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

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Abstract

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

Key words: (Na,K)ATPase; β-Subunit; Disulfide bond; Site-directed mutagenesis

1. Introduction

The ion-transporting (Na,K)ATPase of animal plasma membranes consists of α- and β-subunits: all the known catalytic functions of the enzyme are associated with the α-subunit [1]. Although the functional role of the β-subunit, which is glycosylated, is not yet fully understood, there is evidence that it is required for biogenesis of the enzyme [2,3]. The β-subunit stabilizes the nascent α-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 β-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 $\beta1$) contains seven conserved cysteine residues. One (Cys^{46}) 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^{127}–Cys^{150}, Cys^{160}–Cys^{176} and Cys^{215}–Cys^{278} [12,13] (numbering refers to *Torpedo californica* (Na,K)ATPase β-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 β-subunit in which the Cys^{127}–Cys^{150} bond was disrupted could assemble with the α-subunit, but the resulting $\alpha\beta$-complex showed little enzyme activity. On the other hand, disruption of either the Cys^{160}–Cys^{176} or Cys^{215}–Cys^{278} bond produced the β-subunit that lacks the ability to form a complex with the α-subunit. It is concluded that the highly conserved disulfide bonds of the β-subunit are important for the correct assembly with the α-subunit.

2. Materials and methods

2.1. Materials

Rabbit antisera raised against the α and β-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 pSPTβ containing the entire coding region of the β-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 β-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 α- or β-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 μg/μl 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 μg/ml each of ampicillin, streptomysin and cefmonexime, and 15 μg/ml of nystatin. The incubation medium also contained L-[U-^{14}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 × g for 30 min. Anti-α-subunit or anti-β-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 $MgCl_2$, 1 mM ATP and microsomes (20–80 μg 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].

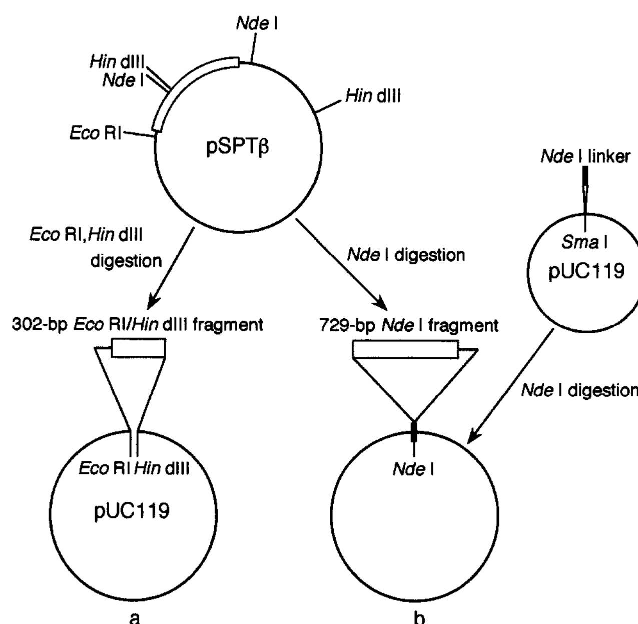

Fig. 1. Construction of template plasmids for mutagenesis. Open and closed boxes indicate the coding sequence for the β-subunit of *T. californica* (Na,K)ATPase and *Nde*I linker, respectively. Plasmid a was used for contrsuction of βC46S. For all the other mutations, plasmid b was used.

