Structural and Functional Features of the Transmembrane Domain of the Na,K-ATPase β Subunit Revealed by Tryptophan Scanning*

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In oligomeric P2-ATPases such as Na,K- and H,K-ATPases, β subunits play a fundamental role in the structural and functional maturation of the catalytic α subunit. In the present study we performed a tryptophan scanning analysis on the transmembrane α-helix of the Na,K-ATPase β1 subunit to investigate its role in the stabilization of the α subunit, the endoplasmic reticulum exit of α-β complexes, and the acquisition of functional properties of the Na,K-ATPase. Single or multiple tryptophan substitutions in the β subunits transmembrane domain had no significant effect on the structural maturation of α subunits expressed in Xenopus oocytes nor on the level of expression of functional Na,K pumps at the cell surface. Furthermore, tryptophan substitutions in regions of the transmembrane α-helix containing two GXXG transmembrane helix interaction motifs or a cysteine residue, which can be cross-linked to transmembrane helix M8 of the α subunit, had no effect on the apparent K⁺ affinity of Na,K-ATPase. On the other hand, substitutions by tryptophan, serine, alanine, or cysteine, but not by phenylalanine of two highly conserved tyrosine residues, Tyr²⁰⁴ and Tyr⁴⁴, on another face of the transmembrane helix, perturb the transport kinetics of Na,K pumps in an additive way. These results indicate that at least two faces of the β subunits transmembrane helix contribute to inter- or intrasubunit interactions and that two tyrosine residues aligned in the β subunits transmembrane α-helix are determinants of intrinsic transport characteristics of Na,K-ATPase.

P-type ATPases represent a family of ubiquitous transporters which are characterized by the formation of a phosphorylated intermediate during the catalytic cycle and which are mainly involved in cation homeostasis. Over 200 members of this family have been identified (1). Of these, only animal Na,K- and H,K-ATPase isozymes and bacterial K-ATPase isozymes, are oligomeric with 1 and 3 subunits, respectively, in addition to the catalytic subunit. Na,K- and H,K-ATPase α subunits have the highest sequence identity among the P-type ATPases. Based on the membrane topology, the catalytic α subunits of Na,K- and H,K-ATPases belong to the group of P2-ATPases (2) which according to the crystal structure of the Ca²⁺-ATPase contains 10 transmembrane (TM)

1 segments (3). The β subunits associated with Na,K- and H,K-ATPases are type II proteins with one TM segment, a short cytoplasmic tail, and a large ectodomain containing several sugar chains and 3 disulfide bridges. So far, three Na,K-ATPase β isoforms and one H,K-ATPase β subunit have been identified.

The unique presence of β subunits in Na,K- and H,K-ATPases remains intriguing both from a functional and evolutionary point of view. At present, we know that the β subunit has two main functions. Of primary importance is its role as a specific chaperone which favors the correct membrane insertion and hence the resistance against proteolysis and cellular degradation of the newly synthesized α subunits of Na,K- and H,K-ATPases (4–7). Since KdpC subunits of the bacterial KdpFABC transporter may have a similar function (8), it has been speculated that the β subunits of Na,K- and H,K-ATPases may be remnants of the bacterial KdpC subunit that have been eliminated in other P2-ATPases (1). Based on topology studies on Na,K- and H,K-ATPase α subunits, we have also suggested that the K⁺ transport function common to all oligomeric P-type ATPases, is associated with a particular amino acid composition that is not compatible with efficient membrane insertion of the α subunits. This has required that during evolution, K⁺ transporting α subunits had to assemble with a helper protein in order to assist their correct membrane integration (9). In addition to their chaperone function, β subunits also influence the cation sensitivity of oligomeric P-type ATPases expressed at the cell surface. The association of the α subunit with different β isoforms (10) or N-terminal truncated β subunits (11, 12) produces Na,K-ATPases with different apparent affinities for Na⁺ and K⁺.

An important issue concerning the structure-function relationship of Na,K- and H,K-ATPases is the identification of the matching interaction sites in the α and the β subunits that are responsible for the chaperone function and/or the transport-modulating effect of the β subunit. Experimental evidence suggests that α and β subunits interact in the extracytoplasmic, the TM, and the cytoplasmic domains. In α subunits of both Na,K-ATPase (13) and H,K-ATPase (14), the most clearly defined interaction site is located in the extracytoplasmic loop between the TM segment M7 and M8. Interaction of the β subunit with this region was shown to be important for the correct membrane insertion and the structural maturation of the Na,K- and H,K-ATPase α subunit (5–7). According to results obtained using the yeast two-hybrid system, the M7 and M8 α-domain interacts with an extracytoplasmic region of the β subunit contained within the 64 amino acids adjacent to the TM domain (13). However, mutational and immunological

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1 The abbreviations used are: TM, transmembrane; NMDG, N-methyl-D-glucamine; ER, endoplasmic reticulum.
studies suggest that other regions in the extracytoplasmic domain of the β subunit such as the 10 most C-terminal amino acids (15) or a YPPYG sequence conserved in all known β subunits (16, 17) might as well participate in α-β interactions and contribute to the stabilization of the α subunit. As suggested by results obtained using chimeras between different β isoforms (12, 18), interactions in the ectodomains of the α and the β subunit might also be responsible for the β subunits effects on the cation sensitivity of Na,K-ATPase.

