Oligomerization and Maturation of Na,K-ATPase: Functional Interaction of the Cytoplasmic NH₂ Terminus of the β Subunit with the α Subunit

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Abstract. Subunit assembly plays an essential role in the maturation of oligomeric proteins. In this study, we have characterized the main structural and functional consequences of the assembly of α and β subunits of Na,K-ATPase. *Xenopus* oocytes injected with α and/or β cRNA were treated with brefeldin A, which permitted the accumulation of individual subunits or α–β complexes in the ER. Only α subunits that are associated with β subunits become resistant to trypsin digestion and cellular degradation. Similarly, assembly with β subunits is necessary and probably sufficient for the catalytic α subunit to acquire its main functional properties at the level of the ER, namely the ability to adopt different ligand-dependent conformations and to hydrolyze ATP in an Na^+- and K^+-dependent, ouabain-inhibitable fashion. Not only the α but also the β subunit undergoes a structural change after assembly, which results in a global increase in its protease resistance. Furthermore, extensive and controlled proteolysis assays on wild-type and NH_2-terminally modified β subunits revealed a K^+-dependent interaction of the cytoplasmic NH_2 terminus of the β subunit with the α subunit, which is likely to be involved in the modulation of the K^+-activation of the Na,K-pump transport activity. Thus, we conclude that the ER assembly process not only establishes the basic structural interactions between individual subunits, which are required for the maturation of oligomeric proteins, but also distinct, functional interactions, which are involved in the regulation of functional properties of mature proteins.

MANY plasma membrane and secretory proteins are oligomeric. The subunits of these proteins are synthesized independently of each other and are inserted into the ER membrane or the lumen during their synthesis. They are subjected to cotranslational modifications, fold, and then oligomerize. Once correctly assembled, the proteins leave the ER and are targeted to their final cellular site of action. Misfolded or unassembled subunits are retained in the ER and are degraded (15). Although it is increasingly clear that the oligomeric state controls the proper function of the protein, little is known about the nature of interactions that are involved in subunit assembly and about the structural and functional consequences of subunit oligomerization. In this study, we investigate several aspects of this question by analyzing the oligomerization and the functional maturation of Na,K-ATPase.

The ubiquitous Na,K-ATPase is responsible for the maintenance of the sodium and potassium gradients between the intra- and extracellular milieu. The enzyme is composed of two heterologous subunits. The α subunit is a large multimembrane-spanning protein, while the β subunit is a smaller type II membrane glycoprotein with one transmembrane domain, a short cytoplasmic NH_2-terminal tail, and a large COOH-terminal ectodomain. Both subunits are cotranslationally inserted into ER membranes, and the β subunit acquires three to seven core sugars and forms three disulphide bridges (for review see 9). In the β subunit, a 16–amino acid stretch in the membrane domain, but not the hydrophilic NH_2 terminus is needed for membrane insertion (24), while in the α subunit, alternating membrane insertion and stop-transfer sequences are included in the first four transmembrane segments (16).

The α subunit possesses the enzymatic properties and the binding sites for cardiac glycosides, and probably forms the pore for cation transport. However, studies in cRNA-injected oocytes or in transfected cells have revealed that β subunits are needed for the expression of functional Na,K-pumps at the cell surface (for review see 8). Association of the β subunit with the newly synthesized, catalytic α subunit at the level of the ER is necessary for its structural maturation, which is characterized by an increase in the trypsin resistance (10) and/or the half-life as well as the ER exit of the α subunit (1, 19). Finally, in addition to its function in the early maturation, the β subunit plays a role in the mature enzyme and affects the

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transport activity, in particular the K^+-activation of Na,K-pumps expressed in the plasma membrane of *Xenopus* oocytes (18, 19, 34) or of yeast (6).

Little is known about the nature of the interaction sites that mediate the various functions of the β subunit. Several lines of evidence indeed suggest that there might be different types of interaction sites in the β subunit, some of which are needed to stabilize a correct α-subunit configuration and others which are implicated in an indirect control of the α subunit's functional properties. Likely candidates for the first category of interaction sites, which we would tentatively call "structural" interaction sites, are a hydrophobic domain in the most COOH-terminal part of the β subunit (2), the transmembrane region (20), and a region between amino acids 126 and 170 (7), while the cytoplasmic NH_2 terminus of the β subunit does not appear to play a role in the cell surface expression of Na,K-pumps (31). On the other hand, studies with chimera, in which the NH_2-terminal part including the transmembrane domain and the COOH-terminal ectodomain of different β subunits were exchanged, suggest that both domains participate in the modulation of the transport properties of the Na,K-ATPase (6, 20) and thus might contain so-called "functional" interaction sites.

In this study, we have investigated the functional consequences of subunit assembly of Na,K-ATPase and have explored whether the short cytoplasmic NH_2-terminal tail of the β subunit interacts with the α subunit and plays a role in the early maturation (structural interaction) and/or in the modifying effect on the K^+-activation of Na,K-pump transport (functional interaction). Our data show that assembly with the β subunit is necessary and sufficient for the catalytic α subunit to acquire its main functional properties at the level of the ER. Furthermore, expression of wild-type or NH_2-terminally modified β subunits alone or together with α subunits in *Xenopus* oocytes confirmed that the NH_2 terminus is not involved in the maturation and the cell surface expression of the Na,K-ATPase. However, a follow-up of the protease sensitivity of individual or α-associated ER-retained β subunits expressed in brefelden A (BFA)[1]-treated oocytes permitted us to reveal that oligomerization of the subunits provokes a conformational change in the β subunit that reflects an interaction with the α subunit of the NH_2-terminal, cytoplasmic β domain likely to be implicated in the K^+ modulation of Na,K-pump transport.

Materials and Methods

cDNA Constructs

The mutant $\beta_1 \Delta N$ was constructed by deletion of 33 amino acids after the first methionine of the *Xenopus* β1 subunit (37) using the PCR method described by Nelson and Long (29). In a first step, a DNA fragment of the linearized $pGEM_2$ vector containing the β1 cDNA was amplified between the sense oligonucleotide GAAGGACAACATGAAAATTTTATTGT-TCTATCTC comprising part of the noncoding sequence, the ATG coding for the first methionine and the sequence coding for the amino acids Lys35 to Leu40 of the β1 subunit, and the antisense oligonucleotide consisting of the primer D of Nelson and Long (1989) followed by the β1 sequence G_{647}–C_{628}. In a second step, the amplified fragments were used to

elongate the inverse DNA strand. In this step, a long annealing time (15 min) and a low temperature (40°C) were chosen to favor the hybridization between the amplified fragment and the template, leading to the formation of a loop. In a third step, the elongated DNA strands were amplified between a sense oligonucleotide corresponding to the SP_6 promoter and the antisense oligonucleotide D. Finally, the amplified mutated fragment was isolated and introduced into a wild-type pGEM2 β1 plasmid by using EcoRI and BamHI restriction sites.

For the introduction of a NH_2-terminal myc epitope EQKLISEEDL (25), β1 (37), and β3 (13) cDNAs were recombined into the vector pSDM1, a derivative of pSD65 that contains a single copy of the myc epitope followed by an NcoI site that encodes an in-frame AUG codon. For the β3 isoform, the NcoI site at the initiator AUG and a ScaI site in the AMP gene were used to recombine the β3 cDNA from pSD5/β3 into pSDM1. For the β1 isoform, the sequence surrounding the initiator AUG was converted to an NcoI site by PCR mutagenesis, and the altered β1 cDNA was recombined into the pSDM1 vector. For a hemagglutinin (HA) YPYDVPDYA (25) epitope-tagged β3 isoform, the β3 cDNA insert was recombined as described above into the vector p64T-HA, a derivative of pSP64T with an amino-terminal HA epitope followed by an NcoI site that encodes an in-frame AUG codon. All inserts and mutants generated by PCR amplification were sequenced by dideoxy sequencing (32).

Expression in Xenopus Oocytes and Immunoprecipitation of α and β Subunits of Na,K-ATPase

cRNAs encoding *Xenopus* α_1 (37), β_1 (37), β_3 (13), $\beta\Delta N$, $\beta_1$myc, $\beta_3$myc, and β_3HA subunits were obtained by in vitro transcription (28) of linearized cDNAs recloned into the plasmid pSD5 (12). Stage V-VI oocytes were obtained from *Xenopus* females (South African Xenopus Facility, Noerdhoek, Republic of South Africa) as described (19). Routinely, 5-7 ng of α_1 and/or 0.2–0.5 ng of β cRNA was injected into oocytes. Oocytes were incubated in modified Barth's medium containing 0.7–1.5 mCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) in the absence or presence of 5 μg/ml of BFA (kindly provided by Sandoz Pharma Ltd., Basel, Switzerland) for times indicated in figure legends. In preliminary experiments, it was determined that the effect of BFA on ER retention of proteins was attained within 1–2 h and was completely reversible after washing of the drug. After the labeling period or after an additional chase period in the presence of 10 mM cold methionine and in the absence or presence of BFA, microsomes were prepared as described below, and the α and β subunits were immunoprecipitated under denaturing or nondenaturing conditions, as described (20), before revelation by SDS-PAGE and fluorography. Denaturing immunoprecipitations were performed with antibodies against the NH_2 terminus of the *Xenopus* α_1 subunit (A7) (1) or the purified Bufo α1 (RN) (11) or with antibodies against the extracytoplasmic domain of the *Xenopus* β_1 (1) or the β_3 subunit (13) to reveal the α, the β_1, or β_3 subunits, respectively. In nondenaturing immunoprecipitations, the α antibody coprecipitated β subunits associated with α subunits. The myc-tagged $\beta_1$myc and $\beta_3$myc or the hemagglutinin-tagged β_3HA were immunoprecipitated under nondenaturing conditions with mAbs 9E10 or 12CA5 (Boehringer Mannheim), respectively, which permitted α subunits associated with β subunits to coprecipitate. Antigen–antibody complexes were absorbed on protein G–Sepharose-CL-4 (Pharmacia Fine Chemicals, Piscataway, NJ) when 9E10 or 12CA5 antibodies were used and on protein A–Sepharose when α, β_1, or β_3 antibodies were used. In some instances, immunoprecipitated β subunits were subjected to endoglycosidase H (Endo H) (Calbiochem-Novabiochem Corp., La Jolla, CA) treatment as described (19). The dissociated immune complexes were separated by SDS-PAGE, and the labeled proteins were detected by fluorography. Quantifications and determinations of the molecular mass of the immunoprecipitated bands were performed with an analytic program for electrophoretic images (Bio-1D) from Vilber Lourmat (Marne LaVallée, France).

