Covalent Cross-links between the γ Subunit (FXYD2) and α and β Subunits of Na,K-ATPase

MODELING THE α-γ INTERACTION*

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This study describes specific intramolecular covalent cross-linking of the γ to α and γ to β subunits of pig kidney Na,K-ATPase and rat γ to α co-expressed in HeLa cells. For this purpose pig γα and γβ sequences were determined by cloning and mass spectrometry. Three bifunctional reagents were used: N-hydroxysuccinimidyld-4-azidosalicylic acid (NHS-ASA), disuccinimidyl tartrate (DST), and 1-ethyl-3-[3(dimethylaminopropyl)carbodiimide (EDC). NHS-ASA induced α-γ, DST induced α-γ and β-γ, and EDC induced primarily β-γ cross-links. Specific proteolytic and Fe²⁺-catalyzed cleavages located NHS-ASA- and DST-induced α-γ cross-links on the cytoplasmic surface of the α subunit, downstream of His303 and upstream of Val360. Additional considerations indicated that the DST-induced and NHS-ASA-induced cross-links involve either Lys347 or Lys292 in the S4 stalk segment. Mutational analysis of the rat γ subunit expressed in HeLa cells showed that the DST-induced cross-link involves Lys347 and Lys346 in the cytoplasmic segment. DST and EDC induced two β-γ cross-links, a major one at the extracellular surface within the segment Gly143–Ser302 of the α subunit and another within Ala1–Arg142. Based on the cross-linking and other data on α-γ proximities, we modeled interactions of the transmembrane a-helix and an unstructured cytoplasmic segment SKRRCGGKHKR of γ with a homology model of the pig α1 subunit. According to the model, the transmembrane segment fits in a groove between M2, M6, and M9, and the cytoplasmic segment interacts with loops L6/7 and L8/9 and stalk S5.

The Na,K-ATPase actively pumps Na⁺ ions out of cells and K⁺ ions into cells and maintains the characteristic transmembrane electrochemical gradients of Na⁺ and K⁺ ions. As could be expected for a protein with such a central physiological role, Na,K-ATPase is closely regulated at several levels.

Recently a unique mode of regulation of the Na,K-ATPase has been described (for reviews, see Refs. 1–3). It involves interactions between the α/β complex and members of a family of seven short single span transmembrane proteins termed the FXYD proteins (4). Four members of the family, FXYD1 (PLM),¹ FXYD2 (γ), FXYD4 (CHIF), and FXYD7, are now known to interact specifically with the Na,K-ATPase and alter the pump kinetics in characteristic and different ways. The FXYD proteins show a highly tissue-specific expression pattern: γ is expressed in kidney, CHIF is expressed in kidney and colon, PLM is expressed in heart and skeletal muscle, and FXYD7 is expressed in brain. In kidney γ is expressed as two splice variants, γa and γb (4, 5). Splicing in other FXYD proteins has not been detected at the protein level. The working hypothesis is that FXYD proteins function as tissue-specific modulators of Na,K-ATPase that adjust or fine-tune its kinetic behavior to the specific needs of the given tissue, cell type, or physiological state (1–3).

Functional interactions between the γ subunit and the Na,K-ATPase have now been studied extensively after co-expression with the α/β subunits in mammalian cells and Xenopus oocytes or by neutralizing interactions with a specific anti-γ antibody (6–10). In cultured mammalian cells γ raises apparent affinity for ATP by shifting the E₁–E₂ conformational equilibrium toward E₂, reduces apparent affinity for cytoplasmic Na⁺ by making cytoplasmic K⁺ a better competitor (8–10), and slightly reduces extracellular K⁺ affinity (11). Anti-γC (directed against the sequence KHQQVNEDEL at the C terminus of rat γ) abrogates the effect of γ on the apparent ATP affinity in renal Na,K-ATPase or HeLa cells transfected with γ but not that on the K⁺ to Na⁺ antagonism (7, 8, 10). In oocytes, CHIF raises apparent affinity for cell Na⁺ and evokes a small increase in the extracellular K⁺ affinity, which varies with voltage (6). In HeLa and HEK293 cells and Xenopus oocytes small or insignificant differences in functional effects are found between γb and γa (Refs. 10 and 12, but see Ref. 13). In Xenopus oocytes (12) and mammalian cells (14) CHIF raises the apparent affinity for cell Na⁺ by 2–3-fold, the reverse effect to that of γ. In HeLa cells, CHIF

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The abbreviations used are: PLM, phospholemman; NHS-ASA, N-hydroxysuccinimidyld-4-azidosalicylic acid; DST, disuccinimidyl tartrate; EDC, 1-ethyl-3-[3(dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; TM, transmembrane; CHIF, corticosteroid hormone-induced factor; MES, 4-morpholineethanesulfonic acid; PNGase, peptide-N-glycosidase; Tricine, N-[2-hydroxy-1,1-bist(hydroxymethyl)ethyl]glycine.