Controlled proteolysis assays performed on Na,K-ATPase β subunits, indicate that α and β subunits also interact in the cytoplasmic domain (11). A mutational analysis indicates that these interactions are not necessary for the structural maturation of the α subunit (11, 12), nor do they directly influence apparent Na+ or K+ affinities of Na,K-ATPase (12, 19). On the other hand, it cannot be excluded that cytoplasmic α-β interactions contribute to some discrete steps in the catalytic cycle of Na,K-ATPase as suggested by Na+ occlusion and electrogenic binding assays (20).

Interactions in the TM domains of the α and β subunits are the least well understood both in molecular and functional terms. So far, cross-linking experiments have provided evidence that the TM domain of the β subunit may be in contact with M8 of the α subunit (21, 22), but nothing is known on the functional implications of this possible intersubunit interaction.

In this study, we have aimed to identify amino acid residues in the β TM domain that interact with the α subunit and to determine the putative functional role of this interaction by using a tryptophan scanning analysis. Tryptophan scanning has previously been used to determine structural features of integral membrane proteins (23–25). Tryptophan was chosen because of its moderately hydrophobic properties and its large size. The results of previous studies are consistent with the hypothesis that the TM domain of the Na,K-ATPase as suggested by Na+,K+ affinities of Na,K-ATPase (20) or K+ affinities of Na,K-ATPase (12, 19). On the other hand, it cannot be excluded that cytoplasmic α-β interactions contribute to some discrete steps in the catalytic cycle of Na,K-ATPase as suggested by Na+ occlusion and electrogenic binding assays (20).

Site-directed Mutagenesis—Amino acids Ile16 to Glu56 of Xenopus Na,K-ATPase β (β1NK) were individually replaced by tryptophan residues using the polymerase chain reaction method described by Nelson and Long (26). Briefly, fragments of β1NK contained in a pSD5 vector (pSD5β1NK) were first amplified by polymerase chain reaction using sense oligonucleotides containing mutated sequences coding for tryptophan and an antisense oligonucleotide of nucleotides 628–420 tagged by primer D of Nelson and Long (26) and using pSD5 NK as a template. Nhel and PvuII restriction sites were used to introduce the amplified fragment into the pSD5 vector containing NK/HH β subunit was prepared as described previously (27). NK/HH Y40W/Y44W was prepared by amplyfying a fragment of β1NK as described above using a sense oligonucleotide containing both mutated sequences, an antisense oligonucleotide encoding nucleotides 420–440 tailed by primer D of Nelson and Long (26) and using pSD5 DS NK as a template. Nhel and Stul restriction sites were used to introduce the amplified fragment into the pSD5 vector containing NK/HH. β1 Y43W/Y47W was prepared by amplyfying a fragment of β3NK contained in a pSD5 vector (pSD5β3NK) using a sense oligonucleotide containing both mutated sequences, an antisense oligonucleotide encoding nucleotides 301–320 tailed by primer D of Nelson and Long (26) and using pSD5β3NK as a template. The mutated DNA fragment was introduced into wild type pSD5β3NK using Nhel and Stul restriction sites. The oligonucleotide sequences of all constructs were confirmed by dideoxy sequencing. cRNAs coding for Bufo Na,K-ATPase a1 (28), Xenopus Na,K-ATPase a1 (29), Xenopus β1 (β29), β3 (30 subunits, and β subunit mutants were obtained by in vitro transcription (31).

The Transmembrane Domain of the Na,K-ATPase

β Subunits—Oocytes were obtained from Xenopus females as described (4). Routinely, 7 ng of Bufo marinus or α 0.8 ng of Xenopus β subunit cRNAs were injected into oocytes. Oocytes were incubated in modified Barth’s medium containing [35S]methionine (0.5 μCi/ml) for 6 h and then subjected to 24 and 72 h chase periods in the presence of 10 mM cold methionine. Digitonin extracts were prepared as described (11) and the α subunit was immunoprecipitated using a Bufo a1 subunit antibody (32) under nondenaturing conditions as described (11) allowing co-immunoprecipitation of the associated β subunit. The dissociated immune complexes were separated by SDS-polyacrylamide gel electrophoresis and labeled proteins were detected by fluorography. Quantification of immunoprecipitated bands was performed with a laser densitometer (LKB Ultrascan 2202).

Expression in Xenopus Oocytes and Immunoprecipitation of α and β Subunits—Oocytes were obtained from Xenopus females as described (4). Routinely, 7 ng of Bufo marinus or α 0.8 ng of Xenopus subunit cRNAs were injected into oocytes. Oocytes were incubated in modified Barth’s medium containing [35S]methionine (0.5 μCi/ml) for 6 h and then subjected to 24 and 72 h chase periods in the presence of 10 mM cold methionine. Digitonin extracts were prepared as described (11) and the α subunit was immunoprecipitated using a Bufo a1 subunit antibody (32) under non-denaturing conditions as described (11) allowing co-immunoprecipitation of the associated β subunit. The dissociated immune complexes were separated by SDS-polyacrylamide gel electrophoresis and labeled proteins were detected by fluorography. Quantification of immunoprecipitated bands was performed with a laser densitometer (LKB Ultrascan 2202).