Cell Extraction, Preparation of Microsomes, and Cellular Fractionation of Xenopus Oocytes

For the preparation of microsomes, oocytes were homogenized by 15 strokes in a glass Teflon homogenizer in a buffer (25 μl per oocyte) containing (in mM) 83 NaCl, 1 $MgCl_2$, 10 Hepes, pH 7.9, 0.5 PMSF, and 5 μg/ml (each) of antipain, pepstatin, and leupeptin (Sigma Chemical Co., St. Louis, MO). Homogenates were first centrifuged twice at 1,000 g for 10 min at 4°C to remove yolk granules and then at 10,000 g for 20 min to yield

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1. *Abbreviations used in this paper*: BFA, brefeldin A; Endo H, endoglycosidase H; HA, hemagglutinin.

a microsomal pellet (P_1) in which 2–3 μg protein per oocyte were recovered. Preliminary experiments revealed that >90% of the total α and β subunit population from cRNA-injected oocytes were recovered in P_1. Less than 10% of the subunits were found in a secondary microsomal fraction (P_2) produced by high speed (165,000 g, 90 min) centrifugation of the supernatant of P_1, although ∼5.5 μg protein per oocyte was recovered. Finally, <0.5% of the total subunit population was recovered in the high speed supernatant (∼10 μg protein per oocyte). Na,K-ATPase activity measurements (see below) and residence of Bip protein (data not shown) indicate that P_1 is enriched in plasma membranes as well as in ER, and therefore this microsomal fraction was used for most studies. In some instances, oocyte homogenates were fractionated by density centrifugation on 12–50% (wt/vol) linear sucrose gradients as previously described (19, 30). 12 fractions of sucrose densities between 1.05 and 1.17 were collected from the top of the gradient and were either recentrifuged at 228,000 g for 3 h or directly subjected to immunoprecipitations (see above).

Proteolysis of α and β Subunits

To test the trypsin sensitivity of newly synthesized α subunits, fractions 8 to 11 of sucrose density gradients that are enriched in ER membranes (19) were subjected to controlled trypsinolysis. Fractions were incubated for 5 min on ice with trypsin (type XI; Sigma Chemical Co.) at a trypsin/protein ratio of ∼0.5 and 150 mM K-acetate before addition of a fivefold (wt/wt) excess over trypsin of soybean trypsin inhibitor (Sigma Chemical Co.). The reaction mixture was left on ice for 10 min before addition of SDS to a final concentration of 3.7% and immunoprecipitation with α antibodies. A controlled trypsinolysis assay was also used to follow the ability of newly synthesized α subunits to perform ligand-dependent conformational changes. In this case, P_1 fractions were prepared from oocytes that were injected with α or α plus β cRNA and labeled for 24 h in the presence of BFA. Aliquots (5–12 μg protein) of P_1 fractions resuspended in buffer A, containing (in mM) 30 DL-histidine, 5 EDTA, and 18 Tris base, pH 7.4, were incubated either with 15 mM NaCl and 15 mM KCl or with 7.5 mM $MgCl_2$, 7.5 mM Tris-P_i, and 1 mM ouabain for 20 min at 25°C before addition of different concentrations of trypsin (total volume, 50 μl). Trypsinolysis proceeded for 60 min at 25°C for β-associated α subunits and for 5 min on ice for unassembled α subunits, and then was stopped with a fivefold (wt/wt) excess of soybean inhibitor over trypsin. After 10 min at 25°C, SDS was added and the sample was immunoprecipitated with the RN antibody. For proteolysis assays on α subunits, we verified in preliminary experiments that the β subunit was not limiting for the formation of α–β complexes.

To follow conformational changes of newly synthesized β subunits upon assembly with α subunits, aliquots (5–12 μg protein) of microsomal fractions were subjected to proteolysis under the following conditions: (a) trypsin or α-chymotrypsin (type II; Sigma Chemical Co.) at protease/protein ratios between 0.01 and 3.5 for 2 h at 25°C in the presence of different ligands; and (b) chymotrypsin at a chymotrypsin/protein ratio of 50 for 60 min on ice or for 2 h at 25°C. To stop proteolysis, a fivefold (wt/wt) excess of soybean inhibitor or 5 mM PMSF was added to samples treated with trypsin or chymotrypsin, respectively, and the samples were left on ice or at 25°C for 10 min before addition of SDS to a final concentration of 3.7%. Samples were then immunoprecipitated with $β_1$ antibodies (see above) and subjected to SDS-PAGE in the presence or absence of 2% β-mercaptoethanol. Determinations of the molecular mass of the immunoprecipitated β subunits were performed on samples loaded in different arrangements on the gel to exclude artifacts due to gel "smiling." For proteolysis assays of the β subunit, we verified in preliminary experiments that the α subunit was not limiting for the formation of α–β complexes.

Na,K-ATPase Measurements

The Na,K-ATPase activity was measured in P_1 fractions obtained from oocytes injected with α cRNA alone or together with different β cRNAs and incubated for 24 h in the presence of BFA or for 3 d in the absence of BFA. Na,K-ATPase activity was measured in triplicate by an enzyme-linked assay, according to Schoner et al. (33), in which the resynthesis of ATP consumed by the ATPase is coupled by the pyruvate and lactate dehydrogenase reactions to NADH oxidation. The oxidation rate of NADH was recorded at 340 nM wavelength in the automated enzyme kinetic accessory of a DU-64 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). Activity measurements were done in the presence of 0.15 mg SDS per mg of protein, a detergent concentration that was determined to give maximal activation of latent pumps. Specific Na,K-ATPase activity

was calculated from the difference between samples incubated in the absence or presence of 1 mM ouabain. Specific Na,K-ATPase activity accounted for 2–14% of the total ATPase activity. Statistical analysis was done by unpaired t test.

Ouabain Binding and ^{86}Rb Uptake Measurements

Ouabain binding to oocytes and ^{86}Rb uptake measurements were essentially done as previously described (20) in oocytes injected with α and β cRNA and incubated for 3 d. For both measurements, oocytes were preincubated for 1 h in a nominally K⁺-free solution to load the oocytes with Na⁺. Ouabain binding was performed in solution A containing (in mM) 90 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes, pH 7.4, and supplemented with 0.4 μM [21, 22-^{3}H] ouabain (Amersham Corp.) for 20 min at room temperature. Nonspecific binding, measured in the presence of an 1,000-fold excess of cold ouabain, amounted to 3–7% of the total binding. Statistical analysis was done by unpaired t test. ^{86}Rb uptake was performed in oocytes injected with Bufo α (17) and Xenopus β1 wild-type β or β1ΔN cRNA in solution A without NaCl but supplemented with 5 mM $BaCl_2$, 5 μCi/ml of $^{86}RbCl$ (Amersham Corp.) and varying concentrations of KCl for 12 min. During the preincubation of the oocytes and the ^{86}Rb uptake, 1 μM ouabain was added, which permitted the complete inhibition of the endogenous, oocyte Na,K-pumps (17). Individual oocytes were solubilized with 5% SDS before counting. The parameters of the Hill equation $V = V_{max}/[1+(K_{1/2}/CK)^{n_H}]$ were fitted to the data of the ^{86}Rb uptake (V) induced by various concentrations of K⁺ (CK) and yielded least-square estimates of the maximal uptake (V_{max}), the half-maximal activation constant ($K_{1/2}$), and the Hill coefficient (^nH).

Results

ER Assembly and Functional Maturation of the α-Subunit

A first aim of this study was to determine whether the structural change in the α subunit, which occurs upon assembly with the β subunit and which is reflected by an increased trypsin resistance (10) and an increased half-life (1), coincides with the acquisition of its functional properties. To assess this question, we made use of BFA, which, in most somatic cells, blocks the exit of proteins out of the ER, causes vesiculation of the Golgi cisternae, and leads to redistribution of resident Golgi membrane proteins to the ER (27).

