , Tyr $^{248}$ , Gly $^{249}$ , Tyr $^{256}$ , Pro $^{259}$ , Leu $^{260}$  and Asn $^{267}$ ) are not only conserved among  $\beta$ 1 and  $\beta$ 2 isoforms of the (Na,K)ATPase  $\beta$ -subunit [26,27] but are also identical to the corresponding residues of the  $\beta$ -subunit of (H,K)ATPase [28–32]. The  $\beta$ -subunit of (H,K)ATPase has been shown to assemble with the  $\alpha$ -subunit of (Na,K)ATPase to form a stable, trypsin-resistant  $\alpha\beta$ -complex [33,34], probably because of the similarity of the sequences of the Cys $^{160}$ –Cys $^{176}$  and Cys $^{215}$ –Cys $^{278}$  loops between the  $\beta$ -subunits of the two ATPases. On the other hand, the sequence of the Cys $^{127}$ –Cys $^{150}$  loop of isoform  $\beta$ 1 is only 33% conserved among the species examined and there is only one residue

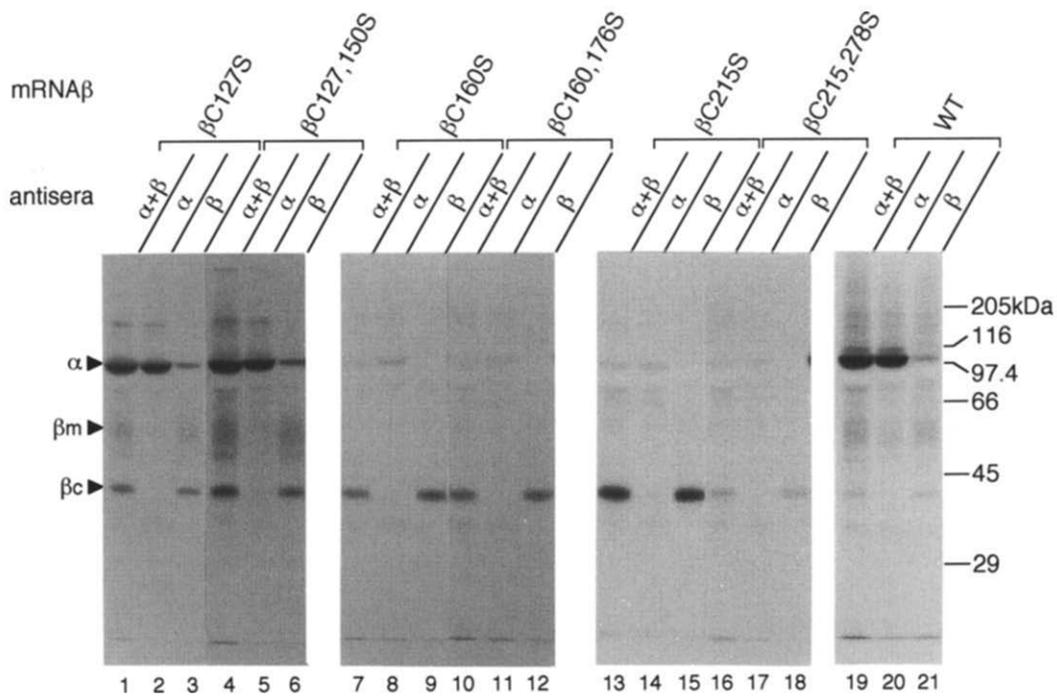


Fig. 3. Translation products produced in *Xenopus* oocytes injected with wild-type mRNA $\alpha$  (10 ng/oocyte) plus mutant mRNA $\beta$  (10 ng/oocyte). The mutant mRNA $\beta$ s injected were  $\beta$ C127S (lanes 1–3),  $\beta$ C127/150S (lanes 4–6),  $\beta$ C160S (lanes 7–9),  $\beta$ C160/176S (lanes 10–12),  $\beta$ C215S (lanes 13–15), and  $\beta$ C215/278S (lanes 16–18). For lanes 19–21, wild-type mRNA $\beta$  was injected together with mRNA $\alpha$ . The translation products were immunoprecipitated and analyzed as described in Fig. 2.