Na,K Pump Current Measurements and Determination of Apparent K+ and Na+ Affinities—Na,K pump activity was measured as the K+-induced outward current using the two-electrode voltage clamp method as described earlier (10). Current measurements were performed 3 days after injection of oocytes with Bufo a1 and either wild type or mutant β cRNAs. One day before performing measurements, oocytes were loaded with 3 mM Ba 2+ in a K+-free solution containing 200 mM ouabain, a concentration that inhibits the endogenous Na,K pumps but not the ouabain-resistant exogenous Bufo Na,K pumps (33).

The K+ activation of the Na,K pump current was determined in a Na+-containing solution (80 mM sodium gluconate, 0.82 mM MgCl2, 0.41 mM CaCl2, 10 mM N-methyl-D-glucamine (NMDG)-HEPES, 5 mM BaCl2, 10 mM tetrathylammonium chloride, pH 7.4) or in a nominally Na+-free solution containing 140 mM sucrose. The current induced by increasing concentrations of K+ (0.5, 1.0, 3.3, and 10 mM K+ in the presence of Na+ and 0.02, 0.1, 0.5, and 5.0 mM K+ in the absence of Na+) was measured either at ~50 mV or during a series of nine 200-ms voltage steps ranging from −130 to +30 mV. To determine the kinetic parameters such as maximal currents (I\text{max}) and half-activation constants (K1/2) for K+ the Hill equation was fitted to the data of the current (I) induced by various K+ concentrations (K) using a least square method: I = I\text{max}K1/2 ((K/K1/2)\text{H})/((K/K1/2)\text{H}+1). According to previous studies (10), the Hill coefficient (nH) was set to a value of 1.6 for experiments performed in the presence of external Na+. Measurements of the half-activation constant for internal Na+ was performed as previously described (12). Briefly, in addition to Na,K-ATPase α and β cRNAs, oocytes were injected with cRNAs coding for α, β, and γ subunits (0.3 ng of each subunit/oocyte) of the rat epithelial Na+ channel, rENaC (34), and were incubated for 3 days in a modified Barth’s solution containing 10 mM Na+. One day before measurements, oocytes were incubated in a Na+-free solution (50 mM NMDG-Cl, 40 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM NMDG-HEPES, pH 7.4) in order to render the Na+ current using the same method as described above.

Intracellular Na+ concentrations were calculated from the reversal potential of the amiloride-sensitive current obtained from a pair of I-V curves recorded with and without amiloride in a solution containing 5 mM Na+ (5 mM sodium gluconate, 0.5 mM MgCl2, 2.5 mM BaCl2, 95 mM NMDG-Cl, 10 mM NMDG-HEPES, pH 7.4). At each intracellular Na+ concentration ([Na]i) the Na,K pump K+ - activated current (I\text{K}) was...
measured in the presence of 20 μM amiloride and in the absence or presence of external Na⁺ (see above) by addition of 5 or 10 mM K⁺. Maximal pump currents (I_{max,Na}) and half-activation constants for Na⁺ (K_{1/2,Na}) were determined by fitting the Hill equation [I = I_{max,Na} / (1 + (K_{1/2,Na}/[Na])^n)] to the measured I_Na and [Na] values. Six to eight pairs of measurements of [Na], and of the Na,K pump K⁺-activated current were performed successively on each oocyte at −50 mV. Between each pair of measurements, the oocytes were allowed to increase their intracellular Na⁺ concentration by exposure to a 100 mM Na⁺ solution (100 mM sodium gluconate, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM Na-HEPES, pH 7.4) in the absence of amiloride and at a holding potential of −50 to −100 mV.

Measurements of the apparent affinity for external Na⁺ were performed using ouabain-sensitive Xenopus α1 subunits by measuring the inhibition of the K⁺-induced current by external Na⁺. 3 days after oocyte injection and 1 day before measurements, oocytes were loaded with Na⁺ in a K⁺-free solution. The Na,K pump current induced by 1 mM K⁺ was measured in a nominally Na⁺-free solution (120 mM NMDG-gluconate, 0.82 mM MgCl₂, 0.41 mM CaCl₂, 10 mM NMDG-HEPES, 5 mM BaCl₂, 10 mM tetraethylammonium chloride, pH 7.4) and in the presence of 10, 30, 60, and 120 mM Na⁺ (NMDG-gluconate was replaced by sodium gluconate) during a series of nine 200-ms voltage steps ranging from −130 to +30 mV. The Na,K pump was then blocked by the addition of 100 μM ouabain and the same series of measurements was repeated. Currents specific for the Na,K pump could be deduced by subtraction of the currents observed in the presence of ouabain from that observed in its absence. A nonsaturating concentration of K⁺ (1 mM) was chosen in order to reveal the competition of external Na⁺ with K⁺ ions for extracellular cation-binding sites. At each external Na⁺ concentration, the averaged endogenous, ouabain-sensitive Na,K pump current was subtracted from total ouabain-sensitive currents measured in oocytes expressing exogenous Na,K pumps.

The decrease in the Na,K pump current produced by exposure to external Na⁺ was used to determine the half-inhibition constant for external Na⁺ (K_{1/2,Na}) by fitting the Hill equation [I = I_{max,Na} / (1 − 1/2 + (K_{1/2,Na}/[Na])^n)] to the data of the current (I) observed at each concentration of external Na⁺. Means of the Na,K pump currents produced in oocytes expressing α subunits and wild type or mutant β subunits were compared by unpaired Student’s t test.