3. Results

As mentioned earlier, Cys[46] of isoform β1 of the β-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[46] with serine (βC46S) on its ability to assemble with the α-subunit. To this end, *Xenopus* oocytes were injected with both βC46S mRNAβ and wild-type mRNAα (mRNAα and mRNAβ refer to mRNAs for the α- and β-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 β-subunit (both fully glycosylated βm and core glycosylated βc) and the wild-type α-subunit were synthesized in the oocytes (lanes 1–3) in nearly the same fashion as upon injection of wild-type mRNAβ and wild-type mRNAα (lanes 4–6). In conformation of our previous finding [6], much larger amounts of the α-subunit were detected in oocytes injected with both mRNAα and βC46S mRNAβ than in those injected with mRNAα alone (data not shown, but see Fig. 4, lane 10), indicating that this mutant β-subunit could stabilize the α-subunit. Anti-β-subunit antiserum could precipitate not only the β-subunit but also the α-subunit, though only slightly (lanes 3 and 6). This poor precipitation of the α-subunit is thought to be due to the dissociation of the αβ-complex by the 1% Triton X-100 used for immunoprecipitation. These results suggest that the βC46S mutant subunit could assemble with the α-subunit and thereby stabilize it. As shown in Table 1, ouabain-sensitive ATPase activity of microsomes from the oocytes injected with both wild-type mRNAα and βC46S mRNAβ was comparable with that obtained after injection of both wild- type mRNAα and wild-type mRNAβ. These activities were significantly higher than the value determined for the uninjected control. We conclude that the βC46S mutant has no functional defect. This is in agreement with the finding that the β-subunit isoforms, β2 and β3, that lack

Table 1
(Na,K)ATPase activity of *Xenopus* oocytes injected with wild-type or mutant mRNAβ together with wild-type mRNAα

mRNAβ injected	Ouabain-sensitive ATPase activity (μmol P_i/mg protein/h)		
	Exp.1	Exp. 2	Exp. 3
Wild-type	4.2 (2.1)	3.3 (1.0)	2.5 (0.7)
βC46S	4.9 (2.8)	4.0 (1.7)	3.2 (1.4)
βC127S	2.4 (0.3)	2.3 (0)	1.6 (−0.2)
βC127/150S	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 mRNAβ (10 ng/oocyte) together with wild-type mRNAα (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.

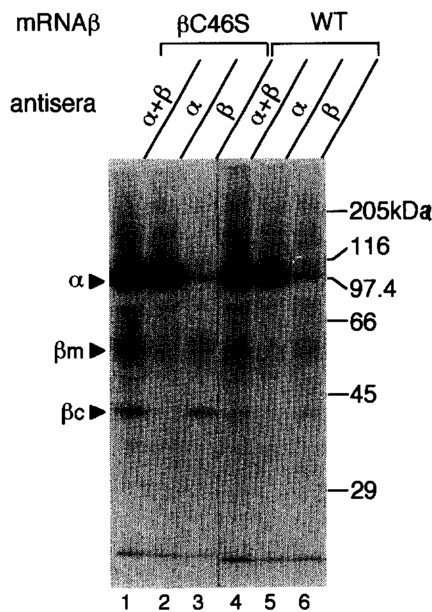

Fig. 2. Functional expression of βC46S mutant. βC46S mRNAβ (10 ng/oocyte) (lanes 1–3) or wild-type mRNAβ (10 ng/oocyte) (lane 4–6) of *T. californica* (Na,K)ATPase was injected into *Xenopus* oocytes together with wild-type mRNAα (10 ng/oocyte). After incubation at 19°C for 3 days in the presence of [^{14}C]leucine, the oocytes were homogenized in the presence of 1% Triton X-100. The labeled translation products were immunoprecipitated with anti-α-subunit antiserum (lanes 2 and 5), anti-β-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. βm and βc indicate fully glycosylated and core glycosylated β-subunit, respectively.

the corresponding cysteine residue in the transmembrane segment, can assemble with the α-subunit to form the fully active αβ-complex [17,18].