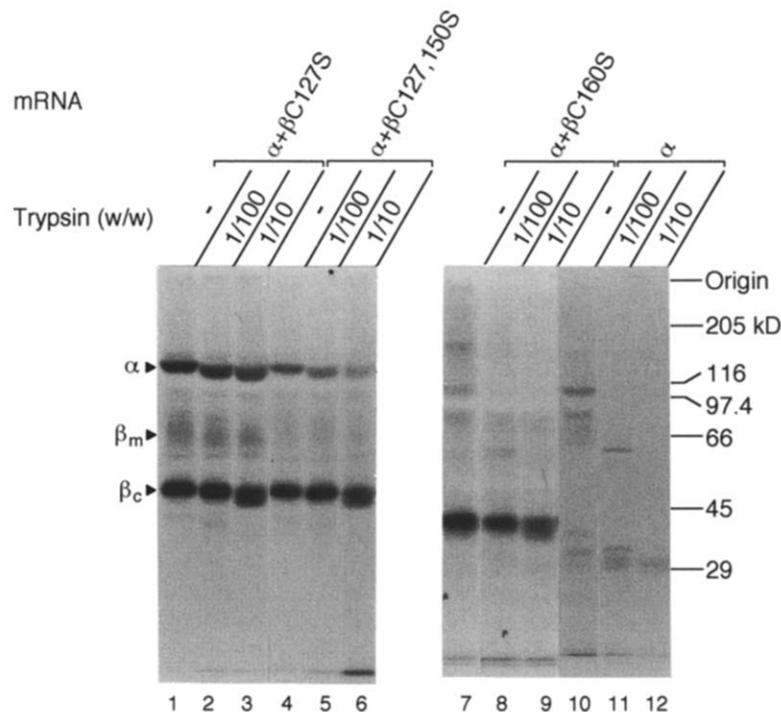


Fig. 4. Trypsin sensitivity of the  $\alpha$ -subunit expressed in *Xenopus* oocytes injected with wild-type mRNA $\alpha$  (10 ng/oocyte) together with mutant mRNA $\beta$  (10 ng/oocyte). Lanes: 1–3,  $\beta$ C127S mRNA $\beta$  plus mRNA $\alpha$ ; 4–6,  $\beta$ C127/150S mRNA $\beta$  plus mRNA $\alpha$ ; 7–9,  $\beta$ C160S mRNA $\beta$  plus mRNA $\alpha$ ; 10–12, mRNA $\alpha$  alone. Microsomes prepared from each set of oocytes were digested with trypsin (Worthington; L-1-tosylamide- $\alpha$ -phenylethyl-chloromethyl ketone-treated) at a trypsin : protein ratio (w/w) of 0 (lanes 1, 4, 7 and 10), 1/100 (lanes 2, 5, 8 and 11), and 1/10 (lanes 3, 6, 9 and 12) for 60 min on ice. The digestion was stopped by adding soybean trypsin inhibitor at a trypsin : inhibitor ratio (w/w) of 1/10 and microsomes were analyzed by immunoprecipitation with a mixture of anti- $\alpha$ -subunit and anti- $\beta$ -subunit antisera followed by SDS-PAGE and fluorography.