To test the effect of BFA in Xenopus oocytes, we injected oocytes with Xenopus $α_1$ and $β_1$ cRNA, labeled the oocytes in the absence or presence of BFA, and subjected them to cell fractionation by sucrose density centrifugation. The α and the β subunits synthesized in the absence of BFA during a continuous pulse of 17 h distributed, as expected, in both ER- and plasma membrane–enriched fractions (Fig. 1, A and C) that we have previously characterized by the presence of specific markers (30). Typically, the β subunit was in the core-glycosylated (Endo H–sensitive) form in ER-enriched fractions and in the fully glycosylated (Endo H–resistant) form in plasma membrane–enriched fractions (Fig. 1 C). Significantly, only the ER population of α and core-glycosylated β subunits was observed in BFA-treated oocytes (Fig. 1, B and D). In agreement, no increase in the cell surface expression of Na,K-pumps could be revealed by ouabain binding in BFA-treated oocytes (data not shown). The fact that β subunits are exclusively recovered as Endo H–sensitive, core-glycosylated forms indicates that in Xenopus oocytes, BFA does not lead to redistribution to the ER of enzymes that are involved in complex-type glycosylation or alternatively that these enzymes are inactive in the presence of
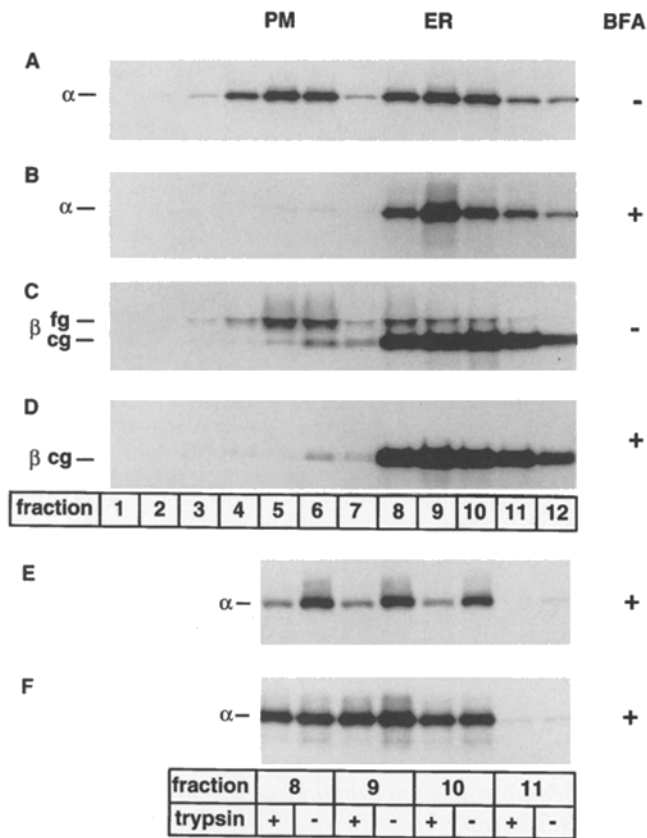
Figure 1. ER retention of Na,K-ATPase subunits in the presence of BFA and functional maturation of the α subunit. Oocytes were injected with 5 ng α_1 cRNA or 5 ng α_1 cRNA plus 0.2 ng *Xenopus* β_1 cRNA and incubated for 17 h with [^{35}S]methionine in the absence or presence of BFA. Oocytes were then subjected to cell fractionation on sucrose gradients, and the α and the β subunits were immunoprecipitated under denaturing conditions from 12 collected fractions. *A–D* show immunoprecipitations of α (*A* and *B*) and β (*C* and *D*) subunits from sucrose gradient fractions prepared from control (*A* and *C*) or BFA-treated (*B* and *D*) oocytes injected with α plus β cRNA. *E* and *F* show immunoprecipitations of α subunits from sucrose gradient fractions prepared from BFA-treated oocytes injected with α cRNA alone (*E*) or with α plus β cRNA (*F*). Aliquots of fractions 8–11 were treated or not with trypsin as described in Materials and Methods before immunoprecipitation.

Figure 2. α subunits become able to adopt ligand-dependent conformations after assembly with the β subunit. Oocytes were injected with 5 ng *Xenopus* α_1 cRNA or with 5 ng α_1 plus 0.2 ng β_1 cRNA and incubated for 24 h with [^{35}S]methionine in the presence of BFA. P_1 microsomes were prepared and subjected to controlled trypsinolysis in the presence of different ligands before immunoprecipitation with an α antibody. (*A*) Tryptic cleavage of individual α subunits. Aliquots of P_1 fractions from oocytes injected with a cRNA alone were preincubated for 20 min at 25°C with 100 or 15 mM KCl or NaCl; 7.5 mM Tris-Pi and 1 mM ouabain (*ouab*); and/or 7.5 mM $MgCl_2$ before trypsinolysis was performed at a trypsin/protein ratio of 0.01 for 5 min at 4°C. (*B*) Ligand-dependent tryptic cleavage of β-assembled α subunits. Aliquots of P_1 fractions from oocytes injected with α and β cRNA were preincubated for 20 min at 25°C with 15 mM NaCl (lanes *1–6*), 15 mM KCl (lanes *7–11*), or 7.5 mM $MgCl_2$, 7.5 mM Tris/Pi, and 1 mM ouabain (lanes *12–16*) before trypsinolysis was performed for 1 h at 25°C at trypsin/protein ratios ranging from 0.01–0.5. Shown are immunoprecipitations with an α antibody. The sample shown in lane *6* was complemented with 7.5 mM choline chloride. *st*, protein markers of known molecular mass.

BFA. In any case, the results indicate that BFA inhibits ER exit of proteins in *Xenopus* oocytes and justify the use of this drug to study the direct consequences of the assembly process in the ER on the properties of Na,K-ATPase subunits.

Fig. 1 *E* shows that individual α subunits from ER membranes of BFA-treated oocytes were highly trypsin sensitive, while β-assembled α subunits became trypsin resistant (Fig. 1 *F*). This result confirms that the structural maturation of the α subunit is a direct consequence of the assembly with the β subunit in the ER. To monitor the functional maturation of the catalytic α subunit after subunit assembly, we first assessed the ability of individual and β-assembled α subunits to undergo ligand-dependent conformational changes, which is a characteristic feature of functional Na,K-ATPases (for review see 21). As a bio-
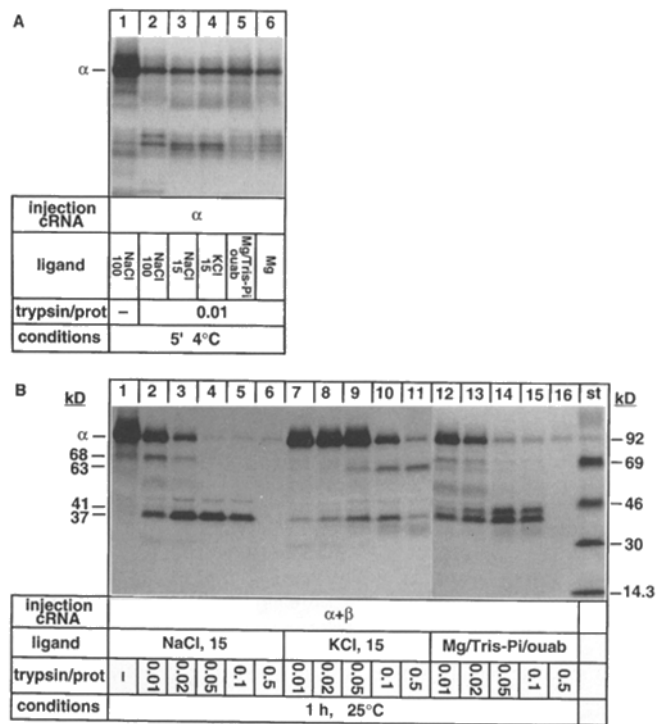
chemical tool to follow the conformational transitions, we adapted a controlled trypsinolysis assay, which has previously been used for the same purpose in purified enzyme preparations (22). The assay is based on the prediction that proteins in different conformations exhibit distinct tryptic sites and that digestion with trypsin would produce distinct tryptic patterns according to the conformation adopted by the protein.