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has no effect on the affinity for external K\(^+\) or ATP (14), whereas in Xenopus oocytes an increased K\(_{0.5}\) to external K\(^+\) was observed at high voltages and in the presence of external Na\(^+\) (12). The opposite functional effects of CHIF and γ on the apparent Na\(^+\) affinity are consistent with their different patterns of expression along the nephron and physiological roles (for reviews, see Refs. 1–3).

When expressed in Xenopus oocytes PLM interacts with both the α1β1 and α2β1 isoforms and decreases the internal Na\(^+\) affinity of the pump by about 2-fold and external K\(^+\) affinity by a small amount (15), whereas use of an anti-PLM antibody on chorioid plexus membranes suggested that PLM might increase Na,K-ATPase activity (16). FXYD7 is the fourth family member whose functional interaction with the Na,K-ATPase has been demonstrated. In Xenopus oocytes FXYD7 decreases the apparent K\(^+\) affinity of the pump when expressed with α1β1 or α2β1 but not with α3β1 (2).

By comparison with functional studies there is little information on structural interactions of FXYD proteins with α/β subunits. It is, however, becoming clear that there are multiple sites of interaction, involving both transmembrane segments and the extramembrane domains. The fact that the anti-γC abrogates the effect of γ on the apparent ATP affinity but not that on the K\(^+\) to Na\(^+\) antagonism (8, 10) provided an initial indication. In addition, in HeLa cells expression of γ with either C- or N-terminal truncated sequences removes the effect on ATP affinity but does not affect the K\(^+\) to Na\(^+\) antagonism (17). In a recent systematic study of roles of the different segments, a series of γ/CHIF chimeric molecules was prepared in which extracellular, transmembrane, and cytoplasmic sequences were interchanged (18). It was found that both the stability of the FXYD-α/β complex in detergent and the effects on the apparent Na\(^+\) affinity were determined by the origin of the transmembrane segment. Interestingly, however, different residues appear to be involved in the stability and functional effects of the transmembrane segments. The functional role of the transmembrane segments has been confirmed in a study showing that peptides corresponding to the transmembrane segment of γ reduce apparent Na\(^+\) affinity of the α/β complex in HeLa cell membranes as found previously for full-length transfected γ (19). In short, extramembrane segments mediate the effect of γ on apparent ATP affinity, whereas transmembrane segments mediate the effect on cation affinities.

Where do FXYD proteins interact with the α/β complex? On the basis of cryoelectron microscopy of renal Na,K-ATPase electron densities were assigned as transmembrane helices of α, β, and γ subunits. The γ subunit helix was proposed to lie in a groove bounded by M2, M6, and M9 of the α subunit (20). A denaturation study suggested that γ might interact in the M8–M10 region (21). Recently a role for M9 of the α subunit has been inferred from effects of mutants in M9 on stability of α/β-γ, α/β-CHIF, or α/β-FXYD7 complexes and their functional consequences studied in Xenopus oocytes (22). Lou\(^{564}\) and Phe\(^{967}\) were important for stability of the complexes, whereas Phe\(^{556}\) and Gln\(^{863}\) were required for mediation of effects of the FXYD protein on K\(^+\) affinity. Thus, stabilizing and functional interactions are separable as found also in mutational studies in CHIF/γ chimera (23). Interestingly the Phe\(^{556}\) and Gln\(^{863}\) mutations did not alter effects of γ and CHIF on Na\(^+\) affinity, implying that still other interactions in the transmembrane segment mediate these effects. Modeling of the FXYD helix was consistent with docking in the groove between M2, M6, and M9.

The present work has utilized a different approach, namely covalent cross-linking, to obtain direct evidence for proximities between the γ subunit and α and β subunits of pig kidney Na,K-ATPase and in HeLa cells expressing rat α and γ subunits. We have used a variety of bifunctional reagents with different chemical specificities and arm lengths, optimized cross-linking efficiency, and determined the approximate positions of observed α-γ and γ-β cross-links. Based on the inferred position of α-γ cross-links in the cytoplasmic domains of the α and γ subunits, we have modeled the α-γ interactions.