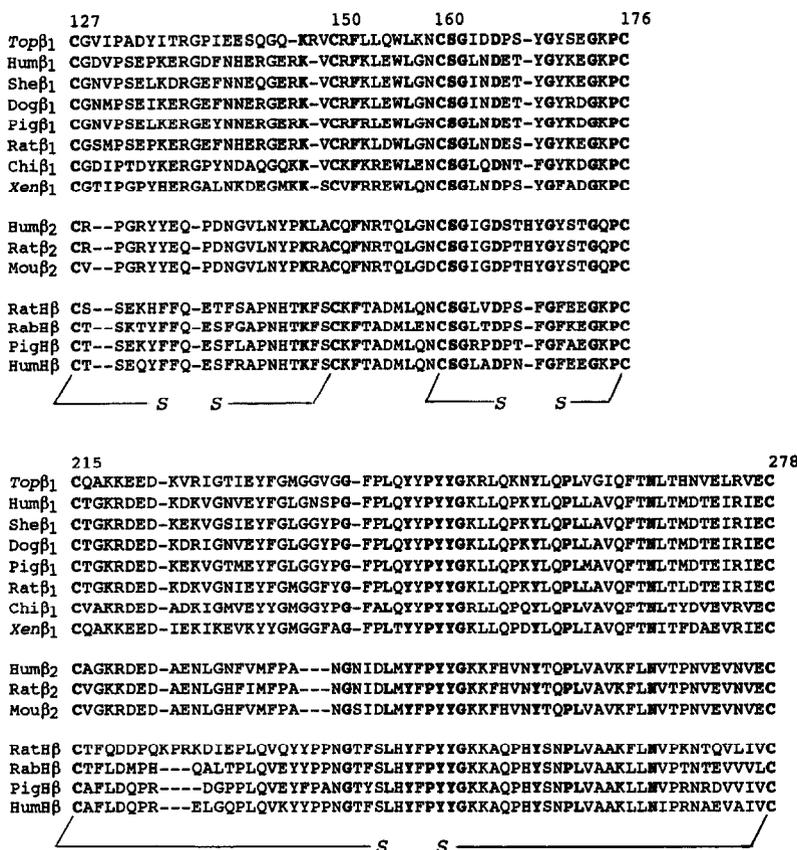


Fig. 5. Alignment of amino acid sequences within disulfide loops in the  $\beta$ -subunit of (Na,K)ATPase and (H,K)ATPase. (Top panel *Torpedo*; Hum, human; She, sheep; Dog, dog; Pig, pig; Rat, rat; Chi, chicken; Mou, mouse; Rab, rabbit; Xen, *Xenopus*.  $\beta 1$  and  $\beta 2$  indicate isoforms  $\beta 1$  and  $\beta 2$ , respectively, of the  $\beta$ -subunit of (Na,K)ATPase.  $H\beta$  indicates the  $\beta$ -subunit of (H,K)ATPase. Numbering refers to isoform  $\beta 1$  of the  $\beta$ -subunit of *T. californica* (Na,K)ATPase. Residues identical in all the sequences shown are indicated by bold letters.

(Lys<sup>147</sup>) that is identical in the  $\beta$ -subunit ( $\beta 1$  and  $\beta 2$ ) of (Na,K)ATPase and the  $\beta$ -subunit of (H,K)ATPase.

The reason why the stable, trypsin-resistant complex of the  $\beta C127S$  or  $\beta C127/150S$  subunit with wild-type  $\alpha$ -subunit possesses no catalytic activity is not clear, but a likely possibility is that the interaction of the Cys<sup>127</sup>–Cys<sup>150</sup> loop with the  $\alpha$ -subunit is critical for the catalytic activity. This possibility is also supported by the finding that the  $\beta$ -subunit of (H,K)ATPase, the sequence of which in this loop is quite different from that of the  $\beta$ -subunit of (Na,K)ATPase, forms a stable complex with the  $\alpha$ -subunit of (Na,K)ATPase, but this hybrid is catalytically inactive [34]. (It is to be noted that this hybrid exhibits a low pump activity according to Horisberger et al. [33]). Probably a specific sequence in the Cys<sup>127</sup>–Cys<sup>150</sup> loop in the (Na,K)ATPase  $\beta$ -subunit is required for the functional assembly of the (Na,K)ATPase  $\alpha$ - and  $\beta$ -subunits. The reduction of disulfide bond(s) of the  $\beta$ -subunit results in the inactivation of (Na,K)ATPase [14,15] and (H,K)ATPase [35]. The fact that the Cys<sup>127</sup>–Cys<sup>150</sup> bond is least stable upon inactivation [15] also suggests that this disulfide bond is important in keeping the active structure of the  $\alpha\beta$ -complex.

As can be seen in Fig. 3, the  $\beta$ -subunit mutants lacking the ability to assemble with the  $\alpha$ -subunit were scarcely fully glycosylated ( $\beta m$ ) but remained in the core glycosylated state ( $\beta c$ ). This suggests that the  $\beta$ -subunit, which is not assembled with the  $\alpha$ -subunit, is not transported from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane [11,36]. It is likely that the mutations prevent the correct folding of the  $\beta$ -subunit and thus inhibit its intracellular transport as well as assembly with the  $\alpha$ -subunit. A detailed analysis of this possibility is in progress in our laboratory.

*Acknowledgements:* This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan.

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