To analyze the ability of the α subunits to perform ligand-dependent conformational changes, we performed trypsinolysis of individual or β-associated α subunits in the presence of either NaCl, KCl, or Mg/Tris-P_i/ouabain. Individual α subunits were digested up to 80% after incubation of microsomes of BFA-treated oocytes for 5 min at 4°C at a trypsin/protein ratio of 0.01. Although some tryptic fragments could be revealed, there were no striking dif-
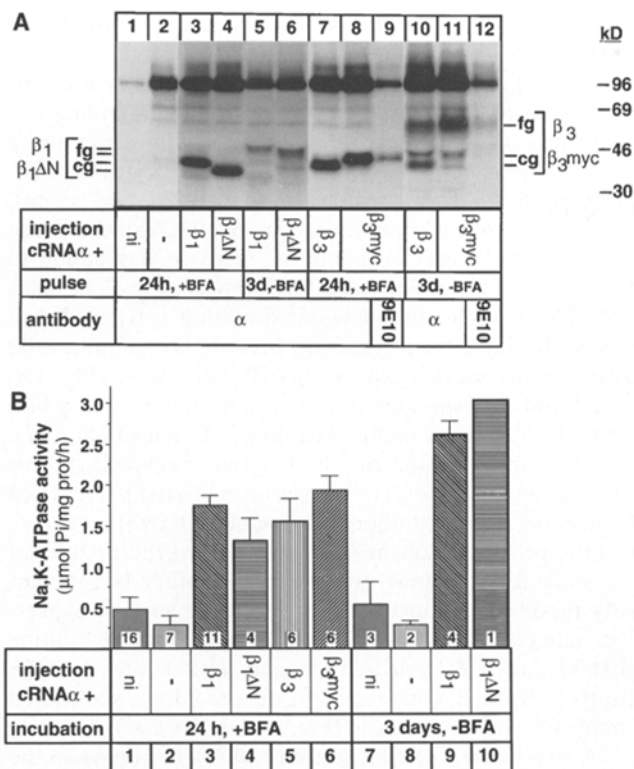
Figure 3. The NH₂ terminus of the β subunit is not involved in the functional maturation of the α subunit. Oocytes were not injected or injected with 7 ng *Xenopus* α₁ cRNA alone, or with 7 ng α₁ plus 0.2 ng *Xenopus* β₁, 0.5 ng β₁ΔN, 0.5 ng β₃, or 0.5 ng β₃myc cRNA and incubated for 24 h or 3 d with or without [³⁵S]methionine in the presence or absence of BFA. Microsomes were prepared and were either subjected to immunoprecipitation under nondenaturing conditions or used for Na,K-ATPase activity measurements. (*A*) Immunoprecipitations of α–β complexes synthesized during a 24-h pulse-labeling of oocytes in the presence of BFA (lanes *1–4* and *7–9*) or during a 3-d pulse in the absence of BFA (lanes *5, 6,* and *10–12*). Nondenaturing immunoprecipitations were performed with an α antibody (lanes *1–8, 10,* and *11*) or with a myc antibody (9E10) (lanes *9* and *12*). *fg* and *cg*, fully- and core-glycosylated β subunits, respectively. (*B*) Na,K-ATPase activity of α subunits and α–β complexes synthesized during a 24-h incubation of oocytes in the presence of BFA (lanes *1–6*) or during a 3-d incubation in the absence of BFA (lanes *7–10*). Numbers of experiments performed on different batches of oocytes are given in the bars. Na,K-ATPase activities measured in noninjected and α cRNA-injected oocytes are statistically not different (*P* > 0.1). On the other hand, Na,K-ATPase activities measured in oocytes expressing the various α–β complexes are significantly different from those measured in oocytes expressing α subunits alone (*P* < 0.001).

ferences in the tryptic digestion pattern in the presence of the different ligands (Fig. 2 *A*, lanes *1–6*). Characteristically, despite their slow cellular degradation (1), the endogenous, oocyte α subunits that are synthesized in excess over β subunits (10) showed a similar trypsin sensitivity (data not shown), indicating that the sensitivity to cellular proteases and the trypsin sensitivity are not obligatorily linked.

The properties of α subunits synthesized together with β subunits were different. Characteristically, the β-associ-

ated α subunits needed incubation at 25°C for 1 h to become visibly digested and were more trypsin resistant in the presence of KCl than of NaCl or Mg/Tris-P$_i$/ouabain (Fig. 2 *B*). In agreement with previous observations on purified enzyme preparations (22), the trypsin resistance was generally more pronounced in the presence of 100 mM than of 15 mM NaCl or KCl (data not shown). A kinetic analysis of the production of the proteolytic patterns of the α subunit in the presence of the different ligands clearly showed that distinct tryptic fragments were produced in the presence of Na⁺ (Fig. 2 *B*, lanes *1–6*, 68-kD fragment) or of K⁺ (lanes *7–11*, 63-kD fragment). Mg/Tris-P$_i$/ouabain also produced characteristic tryptic fragments (lanes *12–16*, 41-kD fragment) that were distinct from those produced in the presence of each of these ligands alone (data not shown). Finally, the ligand-dependent tryptic patterns of the α subunit were similar when trypsinolysis was performed on microsomes of oocytes that were not treated with BFA (data not shown). Thus, altogether the trypsinolysis data indicate that in contrast to individual α subunits, which cannot adopt different conformations necessary for ion transport, α subunits that associate with β subunits acquire these functional properties at the level of the ER.

This result was also supported by our second functional test in which we assessed the Na⁺- and K⁺-dependent ouabain-inhibitable ATPase activity in microsomes of BFA-treated oocytes expressing α subunits alone or α plus β subunits. Despite a significant accumulation of newly synthesized, exogenous α subunits in the ER (Fig. 3 *A*, compare lanes *1* and *2*), a low and similar Na,K-ATPase activity was measured in microsomes prepared from noninjected and α cRNA-injected oocytes treated with BFA (Fig. 3 *B*, lanes *1* and *2*). This result confirms that α subunits alone have no Na⁺- and K⁺-dependent hydrolytic activity. Significantly, in BFA-treated oocytes that expressed α–β complexes containing exclusively core-glycosylated β subunits (Fig. 3 *A*, lane *3*), the Na,K-ATPase activity increased by three- to sevenfold after a 24-h incubation (Fig. 3 *B*, lane *3*). In nontreated oocytes that expressed α–β complexes containing mainly fully glycosylated β subunits (Fig. 3 *A*, lane *5*), the Na,K-ATPase activity increased up to 12-fold after a 3-d incubation (Fig. 3 *B*, lanes *7–9*). Since we have previously shown that glycosylation of the β subunit does not influence the enzyme activity of Na,K-ATPase (38), these data indicate that α subunits become functionally active, e.g., acquire Na,K-ATPase activity upon assembly with β subunits at the level of or close to the ER.

The NH₂ Terminus of the β Subunit Is Not Involved in the Structural and Functional Maturation of the α Subunit

To elucidate whether the NH₂ terminus of the β subunit plays a role in the early functional maturation of the Na,K-ATPase, we compared the ability of *Xenopus* wild-type β₁ and β₃ subunits and of two NH₂-terminally modified β subunits to form α–β complexes with Na,K-ATPase activity. All β subunits including a NH₂-terminally deleted β₁ (β₁ΔN) and a myc-tagged β₃ (β₃myc) subunit, synthesized in the presence of BFA, associated efficiently with α subunits as shown by the coprecipitation with an α antibody
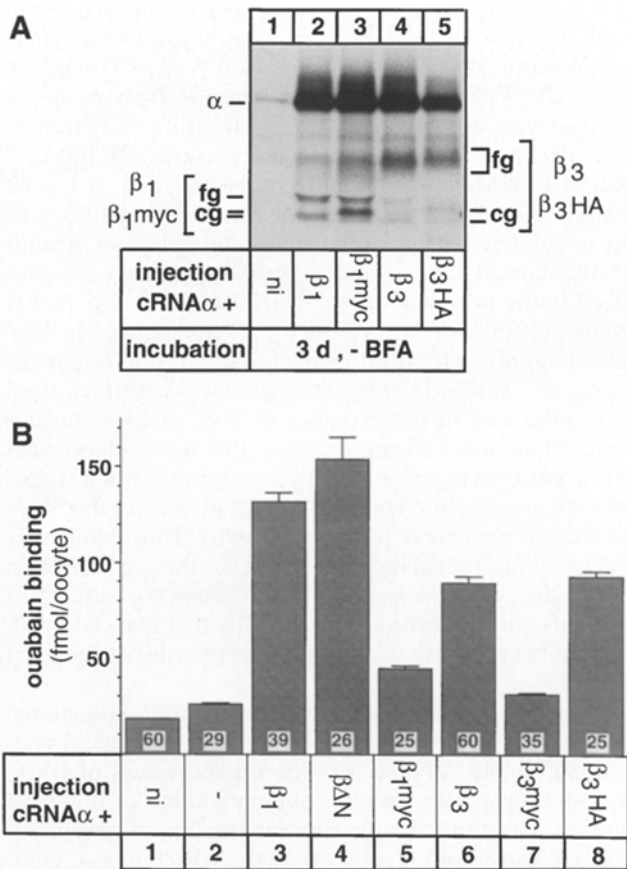
Figure 4. Cell surface expression of α–β complexes. Oocytes were not injected or injected with 7 ng Xenopus α_1 cRNA alone, or with 7 ng α_1 plus 0.2 ng Xenopus β_1, 0.5 ng β_1ΔN, 0.5 ng $\beta_1$myc, 0.5 ng β_3, 0.5 ng $\beta_3$myc, or 0.5 ng β_3HA cRNA and incubated for 3 d with or without [^{35}S]methionine in the sence of BFA. (A) Nondenaturing immunoprecipitations with an α antibody of microsomes prepared from oocytes injected with α cRNA plus different β cRNAs and pulse labeled for 3 d. fg and cg, fully- and core-glycosylated β subunits, respectively. ni, noninjected oocytes. (B) Ouabain binding performed as described in Materials and Methods on oocytes injected with α cRNA alone or with α plus different β cRNAs and incubated for 3 d. Numbers of oocytes tested are indicated in the bars. One to three different batches of oocytes were used for the measurements. Lane 3 vs lane 4, $P > 0.05$; lane 3 vs lane 5, $P < 0.001$; lane 6 vs lane 7, $P < 0.001$; lane 6 vs lane 8, $P > 0.1$.

(Fig. 3 A, lanes 3–8, 10, and 11). An antibody against the myc epitope was able to coprecipitate the α subunit associated with the myc-tagged β_3 subunit (lanes 9 and 12). No significant differences in the Na,K-ATPase activity were observed for the α-β_1, α-β_3, α-β_1ΔN, or α-$\beta_3$myc complexes formed in the presence (Fig. 3 B, lanes 3–6) or absence of BFA (lanes 9 and 10). These data indicate that association of different β subunits can promote the functional maturation of the α subunit and that the NH$_2$ terminus of the β subunit is not crucially important for the functional maturation of the α subunit. This latter observation was also supported by the fact that the α subunit of α-β_1ΔN complexes produced similar ligand-dependent tryptic patterns as that of α–wild-type β_1 complexes (data not

shown) reflecting its ability to perform conformational changes.