To study the roles of the three extracellular disulfide bonds of the β-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: βC127S, βC127/150S, β160S, β160/ 178S, βC215S and βC215/276S. As shown in Fig. 3, in oocytes injected with βC127S or βC127/150S mRNAβ together with wild-type mRNAα, anti-β-subunit antiserum precipitated the α-subunit though only slightly (for the same reason as mentioned above) (lanes 3 and 6). The amounts of the α-subunit precipitated with anti-α-subunit antiserum (lanes 2 and 5) were larger and nearly the same as detected in the oocytes co-injected with wild-type mRNAβ (lane 20). It is, therefore, evident that the βC127S and βC127S/150S subunits, like the wild-type β-subunit, could assemble with and stabilize the α-subunit. However, microsomes from the oocytes injected with bC127S or βC127/150S mRNAβ, together with wild-type mRNAα, showed practically no ouabain-sensitive ATPase activity (Table 1). In contrast to βC127S and βC127/150S, the products of the other four mutant RNAs (βC160, βC160/176S, βC215 and βC215/ 278S) could neither assemble with nor stabilize the α-subunit (lanes 7–18). The faint α-subunit bands detected in the oocytes injected with these four mutant mRNAβs together with mRNAα (lanes 7, 8, 10, 11, 13, 14, 16 and 17) were due to the α-subunit that had been extensively degraded because of the lack of stabilization by the β-subunit. The oocytes injected with mRNAα alone also showed a similar, faint band of the α-subunit (see Fig. 4, lane 10). No ouabain-sensitive ATPase activity could be detected in microsomes from the oocytes injected with the mutant mRNAβ together with wild-type mRNAα (data not shown).

As shown in Fig. 4, the α-subunit in the oocytes injected with wilde-type mRNAα plus βC160S mRNA was completely digested by trypsinolysis even at a protein:trypsin ratio (w/w) of 100:1 (lanes 7–9). The α-subunit in the oocytes injected with mRNAα plus βC127S or βC127/150S mRNAβ, on the other hand, was resistant to trypsin (lanes 1–6). It is thus evident that the assembly-competent β-subunit can confer trypsinresistance on the α-subunit.

4. Discussion

The results described above indicate that the intactness of the structure of the C-terminal portion of the β-subunit, including the Cys^{160}–Cys^{176} and Cys^{215}–Cys^{278} loops, is essential for the subunit to assemble with and stabilize the α-subunit, whereas disruption of the Cys^{127}–Cys^{150} bond does not affect the ability of the β-subunit to assemble with and stabilize the α-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 β1 of the β-subunit are significantly conserved among *Torpedo* [16], human [19], sheep [20], dog [21], pig [22], rat [23], chicken [24] and *Xenopus* [25]. It can bee 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 β1 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 β1 and β2 isoforms of the (Na,K)ATPase β-subunit [26,27] but are also identical to the corresponding residues of the β-subunit of (H,K)ATPase [28–32]. The β-subunit of (H,K)ATPase has been shown to assemble with the α-subunit of (Na,K)ATPase to form a stable, trypsin-resistant αβ-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 β-subunits of the two ATPases. On the other hand, the sequence of the Cys^{127}– Cys^{150} loop of isoform β1 is only 33% conserved among the species examined and there is only one residue