Na,K-ATPase Activity Measurements—Na,K-ATPase activity was measured in micromolar fractions prepared as previously described (11) from oocytes expressing Xenopus α subunits and wild type β1 subunits or β1 Y40W/Y44W mutants. Before activity measurements, samples were freeze-thawed twice in liquid nitrogen. Na,K-ATPase activity was measured in triplicate by an enzyme-linked assay, according to Schoner et al., 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). The substrate concentrations of the reaction mixture were 5 mM KCl, 100 mM NaCl, 4 mM ATP, and 4 mM MgCl₂. To reduce nonspecific, mitochondrial ATPase activities, 5 mM NaN₃ were added to the reaction mixture. Activity measurements were done in the presence or absence of 10⁻⁷–10⁻⁴ M orthovanadate. The specific enzyme activity was calculated as the difference between samples incubated in the presence or absence of 1 mM ouabain. In the absence of vanadate, ouabain-sensitive activities represented 20–30% of the total enzyme activity. Statistical analysis was done by unpaired Student’s t test.

RESULTS

Structural Features of the Na,K-ATPase β Subunits TM Domain—Fig. 1A shows the membrane-spanning domain of the β1 subunit of Xenopus Na,K-ATPase, as previously defined (19). The TM domain of the Xenopus β1 subunit comprises 23 amino acids and shows a sequence identity of 43% with Xenopus β2, 65% with β3, and 43% with the β subunit of chicken H,K-ATPase. Secondary structure prediction on the β1 TM domain revealed an α-helical structure with at least two distinct faces (Fig. 1B). One face is characterized by three aligned glycine residues (Gly₄⁵, Gly₄⁶, and Gly₅₃) forming a cleft in the α-helix. The two tyrosine residues are conserved in all known β subunits whereas the three glycine residues are only found in Na,K-ATPase β1 isoforms and certain β3 isoforms. Other Na,K-ATPase β isoforms and H,K-ATPase β subunits contain 1 or 2 glycine residues corresponding to either Gly₄⁵ and/or Gly₄⁶. Three phenylalanine residues (Phe₁⁵, Phe₁⁶, and Phe₁⁷) are present in the TM domain of all Na,K-ATPase β isoforms whereas the H,K-ATPase β subunit contains a conservative substitution of tyrosine for Phe₁⁷. Na,K-ATPase β2 and β3 isoforms contain a fourth phenylalanine residue which is replaced by a cysteine residue (Cys₂⁵) in β1 isoforms (Fig. 1, A and B). In order to identify the amino acid residues in the β TM domain that interact with the α subunit and to determine the putative functional role of this interaction, amino acids encompassing Ile₂⁶ to Gln₂⁶ were substituted individually or in combination by tryptophan.

Stabilization of Na,K-ATPase α Subunits by β Subunit Tryptophan Mutants—We first tested whether tryptophan substitutions in the TM domain of the β subunit might interfere with the structural maturation of the α subunit. Bufo α subunits were expressed in Xenopus oocytes alone or together with Xenopus wild type or mutant β1 subunits and the cellular expression of the α-β complexes was followed by immunoprecipitation of metabolically labeled proteins after a pulse and various chase periods. As expected, α subunits expressed without β subunits were degraded during a 48-h chase period (Fig. 2, lanes 10 and 11). Similar to wild type β1 subunits (Fig. 2, lanes 1–3), all β-mutants assembled with and stabilized α-subunits as illustrated for the triple glycine mutant G45W/G46W/G52W (lanes 7–9) and the double tyrosine mutant Y40W/Y46W (lanes 7–9). In addition, all β subunit mutants assembled with α subunits became fully glycosylated during the chase periods (lanes 5, 6, 8, and 9) indicating that the α-β complexes were able to leave the ER. Thus, these results show that introduction of tryptophan residues into the β1 TM domain does not significantly affect the structural maturation of the α subunit.

Substitution of Tyr₄⁰ and Tyr₄⁴ in the β Subunits TM Domain by Tryptophan Modifies the K⁺ Activation Kinetics of
**FIG. 2.** Assembly, stabilization, and intracellular transport of Na,K-ATPase α-β complexes containing tryptophan mutations in the β subunit’s TM domain. Xenopus oocytes were injected with 7 ng of Bufo αNK cRNA alone (lanes 10 and 11) or together with 0.8 ng of either Xenopus wild type (β1wt) (lanes 1–3) or mutant β1 (lanes 4–9) cRNAs. After a 6-h pulse period with [35S]methionine (lanes 1, 4, 7, and 10) and after 24 h (lanes 5, 8, and 11) and 72 h (lanes 3, 6, and 9) chase periods, digitonin extracts were prepared and immunoprecipitations were performed using an anti-α subunit antibody under non-denaturing conditions which allowed co-immunoprecipitation of associated β subunits. α and β subunits were revealed by SDS-polyacrylamide gel electrophoresis and fluorography. One out of two to four similar experiments is shown. cg, core glycosylated β subunit; fg, fully glycosylated β subunit.