To test whether the different α–β complexes are correctly transported to the cell surface, ouabain binding was performed on intact oocytes after cRNA injection and a 3-d incubation in the absence of BFA. In oocytes expressing α-β_1 or α-βΔN, ouabain binding increased significantly compared to noninjected or α cRNA-injected oocytes (Fig. 4 B, lanes 1–4), and the increase was similar to the increase in the Na,K-ATPase activity (Fig. 3 B, lanes 7–9). Surprisingly, in oocytes expressing α-$\beta_3$myc complexes, the increase in ouabain binding was significantly lower than in oocytes expressing α-β_3 complexes (Fig. 4 B, lanes 6 and 7) despite the presence of a similar level of biosynthetically labeled complexes (Fig. 3 A, lanes 10 and 11). To assess the question of whether the lower cell surface expression of α-$\beta_3$myc complexes is due to the presence of the myc tag itself or rather to a modulation by the myc tag of some properties of the NH$_2$ terminus of the β_3 subunit necessary for transport, we tested two other NH$_2$-terminally modified β constructs for their cell surface expression, namely a β_3 construct tagged with an HA epitope (β_3HA) and a β_1 construct tagged with a myc epitope ($\beta_1$myc). Both β constructs produced fully glycosylated complexes with α subunits (Fig. 4 A, lanes 1–5), but only β_3HA produced a similar number of Na,K-pumps at the cell surface than the wild-type β subunit (Fig. 4 B, compare lanes 6 and 8). Similarly to that of α-$\beta_3$myc complexes, the cell surface expression of α-$\beta_1$myc complexes was significantly impeded (compare lanes 3 and 5). Although, actually, we have no explanation for the particular behavior of myc-tagged β subunits, our results suggest that the myc tag itself might be responsible for the inefficient cell surface expression of α–β complexes. It cannot, however, be ruled out completely that the myc epitope, in contrast to the HA epitope, imposes a conformational change to the NH$_2$ terminus of the β subunit, which leads to intracellular retention of the α–β complexes beyond a trans-Golgi compartment.

Interaction of the NH$_2$ Terminus of the β Subunit with the α Subunit and Its Functional Consequences

Since the cytoplasmic NH$_2$ terminus of the β subunit does not play a role in the early maturation and the cell surface expression of the Na,K-ATPase, the question remains whether this domain indeed interacts with the α subunit and might mediate the regulatory function of the β subunit in the transport activity of mature pumps. To test this hypothesis, we expressed α subunits together with wild-type or NH$_2$-terminally deleted β subunits in oocytes and measured the transport properties of the α–β complexes expressed at the cell surface, in particular the K$^+$-activation of the ^{86}Rb uptake mediated by the Na,K-pumps. Fig. 5 shows that the apparent K$^+$-affinity of the Na,K-pump–mediated ^{86}Rb uptake is about four times lower for α-β_1ΔN complexes (apparent $K_{1/2}$ for K$^+$: 1.6 mM) than for α–β wild-type complexes (apparent $K_{1/2}$ for K$^+$: 0.4 mM). Thus, indeed, the cytoplasmic NH$_2$ terminus of the β subunit appears to be involved in the modulation of the K$^+$-activation of the Na,K-ATPase transport activity.

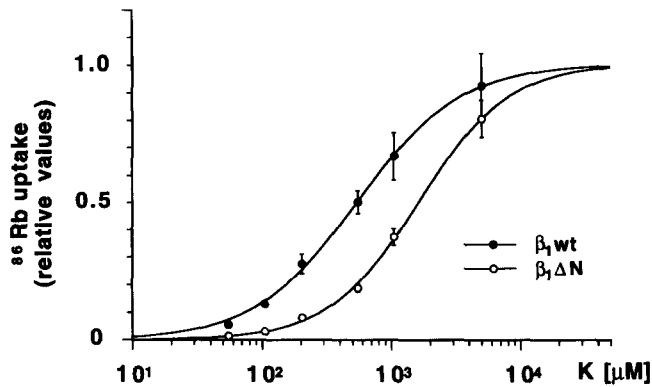The question arose whether the predicted functional in-

Figure 5. NH$_2$-terminally deleted β subunits affect the K$^+$-activation of Na,K-pumps. Oocytes were injected with 9 ng ufo α$_1$ cRNA plus 0.3 ng *Xenopus* β$_1$ (β$_1$wt, ○) or 0.5 ng β$_1$ΔN cRNA (●) and incubated for 3 d. ^{86}Rb uptake into individual oocytes was measured as described in Materials and Methods at different K$^+$ concentrations in the external medium. Each experimental point represents the mean ± SE of 15–19 oocytes. At close to V$_{max}$ conditions, at 5 mM external K$^+$,^{86}Rb uptake was 119 ± 15 pmol per min per oocyte in oocytes expressing α–βwt complexes and 94.5 ± 7.8 pmol per min per oocyte in oocytes expressing α–β$_1$ΔN complexes. One out of two similar experiments is shown. In comparative studies performed on oocytes expressing ouabain-sensitive *Xenopus* α–β complexes, we have found a good correlation between ouabain binding and transport function for both wild-type and mutant α–β complexes, indicating that there is no significant difference in the molecular activity of wild-type and mutant pumps.

teraction of the NH$_2$ terminus with the α subunit could be revealed as a discrete conformational change occurring in the β subunit after association with the α subunit. To assess this question, we checked for differences in the protease sensitivity and in the digestion patterns of individual and α-assembled β subunits synthesized in BFA-treated oocytes. β subunits synthesized in vivo alone or together with α subunits turned out to be highly resistant to trypsin or chymotrypsin treatment. Only extreme digestion conditions at a chymotrypsin/protein ratio of 50 permitted the production of a significant fragmentation of the individual or α-associated β subunits (Fig. 6, lanes 1–4). Most likely, the digestion of the β subunit under these conditions is not due to a specific action of chymotrypsin but rather to the activity of a nonidentified proteolytic contaminant. Nevertheless, we consistently observed a difference in the production of two small proteolytic fragments in individual and α-associated *Xenopus* β$_1$ subunits, indicating that, indeed, the β subunit undergoes a structural change upon assembly with the α subunit. Protease digestion produced a fragment of ∼14.4-kD in individual β subunits (lane 2) and a fragment of ∼16.5-kD in assembled β subunits (lane 4). In an attempt to locate the proteolytic sites responsible for the production of these fragments, we subjected the digested immunoprecipitated sample to nonreducing gel electrophoresis. In the absence of β-mercaptoethanol, the two small molecular mass fragments disappeared (lanes 5–8), indicating that the cleavage site is located within a region held together by a disulphide bridge. According to the molecular mass of the fragments, they might be derived from the NH$_2$-terminal part of the β subunit by cleavage between Cys126 and Cys149 known to form the first of three
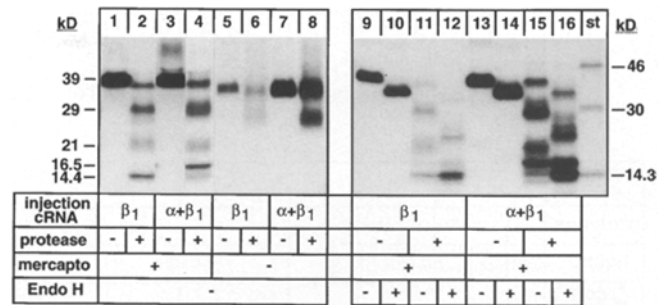

Figure 6. Differential proteolytic cleavage of individual and α-associated β subunits. Oocytes were injected with 0.2 ng *Xenopus* β$_1$ cRNA (lanes 1, 2, 5, 6, and 9–12) or with 7 ng *Xenopus* α$_1$ plus 0.2 ng β$_1$ cRNA (lanes 3, 4, 7, 8, and 13–16) and incubated for 24 h with [^{35}S]methionine in the presence of BFA before preparation of microsomes. Aliquots of microsomes were incubated with chymotrypsin at a protease/protein ratio of 50 in the presence of 100 mM KCl for 60 min at 4°C. β subunits were immunoprecipitated under denaturing conditions and subjected to gel electrophoresis under reducing (lanes 1–4) or nonreducing (lanes 5–8) conditions (mercapto, β-mercaptoethanol). Other immunoprecipitates were subjected or not to endoglycosidase H (Endo H) treatment before gel electrophoresis (lanes 9–16). st, protein markers of known molecular mass.

disulphide bridges in the ectodomain of β subunits (for references see 8) (see Fig. 10). In agreement with this prediction is the fact that, in contrast to the other proteolytic fragments of 29 and 21 kD, the 14.4- and 16.5-kD fragments are not affected by Endo H treatment (Fig. 6, lanes 11, 12, 15, and 16) and thus do not contain sugar chains, as expected from the *Xenopus* β$_1$ model (see Fig. 10). Furthermore, none of the proteolytic fragments produced from an NH$_2$-terminally myc-tagged β$_3$ subunit, which was subjected to similar proteolytic digestion, were recognized by a myc antibody (data not shown), indicating that the NH$_2$ terminus was missing in the cleaved products.