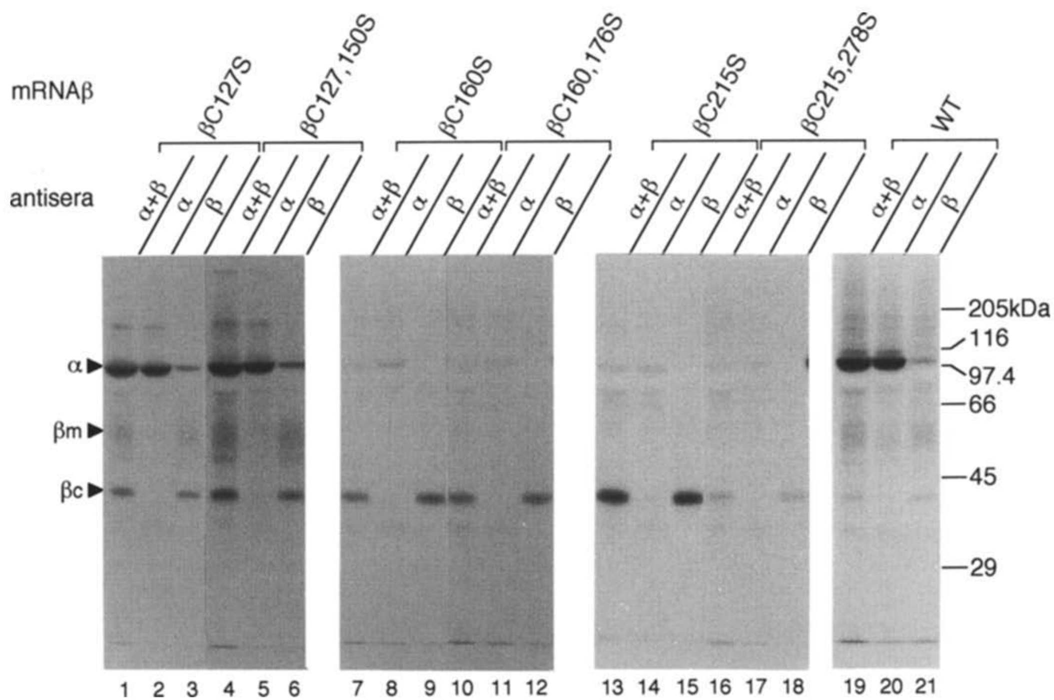

Fig. 3. Translation products produced in *Xenopus* oocytes injected with wild-type mRNAα (10 ng/oocyte) plus mutant mRNAβ (10 ng/oocyte). The mutant mRNAβs injected were βC127S (lanes 1–3), βC127/150S (lanes 4–6), βC160S (lanes 7–9), βC160/176S (lanes 10–12), βC215S (lanes 13–15), and βC215/278S (lanes 16–18). For lanes 19–21, wild-type mRNAβ was injected together with mRNAα. The translation products were immunoprecipitated and analyzed as described in Fig. 2.

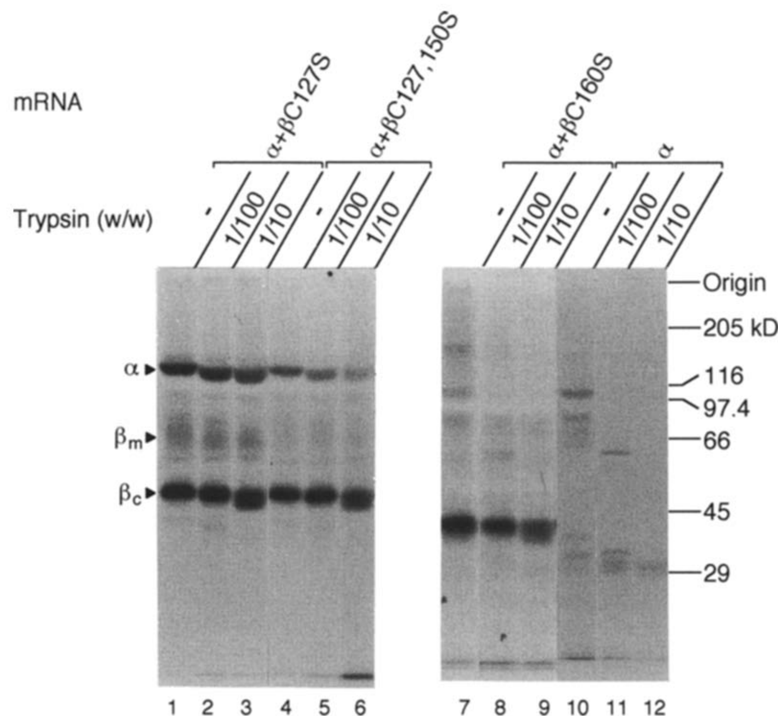

Fig. 4. Trypsin sensitivity of the α-subunit expressed in *Xenopus* oocytes injected with wild-type mRNAα (10 ng/oocyte) together with mutant mRNAβ (10 ng/oocyte). Lanes: 1–3, βC127S mRNAβ plus mRNAα; 4–6, βC127/150S mRNAβ plus mRNAα; 7–9, βC160S mRNAβ plus mRNAα; 10–12, mRNAα alone. Microsomes prepared from each set of oocytes were digested with trypsin (Worthington; L-1-tosylamide-α-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-α-subunit and anti-β-subunit antisera followed by SDS-PAGE and fluorography.