**Na,K Pumps**—To determine the functional expression and the transport properties of Na,K pumps associated with wild type or mutant β subunits, K+-induced outward currents were measured on intact oocytes by the two electrode voltage clamp technique. Maximal pump currents (I\(_{\text{max}}\)) varied but were not consistently different in oocytes expressing wild type or mutant α-β complexes (Fig. 3A), indicating that none of the β tryptophan mutants affected the overall transport activity. However, measurements of the apparent affinities for external K+ revealed a significant increase in the half-activation constants for K\(^+\) (K\(_{1/2}^+\)) for pumps associated with β1 mutants in β1 wt subunits or β1 tryptophan mutants. Three days after injection, maximal K+-induced Na,K pump currents (I\(_{\text{max}}\)) were measured at −50 mV in the presence of external Na+ as described under “Experimental Procedures.” Measurements were performed in the presence of 200 mM ouabain which blocks endogenous, oocyte Na,K pumps but not the exogenous, moderately ouabain-resistant Bufo Na,K pumps. The I\(_{\text{max}}\) value for Na,K pumps containing wild type β1 subunits (691.6 ± 62 nA) or β1 tryptophan mutants were extrapolated from K\(^+\) activation curves permitting estimations of K\(_{1/2}^+\) values shown in B. Shown are mean ± S.E. of data obtained from 14 to 28 oocytes from two to four different Xenopus females. B, apparent affinities for external K+ of Na,K pumps containing wild type β1 subunits or β1 tryptophan mutants. Half-activation constants for external K\(^+\) (K\(_{1/2}^+\)) were determined at −50 mV in the presence of external Na\(^+\) as described under “Experimental Procedures.” The K\(_{1/2}^+\) value for Na,K pumps containing wild type β1 subunits was arbitrary set to 1. Shown are mean ± S.E. of data obtained from 14 to 28 oocytes from two to four different Xenopus females. *, p < 0.01 compared with wild type α-β complexes.

K\(_{1/2}^+\) values determined in the presence of external Na\(^+\) (compare Fig. 4, A and B, lanes 1 and 2). These results indicate that tyrosine mutations produce highly specific effects on discrete steps in the transport cycle.

Since the two tyrosine residues are highly conserved in the TM domains of all β subunits identified so far, we also investigated K\(_{1/2}^+\) values of Na,K pumps associated with tyrosine mutants of other β isoforms, namely of the Na,K-ATPases β3 isofrom. The corresponding tyrosine residues of the Xenopus β3 isoform, Tyr43 and Tyr47, were replaced by tryptophan residues (β3 Y43W/Y47W) and K\(_{1/2}^+\) values of mutant α-β3 complexes were compared with those of wild type α-β3 complexes, both in the presence and absence of external Na\(^+\). As previously reported (10), K\(_{1/2}^+\) values measured in the presence, but not in the absence of external Na\(^+\), were about 2-fold higher for wild type α1-β3 complexes than for wild type α1-β1 complexes (Fig. 4, A and B, compare lanes 1 and 5). Similar to the results obtained with the β1 Y40W/Y44W mutant, the β3 Y43W/Y47W
FIG. 4. Apparent affinities for external K\(^+\) of Na,K pumps associated with \(\beta_1\) or \(\beta_3\) mutants or with \(\beta_{NK/HK}\) chimera. A and B, \(K^+\) activation of wild type and mutant \(\alpha\)-complexes. Oocyte cRNA injection was as described in the legend to Fig. 3. Half-activation constants for external \(K^+\) \(K_{1/2}^+\) were determined at \(-50\) mV in the presence (A) or absence (B) of external Na\(^+\) as described under “Experimental Procedures.” Shown are mean ± S.E. of data obtained from 10 to 20 oocytes from two to three different \(Xenopus\) females. *, \(p < 0.01\) compared with wild type \(\alpha\)-complexes (lane 1); NS, statistically not significantly different from wild type \(\alpha\)-complexes. Inset, maximal Na,K pump currents \(I_{\text{max}}\) extrapolated from the \(K^+\) activation curves. D, voltage dependence of \(K_{1/2}^+\)\(K^+\) values. \(K^+\)-induced currents were measured during a series of nine 200-ms voltage steps ranging from \(-130\) to \(+30\) mV in oocytes injected with \(\alpha\) and wild type \(\beta_1\) (squares) or \(\beta_1\) Y40W/Y44W mutant (triangles) cRNA. \(K_{1/2}^+\) values were determined after each voltage step in the presence of external Na\(^+\) as described under “Experimental Procedures.” Shown are mean ± S.E. of data obtained from 14 oocytes from two to three different \(Xenopus\) females. *, \(p < 0.01\) compared with wild type Na,K pumps. Inset, maximal Na,K pump currents \(I_{\text{max}}\).