Although these data argue that the 14.4- and 16.5-kD fragments are produced from the NH$_2$ terminus of the β subunit, they did not permit us to decide whether the increase in the molecular mass of the fragment from α-associated β subunits was due to a protection of the NH$_2$-terminal or the COOH-terminal part in the 16.5-kD fragment. In this context, it is interesting to note that Lutsenko and Kaplan (27) have recently suggested that a tryptic cleavage site at Arg^{134}-Gly^{135} in the β subunit of microsomal Na,K-ATPase is hidden in the presence of Rb$^+$ but exposed in the presence of Mg/P$_i$. To identify the proteolytic cleavage sites that become protected in the β subunit after association with the α subunit, milder and thus more specific proteolysis conditions were applied. Significantly, when trypsinolysis was performed for 2 h at 25°C at a trypsin/protein ratio of 3.5 in the presence of MgCl$_2$, the individual β subunits were nearly completely digested (Fig. 7 A, lane 3) while the α-associated β subunits were partly resistant to proteolytic attack (lane 6). Similarly, α-associated β subunits resisted better to proteinase K digestion (protease/protein ratio of 1, 1 h, 4°C) in the presence of 1% Triton X-100 than individual β subunits (compare lanes 9 and 12). These data argue that the β subunit undergoes an important structural change upon assembly
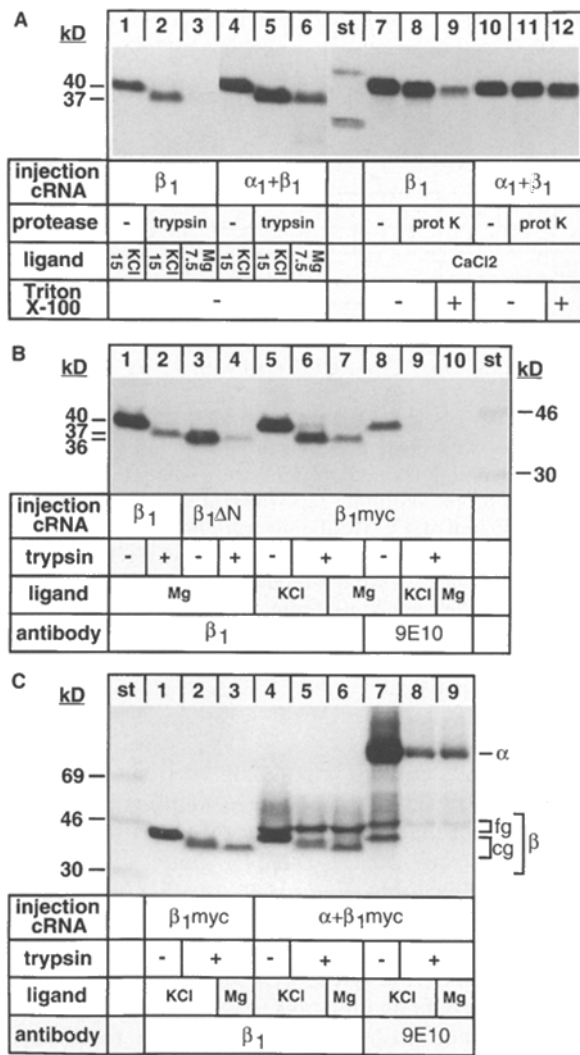
A kD	1	2	3	4	5	6	st	7	8	9	10	11	12
injection cRNA	β1			α1+β1				β1			α1+β1		
protease	-	trypsin		-	trypsin			-	prot K		-	prot K	
ligand	KCl 15	KCl 15	Mg 7.5	KCl 15	KCl 15	Mg 7.5		CaCl2					
Triton X-100	-							-		+	-		+

B kD	1	2	3	4	5	6	7	8	9	10	st	kD
injection cRNA	β1		β1ΔN		β1myc							—46
trypsin	-	+	-	+	-	+	-	+				
ligand	Mg				KCl	Mg		KCl	Mg			—30
antibody	β1						9E10					

C kD	st	1	2	3	4	5	6	7	8	9	
injection cRNA		β1myc			α+β1myc						—α
trypsin		-	+		-	+		-	+		fg cg] β
ligand		KCl	Mg		KCl	Mg		KCl	Mg		
antibody		β1						9E10			

Figure 7. Controlled proteolysis reveals a global conformational change in α-associated β subunits and a specific cleavage of the cytoplasmic NH$_2$ terminus. Oocytes were injected with 0.2 ng *Xenopus* β$_1$ cRNA, with 0.5 ng β$_1$ΔN, with 0.5 ng β$_1$myc, with 7 ng α$_1$ plus 0.2 ng β$_1$ cRNA, or with 7 ng α$_1$ plus 0.5 ng β$_1$myc and incubated for 24 h (*A* and *B*) or 48 h (*C*) with [^{35}S]methionine in the presence (*A*) or absence of BFA (*B* and *C*) before preparation of microsomes. (*A*) Digestion of individual and α-associated β subunits with trypsin and proteinase K. Aliquots of microsomes from BFA-treated oocytes injected with β$_1$ cRNA alone (lanes *1–3*, and *7–9*) or with α$_1$ plus β$_1$ cRNA (lanes *4–6*, and *10–12*) were incubated either without or with trypsin (lanes *1–6*) or proteinase K (lanes *7–12*) before denaturing immunoprecipitation with a β antibody. For trypsin digestion, samples were preincubated for 3 h at 25°C with 15 mM KCl (lanes *1, 2, 4,* and *5*) or 7.5 mM MgCl$_2$ (lanes *3* and *6*) before addition of trypsin at a protease/protein ratio of 3.5 and deoxycholate at a detergent/protein ratio of 0.3 for 2 h at 25°C. Proteinase K digestion was performed at a protease/protein ratio of 1 for 1 h at 4°C in the presence of 10 mM CaCl$_2$ and in the absence (lanes *7, 8, 10,* and *11*) or presence (lanes *9* and *12*) of 1% Triton-X 100. (*B*) Trypsinolysis of individual, NH$_2$-terminally modified β subunits. Aliquots of microsomes from oocytes injected with β$_1$ (lanes *1* and *2*), β$_1$ΔN (lanes *3* and *4*), or β$_1$myc (lanes *5–10*) cRNA were incubated without or with trypsin at a protease/protein ratio of 3.5 for 2 h at 25°C in the presence of 7.5 mM MgCl$_2$ (lanes *1–4, 7, 8,* and *10*) or 15 mM KCl (lanes *5, 6,* and *9*). Immunoprecipitations were performed under

denaturing conditions with a β antibody (lanes *1–7*) or under nondenaturing conditions with the myc antibody 9E10 (lanes *8–10*). (*C*) Trypsinolysis of individual and α-associated fully glycosylated β$_1$myc. Aliquots of microsomes from oocytes injected with β$_1$myc (lanes *1–3*) or with α$_1$ plus β$_1$myc (lanes *4–9*) cRNA were incubated without or with trypsin at a protease to trypsin ratio of 0.1 for 1 h at 25°C in the presence of 15 mM KCl (lanes *1, 2, 4, 5, 7,* and *8*) or 7.5 mM MgCl$_2$ (lanes *3, 6,* and *9*). Immunoprecipitations were performed under denaturing immunoprecipitations with a β antibody (lanes *1–6*) or under nondenaturing conditions with the myc antibody 9E10 (lanes *7–9*).
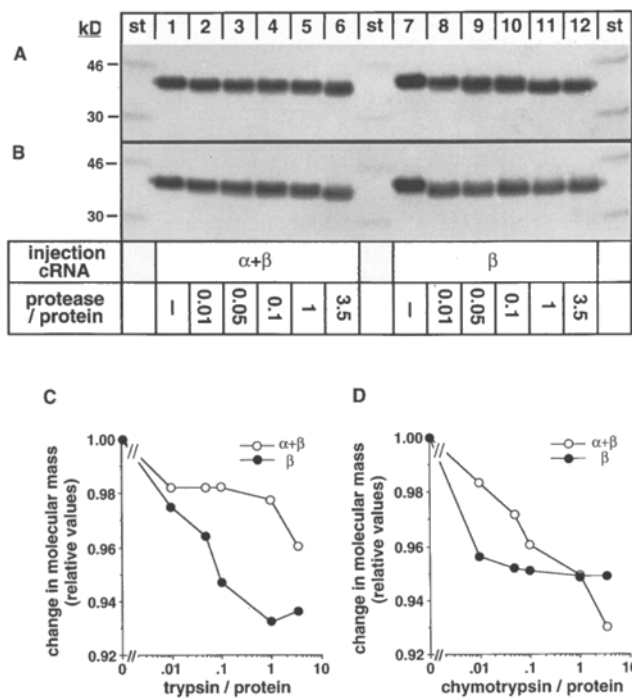
with the α subunit, which is reflected in a general increase in its protease resistance.

Trypsinolysis at a trypsin/protein ratio of 3.5 in the presence of KCl revealed that initial cleavage removed a fragment of ∼3 kD in individual (Fig. 7 *A*, lane *2*) or α-associated (lane *5*) β subunits. To locate this tryptic split, we tested the effect of trypsin digestion on the NH$_2$-terminally modified β subunits, the β$_1$ΔN, the β$_1$myc, and the β$_3$HA constructs. In contrast to the wild-type β$_1$ subunit (Fig. 7 *B*, lanes *1* and *2*), β$_1$ΔN was not cleaved by trypsin to a product of lower molecular mass (lanes *3* and *4*), indicating that the cleavage site was missing in the NH$_2$-terminally deleted β subunit. Similar results were obtained when trypsinolysis was performed in the presence of KCl rather than MgCl$_2$ (data not shown). On the other hand, the β$_1$myc construct showed the characteristic shift of 3 kD after trypsinolysis, and the cleaved product could be revealed by an antibody against the β ectodomain (lanes *5–7*). In contrast, an antibody against the myc epitope only recognized the uncleaved but not the cleaved β subunits (lanes *8–10*), indicating that the NH$_2$-terminally attached myc epitope had been removed during trypsinolysis. Similar results were obtained with the β$_3$HA construct (data not shown). Thus, these data indicate that the 3-kD cleavage in the β subunit, observed at a trypsin/protein ratio of 3.5, is due to a split in the 34-amino acid–containing, cytoplasmic NH$_2$ terminus.