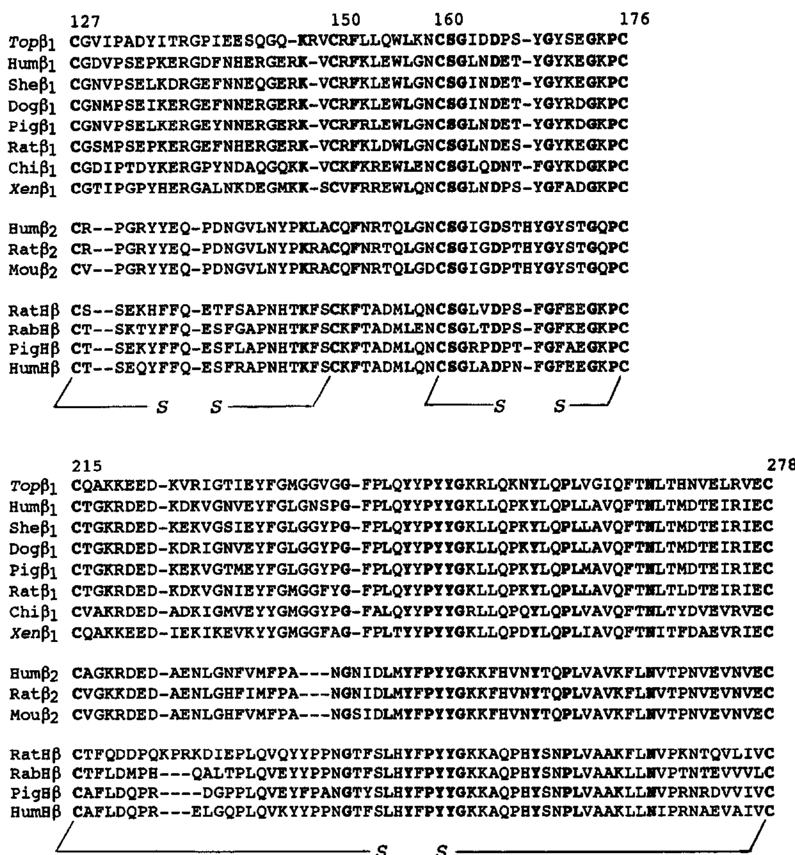

Fig. 5. Alignment of amino acid sequences within disulfide loops in the β-subunit of (Na,K)ATPase and (H,K)ATPase. (Top panel *Torpedo*; Hum, human; She, sheep; Dog, dog; Pig, pig; Rat, rat; Chi, chicken; Mou, mouse; Rab, rabbit; Xen, *Xenopus*. β1 and β2 indicate isoforms β1 and β2, respectively, of the β-subunit of (Na,K)ATPase. Hβ indicates the β-subunit of (H,K)ATPase. Numbering refers to isoform β1 of the β-subunit of *T. californica* (Na,K)ATPase. Residues identical in all the sequences shown are indicated by bold letters.

(Lys^{147}) that is identical in the β-subunit (β1 and β2) of (Na,K)ATPase and the β-subunit of (H,K)ATPase.

The reason why the stable, trypsin-resistant complex of the βC127S or βC127/150S subunit with wild-type α-subunit possesses no catalytic activity is not clear, but a likely possibility is that the interaction of the Cys^{127}–Cys^{150} loop with the α-subunit is critical for the catalytic activity. This possiblity is also supported by the finding that the β-subunit of (H,K)ATPase, the sequence of which in this loop is quite different from that of the β-subunit of (H,K)ATPase, forms a stable complex with the α-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^{127}–Cys^{150} loop in the (Na,K)ATPase β-subunit is required for the functional assembly of the (Na,K)ATPase α- and β-subunits. The reduction of disulfide bond(s) of the β-subunit results in the inactivation of (Na,K)ATPase [14,15] and (H,K)ATPase [35]. The fact that the Cys^{127}–Cys^{150} 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 β-subunit mutants lacking the ability to assemble with the α-subunit were scarcely fully glycosylated (βm) but remained in the core glycosylated state (βc). This suggests that the β-subunit, which is not assembled with the α-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 β-subunit and thus inhibit its intracellular transport as well as assembly with the α-subunit. A detailed analysis of this possibility is in progress in our laboratory.

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