Previous studies have shown that gastric H,K-ATPase \(\beta\) subunits can associate with Na,K-ATPase \(\alpha\) subunits and produce \(\alpha\)-\(\beta_{NK}\) complexes which exhibit higher \(K_{1/2}^+\) values than \(\alpha\)-\(\beta_{NK}\) complexes (10). As suggested by analysis of chimeras between \(\beta_{NK}\) and \(\beta_{HK}\), the reduced apparent \(K^+\) affinity of \(\alpha\)-\(\beta_{HK}\) complexes compared with \(\alpha\)-\(\beta_{NK}\) complexes is mainly mediated by the ectodomain of \(\beta_{HK}\) (12). To test whether the \(K^+\) effect of the TM domain and the ectodomain of \(\beta\) subunits is additive, the two tyrosine residues in the TM domain of a \(\beta_{NK/HK}\) chimera (containing the cytoplasmic and the TM domain of \(\beta_{NK}\) and the ectodomain of \(\beta_{HK}\)) were substituted by tryptophan. The \(\beta\) \(\beta_{NK/HK}\) chimera, rather than the wild type \(\beta_{HK}\), was chosen for these studies since \(\beta_{HK}\) only produces a small population of pumps which are functionally active (12). As previously reported (12), Na,K pumps containing \(\beta\) \(\beta_{NK/HK}\) chimeras produced high \(I_{\text{max}}\) values and the \(K_{1/2}^+\) value was similar to \(\alpha\)-\(\beta_{HK}\) complexes and about 2.5-fold higher than \(I_{\text{max}}\) of \(\alpha\)-\(\beta_{NK}\) pump containing wild type \(\beta_1\) subunits both in the presence and absence of external Na\(^+\) (Fig. 4A and B, compare lanes 1 and 7). Compared with \(\alpha\)-\(\beta_{NK/HK}\) complexes, \(\alpha\)-\(\beta_{HK}\) pumps which were associated with the \(\beta\) \(\beta_{NK/HK}\) chimera containing the Y40W/Y44W mutations exhibited \(K_{1/2}^+\) values which were slightly but significantly increased in the absence of external Na\(^+\) (Fig. 4B, compare lanes 7 and 8). These values were more than two times higher in the presence of external Na\(^+\) (Fig. 4A, compare lanes 7 and 8).

These results suggest that the effect of the \(\beta\) subunits TM domain and ectodomain on the apparent \(K^+\) affinity are complementary. Since the relative increase in \(K_{1/2}^+\) values of Na,K pumps containing double tryptophan mutants of \(\beta_1\) isoforms, \(\beta_3\) isoforms, and \(\beta_{NK/HK}\) chimeras was similar, our results also indicate that the \(K^+\) effect of the TM domain is of general functional relevance.

To further characterize the potential functional interaction of the \(\beta\) subunit with the \(\alpha\) subunit in the TM domain, Tyr\(^{40}\) and Tyr\(^{44}\) were replaced by several amino acids other than tryptophan. \(\alpha\)-\(\beta\) Complexes containing double phenylalanine, serine, cysteine, or alanine \(\beta\)-mutants produced similar Na,K pump currents as wild type Na,K pumps (see inset in Fig. 4C). \(K_{1/2}^+\) values of Na,K pumps associated with double serine, cysteine, or alanine \(\beta\) mutants were significantly higher than that of wild type Na,K pumps (Fig. 4C, compare lane 1 to lanes \(4–6\)), but were lower than that of Na,K pumps associated with the double tryptophan \(\beta_1\) mutant (compare lane 2 to lanes \(4–6\)). On the other hand, a double phenylalanine \(\beta_1\) mutant produced Na,K pumps with a \(K_{1/2}^+\) value which was similar to that of wild type pumps (compare lanes 1 and 3). These results indicate that correctly positioned aromatic side chains, present in tyrosine and phenylalanine but not in serine, cysteine, and alanine residues, are essential for correct interaction of the \(\beta\) subunits TM domain with the \(\alpha\) subunit and the inherent Na,K pump function. The most pronounced, functional perturbations produced by the double tryptophan \(\beta\) mutant could be due to the large side chain of tryptophan which as predicted (23) may favor disruption of helix-helix interactions.
by inducing local structural changes.

To gain further insight into the functional effect of the β subunits TM domain on the transport properties of the Na,K-ATPase α subunit, we next compared the voltage dependence of Na,K pump currents in the presence of external Na or K and wild type β1 subunits or β1 Y40W/Y44W mutants. As previously observed (10), maximal Na,K pump currents of wild type Na,K pumps composed of α1 and β1 subunits are slightly voltage-dependent (Fig. 4D, inset). Na,K pumps associated with β1 Y40W/Y44W mutants exhibited a stronger voltage dependence and produced lower I max values than wild type Na,K pumps, in particular at highly negative membrane potentials (Fig. 4D, inset). K 1/2 K- values of mutant Na,K pumps were significantly higher than those of wild type Na,K pumps over the whole potential range and the difference was particularly pronounced at very negative membrane potentials (Fig. 4D).

Substitution of Tyr 40 and Tyr 44 in the β subunit TM domain may shift the E1-E2 conformational equilibrium to the E2 state. To test this hypothesis, we investigated the effect of Tyr 40/Tyr 44 β mutations on the apparent affinities of Na,K pumps for intra- and extracellular Na and K.

The apparent affinity for intracellular Na of wild type and mutant Na,K pumps was measured as described under “Experimental Procedures.” Fig. 5A shows that Na,K pumps containing β1 Y40W/Y44W mutants had a slightly higher apparent affinity for intracellular Na than wild type Na,K pumps.

An estimate for the apparent affinity for extracellular Na (K 1/2 Na ext) of wild type and mutant Na,K pumps was obtained by measuring the inhibition of Na,K pump currents as a function of increasing concentrations of external Na (Fig. 5B). When measured at +30 mV, inhibition of Na,K pump currents by external Na was similar for Na,K pumps associated with wild type or mutant β subunits. However, when Na,K pump currents were measured at a membrane potential of –130 or –50 mV, external Na had a significantly stronger inhibitory effect on Na,K pumps containing than on those containing the wild type β1 subunit (Fig. 5B). These results indicate that mutant α-β complexes have a higher, voltage-dependent, apparent affinity for external Na than wild type α-β complexes.