To see whether the NH$_2$ terminus was similarly cleaved in β subunits that were processed to the fully glycosylated form after exit of α–β complexes from the ER, we expressed β$_1$myc in oocytes together with α subunits in the absence of BFA and tested the effect of trypsin on the recognition of the β subunit by the myc antibody. The core-glycosylated β$_1$myc synthesized alone (Fig. 7 *C*, lanes *1–3*) or together (lanes *4–6*) with α subunits during a 48-h pulse in the absence of BFA were cleaved by ∼3 kD in the presence of KCl and by ∼3.5 kD in the presence of MgCl$_2$ as revealed with an antibody against the β ectodomain. On the other hand, the fully glycosylated β$_1$myc showed no apparent change in its molecular mass (lanes *4–6*). The myc antibody, as expected, did not recognize the cleaved, core-glycosylated β subunit but still immunoprecipitated to a certain extent the fully glycosylated species and also coprecipitated some α subunits associated with the β subunit (lanes *7–9*). These data suggest that the structural maturation of the β subunit proceeds beyond the ER and might impose a certain trypsin resistance to the NH$_2$ terminus of the β subunit, which is independent of the assembly process.

Figure 8. The cytoplasmic NH_2-terminal tail of the β subunit becomes protected against proteolysis after assembly with the α subunit. Oocytes were injected with 7 ng α_1 plus 0.2 ng β_1 cRNA (lanes *1–6*) or with 0.2 ng β_1 cRNA alone (lanes *7–12*) and incubated for 24 h with [^{35}S]methionine in the presence of BFA. Microsomes were prepared and aliquots were incubated with trypsin (*A*) or chymotrypsin (*B*) at protease/protein ratios between 0.01 and 3.5 for 2 h at 25°C in the presence of 15 mM KCl before immunoprecipitation under denaturing conditions with a β antibody. *C* and *D* show the relative changes of the molecular mass of the β subunits shown in *A* and *B* respectively, as a function of the protease/protein ratio used. The molecular mass of the nondigested β subunit has arbitrarily been set to 1. One out of three similar experiments is shown.

Since softer proteolysis conditions permitted us to selectively cleave the β subunit from the NH_2-terminal side, we performed in a last set of experiments a detailed analysis on the protease sensitivity of individual and α-assembled β subunits to demonstrate the putative functional interaction site in the cytoplasmic tail of the β subunit. When microsomes from BFA-treated oocytes expressing individual or α-assembled β subunits were incubated with increasing concentrations of trypsin or chymotrypsin for 2 h at 25°C in the presence of KCl, the NH_2 termini of individual and α-associated β subunits were progressively cleaved to fragments of lower molecular mass with increasing protease concentrations (Fig. 8). However, for a similar shift in the molecular mass, α-assembled β subunits required ~10 times more protease than individual β subunits (Fig. 8, *A–D*). The results were particularly striking with chymotrypsin. While the individual β subunits were nearly maximally cleaved by 3 kD at the lowest protease concentration used (Fig. 8, *B* [lanes *7–12*] and *D*), the molecular mass of the α-associated β subunits only progressively decreased with increasing protease concentrations (Fig. 8, *B* [lanes *1–6*] and *D*). Interestingly, the NH_2 terminus of the α-assembled β subunit was only efficiently protected when trypsinolysis was performed in the presence of KCl but not
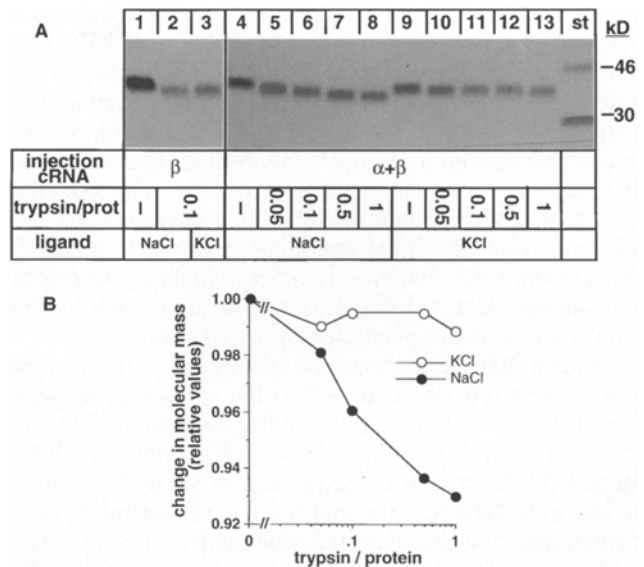
Figure 9. KCl but not NaCl protects the NH_2 terminus of the β subunit from digestion with trypsin. (*A*) Oocytes were injected with 0.2 ng β_1 cRNA alone (lanes *1–3*) or with 7 ng α_1 plus 0.2 ng β_1 cRNA (lanes *4–13*) and incubated for 24 h with [^{35}S]methionine in the presence of BFA. Microsomes were prepared, and aliquots were incubated without trypsin (lanes *1, 4*, and *9*) or with trypsin at indicated trypsin/protein ratios in the presence of 15 mM NaCl (lanes *2* and *5–8*), or 15 mM KCl (lanes *3, 6*, and *10–13*) for 2 h at 25°C before immunoprecipitation with a β antibody. (*B*) Relative changes of the molecular mass of the β subunits shown in *A* as a function of the protease/protein ratio used.

when it was performed in the presence of NaCl (Fig. 9, lanes *4–13*). KCl but not NaCl had also a slight protective effect on the tryptic digestion of individual β subunits (lanes *1–3*), although this effect was much less pronounced than that on assembled β subunits. Thus, these data suggest that the cytoplasmic NH_2 terminus of the β subunit indeed interacts with the α subunit, which leads to a change in its conformation and protects it from proteolytic cleavage. Since the NH_2 terminus of the β subunit is not involved in the structural maturation of the α subunit, it is likely that its interaction with the α subunit is important in the functional modulation of the catalytic subunit.

Discussion

In this study, we document for the first time the multiple effects of subunit assembly on a house-keeping protein, the Na,K-ATPase. In particular, we show that association of the β subunit in or close to the ER is necessary and sufficient for the catalytic α subunit to acquire its structural stability and its main functional properties. Furthermore, our studies reveal that the β subunit itself is subjected to a structural change upon assembly. Finally, we document that the cytoplasmic NH_2 terminus of the β subunit interacts with the α subunit and modulates the K^+-activation of the Na,K-pumps.

Subunit Assembly is Necessary and Sufficient for the Functional Maturation of the Catalytic α Subunit

Individual subunits generally assemble into oligomers at

the level of the ER after extensive folding. Oligomerization is, however, likely to induce an additional structural change that is necessary for the protein to attain its native state and to be transported to its cellular site of action. For many viral and eukaryotic membrane proteins, it has been shown that subunit assembly is involved in their structural maturation, which permits the proteins to escape from the ER quality control. Only assembled subunits can leave the ER and avoid pre-Golgi degradation (for review see 5, 15). On the other hand, only a few examples document how oligomerization influences the functional maturation of proteins. For the pentameric acetylcholine receptor, it was shown that the efficiency of the binding of the specific ligand bungarotoxin is related to the sequential association of the four different yet homologous subunits (14 and references therein). In the case of Na,K-ATPase, we have studied the functional consequences of subunit assembly on the hydrolytic activity, and the ligand-dependent conformational changes, as well as the ouabain binding. The results show that the Na,K-ATPase becomes functionally active at the level of the ER, and this is, to our knowledge, the first demonstration that subunit assembly might be sufficient for the acquisition of the main functional properties of an oligomeric protein.

The basic prerequisite for the cation transport of P-type ATPases is the ligand-dependent adoption of two main conformations, an E_1 and an E_2 conformation (for review see 21). In the case of Na,K-ATPase, the E_1 conformation exposes binding sites for Na^+ to the cytoplasmic face and becomes intermediately phosphorylated from ATP. The change to the E_2 conformation permits the release of Na^+ to the extracellular milieu and the binding of K^+, as well as the interaction with the specific inhibitor ouabain. The transport cycle is closed by an E2 to E1 transition. It has previously been shown on transport-competent, purified enzyme preparations that ligand-dependent conformational changes can be monitored by controlled trypsinolysis, which produces different tryptic fragments of the α subunit in the presence of different ligands and characteristic functional effects (22). In the presence of Na^+, which induces the E_1 conformation, trypsin preferentially cleaves in the first cytoplasmic loop between the transmembrane regions M2 and M3, and in the presence of K^+, which induces the E_2 conformation, trypsin cleaves in the big cytoplasmic loop between M4 and M5.