Vanadate Sensitivity of Na,K-ATPase Containing Wild Type β Subunits or β1 Y40W/Y44W Mutants—The parallel decrease in the apparent affinity for external K (K 1/2 K ext) of wild type and mutant Na,K pumps was obtained by measuring the inhibition of Na,K pump currents as a function of increasing concentrations of external K (Fig. 5B). When measured at +30 mV, inhibition of Na,K pump currents by external K was similar for Na,K pumps associated with wild type or mutant β subunits. However, when Na,K pump currents were measured at a membrane potential of –130 or –50 mV, external K had a significantly stronger inhibitory effect on Na,K pumps containing than on those containing the wild type β1 subunit (Fig. 5B). These results indicate that mutant α-β complexes have a higher, voltage-dependent, apparent affinity for external K than wild type α-β complexes.

The Transmembrane Domain of the Na,K-ATPase α Subunit

**FIG. 5.** Apparent affinities for intracellular and extracellular Na of Na,K-ATPase α-β complexes containing wild type β1 subunits or β1 Y40W/Y44W mutants. A, activation of wild type and mutant Na,K pumps by intracellular Na. Oocytes were injected with Na,K-ATPase α and β cRNAs (7 and 0.8 ng, respectively) together with cRNAs coding for α, β, and γ subunits of the rat epithelial sodium channel (0.3 ng of each). Half-activation constants for intracellular Na (K 1/2 Na int) were determined as described under “Experimental Procedures.” Shown are mean ± S.E. of data obtained from 11 oocytes from 3 different Xenopus females. *, p < 0.01 compared with wild type α-β complexes (lane 1). Inset, maximal Na,K pump currents (I max). B, inhibition of wild type and mutant Na,K pumps by extracellular Na. Oocytes were injected with Xenopus α cRNA and wild type β1 (squares) or β1 Y40W/Y44W mutant (triangles) cRNAs. Na,K pump currents (I) were determined at –130, –50, and +30 mV in the presence of various concentrations of extracellular Na, as described under “Experimental Procedures.” Shown are mean ± S.E. of data obtained from 9 and 11 oocytes from two different Xenopus females for wild type and mutant pumps, respectively. *, p < 0.01 compared with wild type Na,K pumps.
performed in triplicate on microsomes from 2 different batches of oo-
cells. The results shown are mean ± S.E. of two independent experiments
performed in triplicate on microsomes from 2 different batches of oo-
cytes. A statistically significant difference (p < 0.01) between the van-
adate sensitivity of Na,K-ATPase in microsomes from oocytes express-
ing wild type β subunits and β mutants is indicated by an asterisk.

### DISCUSSION

By performing a tryptophan-scanning analysis we have identi-
ﬁed two highly conserved tyrosine residues in the TM domain
of Na,K-ATPase β subunits which are implicated in Na,K pump
function. This result provides compelling evidence for interac-
tions of α and β subunits in the TM domain and highlights the role
of the β subunit as a determinant of intrinsic Na,K-ATPase
transport properties.

Various parameters were tested to assess the functional role
of the Na,K-ATPase β subunits TM domain including its role in
stabilizing the Na,K-ATPase α subunit, in favoring a folding state
that permits ER exit of α-β complexes, and in conveying
particular transport properties to Na,K-ATPase.

All β mutants containing individual or combined tryptophan
substitutions in the TM domain were able to stabilize α sub-
units and to permit ER exit of α-β complexes, indicating that no single amino acid, nor Tyr40/Tyr44' and Gly45/Gly48/Gly54 when mutated in combination are necessary for the structural maturation
of Na,K-ATPase. These results apparently contrast with previous results obtained with chimeras between Na,K- and H,K-ATPase β subunits, which showed that the presence of the TM domain of the Na,K-ATPase β subunit is needed for a
concordant interaction with the Na,K-ATPase α subunit permitting
ER exit of α-β complexes (12). Although it cannot entirely be
excluded that tryptophan substitutions may allow for proper β
assembly, it is more likely that the results obtained in this study
and previous studies indicate that a certain overall integrity rather
than individual amino acids may be necessary for α-β interactions
that mediate some speciﬁc steps in the maturation process.

This conclusion is also supported by results obtained by
Renaut et al. (39) who showed that deletions of 3 amino acids
in the TM domain permits assembly of α-β complexes and their
cell surface expression whereas deletions of 5–11 amino acids
in the TM domain of β subunits only allows for assembly but not
for ER exit of the α-β complexes.

Tryptophan scanning of the TM domain of the Na,K-ATPase
β1 subunit revealed that substitution of 3 amino acids, Ile37,
Tyr40, and Tyr44, perturbs the inherent, apparent afﬁnities for
K+ of associated Na,K-ATPase α subunits. The functional ef-
effects observed with Tyr40 and Tyr44 substitutions are likely to
be a reﬂection of the disruption of a functionally active site.
This is supported by the observations that the two tyrosine
residues are aligned in the TM α-helix, they produce additive
effects, and the functional effects can be reproduced with dif-
f erent amino acids. Moreover, the K+ effect produced by Tyr
replacements in the β subunits TM domain varies depending
on experimental conditions, e.g., in the presence or absence of
external Na+. The cause underlying the K+ effect produced by
substitution of Ile37 with tryptophan is less clear. In membrane
proteins, tryptophan residues are enriched at both ends of TM
domains (40) and preferentially interact near the lipid carboxyl
moiety (41, 42). Introduction of tryptophan, in positions ﬂank-
ing model TM helices, tend to “push” the TM helix into the
membrane (43). In the context of our result, these observations
may indicate that tryptophan substitution of Ile37, located adja-
cent to Lys36 which delimits the TM domain of β1 subunits,
could produce a local conformational perturbation in the α helix
that may be transmitted to the aligned Tyr residues and thereby
produce an indirect functional effect. Consistent with
this idea is that the K+ effect of Ile37 substitutions is observed
both in the presence and absence of external Na+ (data not shown).