In this study, we have developed a similar biochemical approach to probe for the ability of newly synthesized α subunits, which were expressed either alone or together with β subunits in BFA-treated oocytes, to perform ligand-dependent conformations. Our data indicate that individual α subunits are devoid of Na^+- and K^+-dependent ATPase activity and cannot undergo conformational transitions permitting ion transport. More data are needed to reveal whether unassembled α subunits might have certain other functional properties. It has indeed been reported in a recent study that individual α subunits exhibit an ATPase activity that occurs in the absence of Na^+ and K^+ but in the presence of Mg^{2+} and that is not inhibited by ouabain (3). In any case, our data show that association with the β subunit at the level of the ER is required for the acquisition of the normal activity of the enzyme. Only β-associated α subunits are able to hydrolyze ATP in an

Na^+-, K^+-dependent and ouabain-inhibitable fashion and to perform Na^+-, K^+-, or ouabain-dependent conformational transitions as reflected by the production of specific tryptic α fragments in the presence of the different ligands. With respect to this latter property, it should be noted that the tryptic fragments produced from our native α subunits (68 and 63 kD) exhibit different molecular masses than those described for purified enzyme preparations obtained by SDS purification (77 and 58 kD in reference 22). The reason for these discrepancies is not clear, but they could result from discrete local differences in the compactness of α subunits in native membranes and of SDS-purified α subunits. A similar deviation from the "classical" splits has been described for α subunits in proteoliposomes (23 and references therein).

Altogether, these results indicate that the newly formed α-β complexes in the ER exhibit similar structural and functional characteristics than the transport-competent α-β complexes expressed at the cell surface. The question may arise whether these potentially transport-competent Na,K-ATPases are active in the ER in intact cells. Although the ionic conditions of the ER lumen are not defined, one might speculate that they would not favor the transport activity of Na,K-pumps. Furthermore, active α-β complexes, once formed in intact cells, do not accumulate but rapidly leave the ER, which might prevent any functional effect in the ER.

According to our results, the β subunit could be considered as a specific molecular chaperone that is necessary for the correct folding of the newly synthesized α subunit. A major challenge for future studies will be to identify the nature of the interaction sites in the two subunits and to reveal the structural changes in the α subunit that permit its functional maturation. In view of the temporal coincidence of the structural maturation characterized by a global increase in the trypsin sensitivity, and the functional maturation of the α subunit, it is possible that both processes are mechanistically linked and might depend on a primary interaction between extracytoplasmic and transmembrane domains of the two subunits. Likely candidates for these interaction domains that we would tentatively call structural interaction sites are the most COOH-terminal part (2), the transmembrane (6, 20) and adjacent regions (7) in the β subunit, and the extracellular loop between M7 and M8 (7) in the α subunit.

The NH₂ Terminus of the β Subunit Interacts with the α Subunit and Modulates its Apparent K^+ Affinity

Interactions in the cytoplasmic domains of subunits of oligomeric membrane proteins have been described, but, as suggested by mutational studies on viral proteins, these interactions generally have little effect on oligomer stability (for review see 5). As suggested by the present study, interactions in the cytoplasmic domains might instead have consequences on functional properties of the completed oligomer.

Increasing experimental evidence suggests that besides their role in the maturation of the α subunit, β subunits have an additional function. Several studies document that β subunits of Na,K-, as well as of H,K-ATPase, might act as indirect modulators of the transport activity and in par-

A.

B.

~2.6 kD

chymotrypsin

15 kD   14 kD

1   10   20   30   M

1. ARDKAKETDGGWRKFIWNADKKEFLGRTGGSWFK|ILLF..

16 kDa

trypsin

~2.4 - 3 kD

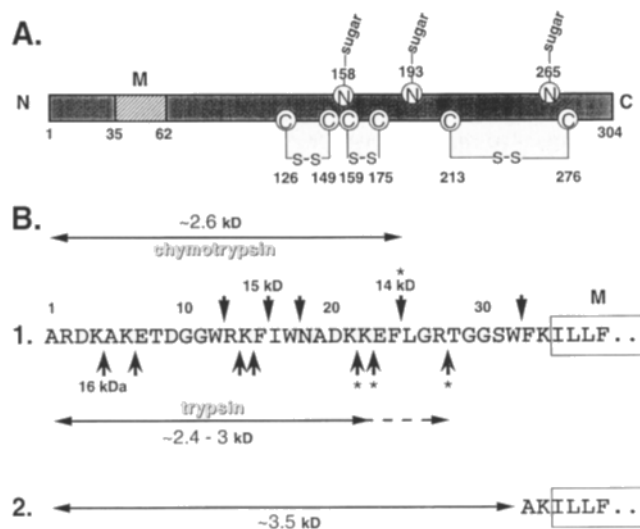**2.** ←————————————————————→ AK|ILLF..
~3.5 kD

Figure 10. Linear model of the β subunit and putative proteolytic cleavage sites in the cytoplasmic NH$_2$-terminal tail. (*A*) Linear model of the *Xenopus* β$_1$ subunit with the cytoplasmic NH$_2$ terminus, the transmembrane domain (*M*), and the COOH-terminal ectodomain. Indicated are the positions of the cysteine (*C*) residues that form disulphide (*S-S*) bridges and the asparagine (*N*) residues that are glycosylated. (*B*) Amino acid sequence of the NH$_2$ terminus of the *Xenopus* wild-type β$_1$ subunit (*1*) and the β$_1$ΔN mutant (*2*). In *B.1*, the putative proteolytic sites for chymotrypsin or trypsin (*arrowheads*) and the approximate molecular mass of the fragments that are removed in the maximally digested β subunits (***) are indicated. Molecular masses above and below arrowheads refer to chymotryptic and tryptic fragments that are produced from splits between the cytoplasmic domain and Arg^{142} in 19-kD membranes (35). In *B.2*, the expected change in the molecular mass of β$_1$ΔN compared to wild-type β$_1$ is indicated. For more details see text.

ticular of the K$^+$-activation of Na,K-pumps (4, 6, 18, 20, 27, 35). Previous studies performed on purified enzyme preparations or using chimeric β subunits have provided indirect evidence that the ectodomain (6, 27) or both the ectodomain and the cytoplasmic plus transmembrane domain (20) might be implicated in the modulatory function of the β subunit. In this study, we provide direct evidence for an interaction of the cytoplasmic NH$_2$ terminus of the β subunit with the α subunit, which depends on the presence of K$^+$ and affects the K$^+$-activation of the Na,K-pumps. The interaction was revealed by a controlled proteolysis assay that shows that the cytoplasmic NH$_2$ terminus of the β subunit becomes protected against degradation when α and β subunits are coexpressed in *Xenopus* oocytes. The link between the interaction of the β NH$_2$ terminus with the α subunit and K$^+$-activation is supported, on the one hand, by the observation that the protection against proteolysis is more pronounced in the presence of K$^+$ than of Na$^+$, and, on the other hand, that α–β complexes that contain a β subunit lacking the NH$_2$ terminus exhibit a different apparent K$^+$-affinity than Na,K-pumps containing wild-type β subunits.

The nature of interaction of the β NH$_2$ terminus with the α subunit is definitely different from that of the ectodomain. Although it occurs soon after synthesis of the subunits at the level of the ER, similar to the interactions

implicated in the structural and functional maturation of the α subunit, the interaction of the cytoplasmic NH$_2$ terminus with the α subunit is not implicated in these processes. Indeed, as previously suggested by Renaud et al. (31), we confirm in this study that NH$_2$-terminally deleted β subunits produce functional Na,K-pumps at the cell surface. Altogether, our data indicate that the cytoplasmic NH$_2$ terminus of the β subunit represents a functional interaction site in opposition to the structural interaction sites mainly needed for the early maturation of the catalytic α subunit.

The involvement of the β NH$_2$ terminus in the K$^+$-activation is supported by our recent observation that NH$_2$-terminally deleted mutants expressed in *Xenopus* oocytes together with α subunits influence the apparent K$^+$-affinity of the Na,K-pump current (Jaisser, F., X. Wang, K. Geering, and J.D. Horisberger, manuscript in preparation). In this study, we were able to show that a stretch of eight amino acids close to the transmembrane domain might be important for the functional effect. An implication of the cytoplasmic NH$_2$ terminus of the β subunit was also proposed recently in Rb$^+$ occlusion (36). These studies were performed on 19-kD membranes that are produced by extensive tryptic digestion of purified Na,K-ATPase preparations and that contain a 19-kD COOH-terminal fragment of the α subunit and an NH$_2$-terminal 16-kD and a COOH-terminal 50-kD fragment of the β subunit. Rb$^+$ and Na$^+$ occlusion as well as ouabain binding are preserved in these preparations. It was shown that at 37°C, but not at 20°C, cleavage of the β NH$_2$ terminus by chymotrypsin from a 16- to a 15-kD fragment (Fig. 10) reduced Rb$^+$ affinity and increased deocclusion rates.

How could the cytoplasmic NH$_2$ terminus affect the binding of extracellular K$^+$? One possibility is that the cytoplasmic NH$_2$ terminus is part of the intramembraneous K$^+$ binding site. Our observation that interaction of the β NH$_2$ terminus with the α subunit is favored in the presence of K$^+$ but not in the presence of Na$^+$ would be in agreement with a direct sensing of K$^+$ by the NH$_2$ terminus. An indirect conformational effect cannot, however, be excluded since, as discussed above, it is indeed likely that several regions determine the functional interaction in the β subunit. It might be envisioned that the binding of K$^+$ either to another site in the β subunit or to the α subunit might indirectly influence a "gating" property of the β NH$_2$ terminus.

In conclusion, our studies demonstrate that the NH$_2$ terminus of the β subunit interacts with the α subunit in a K$^+$-dependent fashion and that this functional interaction is implicated in the modulation of the K$^+$-activation of Na,K-pumps at the cell surface rather than in their maturation. Further studies are needed to define more precisely the identity of the amino acids in the β and the α subunits involved in this interaction and the nature of the functional effects.

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