The change in the apparent K+ afﬁnity of Na,K pumps
containing β subunits with Tyr40 and Tyr44 substitutions are
more prominent when measurements are performed in the
presence rather than in the absence of external Na+ indicating
that substitution of the tyrosine residues in the β subunits TM
domain may not only alter the K+–binding step itself, but also
other discrete steps in the ion transport cycle. In both wild type
and mutant Na,K pumps, a parallel exists between the decrease
in the apparent afﬁnity for external K+ and a gain in the
apparent afﬁnity for external Na+ during hyperpolarization.
This result reﬂects the interplay between Na+ binding and
release and K+ binding to external sites and the voltage
dependence of these steps. Furthermore, the difference in the
apparent afﬁnities for external Na+ between Na,K pumps
associated with β subunit tyrosine mutants and those associated
with wild type β subunits are largest at very negative mem-
brane potentials. This result is consistent with the idea that
Tyr mutations in the β subunits TM domain may favor the
transition from an E2 to a Na+-carrying E1 conformation which
prevails during hyperpolarization. A shift of the E1–E2 equili-
rium to the E1 conformation may also explain the higher
apparent afﬁnity for intracellular Na+ and the lower sensitiv-
ity for vanadate observed for the mutant Na,K pumps. The two
tyrosine residues are highly conserved in the TM domain of all
known β subunits of both Na,K- and H,K-ATPases. Experimen-
tal conﬁrmation is needed to show whether, as expected, these
residues inﬂuence a basic step in the catalytic cycle common to
Na,K and H,K pumps.

The tryptophan scanning analysis of the β1 subunits TM
domain was complemented by substitution of the two function-
ally relevant tyrosine residues by other amino acids. An in-
crease in K+K+ values was also observed in mutant Na,K pumps
containing β subunits in which Tyr40 and Tyr44 in the
TM domain were replaced by serine, cysteine, or alanine resi-
dues. On the other hand, no K+ effect was observed by replac-
ing the tyrosine residues with phenylalanine. These results
indicate that a correctly positioned aromatic moiety present in
The Transmembrane Domain of the Na,K-ATPase β1 Subunit

of the Na,K-ATPase include contacts of the membrane segments of Na,K-ATPases with the β subunit and produce a conformational effect. The primary interactions that specify TM helix packing which mediates subunit interactions and the correct folding of membrane proteins are still largely unknown. Based on energetic considerations, the possible driving forces for TM helix interactions are van der Waals interactions between closely packed helices and interhelical polar interactions. Since TM helices of membrane proteins contain only few polar amino acids and TM helix interactions can be very stable in the absence of any hydrogen bonds or salt bridges, van der Waals interactions may play a major role in governing interhelical interactions. However, recent work has implicated polar residues in membrane helix interactions. For instance, charged residues in the membrane domain are necessary for subunit assembly in the T-cell receptor.

Further, formation of hydrogen bonds, e.g. between Asn residues, mediates strong interactions of TM helices, but not in Na,K-ATPase. Little is known about the role of tyrosine residues in mediating TM helix interactions. Aromatic amino acids such as Tyr, Phe, or Trp may undergo "amino aromatic" interactions in which neutral NH-containing groups tend to be positioned near the aromatic rings and even more favorably "cation-π" interactions (cation-Å interactions) with positively charged residues. Sequence analysis of TM segments of Na,K-ATPase α subunits does not provide conclusive evidence for the existence of a potential interaction site matching the two tyrosine residues in the β subunit. Highly conserved, neutral NH-containing amino acids are indeed present in M5, M7, and M8 of Na,K- and H,K-ATPases but they are unique or not aligned in a putative α-helix to permit accommodation of the two tyrosine residues of the β subunits TM domain. Furthermore, interactions of Tyr^{40} and Tyr^{44} with positively charged residues are likely to influence the intracellular interactions. Aromatic amino acids such as Tyr or Phe are present in Na,K-ATPase α subunits and are directed to the face of M8 of the α subunit as suggested by cross-linking studies.

In conclusion, modeling of the TM helix accompanied by cryptophan scanning reveal that the TM domain of β1 subunits of Na,K-ATPase contain two distinct helix faces which are probably both buried in the interior of the protein and partic...
imate in membrane protein-protein interactions. Whereas no functional role could be attributed to TM α-β interactions in the face of the β subunits TM helix containing the cysteine residue which is closely located to M8 of the α subunit, putative TM interactions in another face of the β subunits TM helix, which are mediated by two highly conserved tyrosine residues, contribute to intrinsic functional properties of oligomeric P-type ATPases. Finally, a putative third face, containing a basic GXXXG motif, may be of potential functional relevance, in permitting β1 subunit dimerization.

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