**Materials and Methods**

**Pig Kidney Na,K-ATPase: Preparations and Detergent Solubilization**

Partially purified Na,K-ATPase from pig kidney outer medulla was prepared as described previously (24). Extensively tryptosinized 19-kDa membranes were prepared as described previously (25). Membranes were solubilized by the non-ionic detergent C\(_{12}E_{10}\) (polyoxyethylene 10-auryl ether) in the presence of Rb\(^+\) plus ouabain or Na\(^+\) plus oligomycin as described previously (14).

**HeLa Cell Expression of γα or γβ**

HeLa cells overexpressing the rat α1 subunit of Na,K-ATPase were kindly provided by Dr. J. B Lingrel, University of Cincinnati College of Medicine, Cincinnati, OH. Cells were transfected with wild type and mutated rat γ constructs subcloned into pIREShyg. Transfection was done using Polyfect (Qiagen) according to the manufacturer's instructions. Colonies stably expressing γ proteins were selected in 400 μg/ml hygromycin B and tested for maximal expression of γ by Western blotting.

**Covalent Cross-linking**

NHS-ASA—Purified pig kidney Na,K-ATPase or 19-kDa membranes were suspended in 10 mM imidoborate, pH 9.5, 130 mM NaCl or 10 mM HEPES, pH 8, 130 mM NaCl to 0.5 mg/ml protein concentration. NHS-ASA (26) dissolved in Me\(_2\)SO was added to 0.25–1 mM in three aliquots and incubated at room temperature in the dark for 30 min. The reaction was quenched by 50 mM unbuffered Tris. The pH of the suspension was restored to near neutral with 100 mM HEPES, pH 7.4, and the suspension was illuminated for 2 min with a xenon lamp (150 watts, fitted with a filter cutting off light below 300 nm). Where indicated, the membranes were pelleted and resuspended in the detergent solubilization buffer: 25 mM imidazole, pH 7.5, 1 mM EDTA and either 10 mM RbCl plus 5 mM ouabain or 20 mM NaCl plus oligomycin 0.1 mg/ml (pig kidney enzyme, 20 mM NaCl plus oligomycin). The membranes were solubilized by addition of concentrated gel sample buffer.

**DST**—Purified pig kidney enzyme was suspended in 10 mM HEPES, pH 8, 250 mM NaCl or 30 mM Rb\(^+\) plus 220 mM choline at 0.5–1 mg/ml protein, or the Na,K-ATPase was solubilized with C\(_{12}E_{10}\) in 10 mM HEPES, pH 8, in the presence of 10 mM RbCl plus 5 mM ouabain or 20 mM NaCl plus oligomycin. HeLa cells membranes were solubilized with C\(_{12}E_{10}\) in the presence of Na\(^+\)/oligomycin. 19-kDa membranes were suspended in 10 mM RbCl, 10 mM Na-HEPES, pH 7.4. DST (27) in Me\(_2\)SO was added to a final concentration of 2 mM followed by incubation for 30 min at room temperature. The reaction was quenched with 50 mM unbuffered Tris.

**EDC**—Purified kidney enzyme was suspended in 100 mM MES, pH 6, 250 mM NaCl or 30 mM Rb\(^+\) plus 220 mM choline at 0.5–1 mg/ml protein. Alternatively the Na,K-ATPase was solubilized with C\(_{12}E_{10}\) in 10 mM HEPES, pH 8, in the presence of 10 mM RbCl plus 5 mM ouabain or 20 mM NaCl plus oligomycin. HeLa cell membranes were solubilized with C\(_{12}E_{10}\) in the presence of Na\(^+\)/oligomycin. 19-kDa membranes were suspended in 10 mM RbCl, 10 mM Na-HEPES, pH 7.4. DST (27) in Me\(_2\)SO was added to a final concentration of 2 mM followed by incubation for 30 min at room temperature. The reaction was quenched with 50 mM unbuffered Tris.

**Deglycosylation**

Cross-linked native enzyme or 19-kDa membranes (30–40 μg) were treated with PNGase F (125 units, New England Biolabs) for 24 h at 37 °C in a medium containing 20 mM Tris-HCl, pH 8.0, 10 mM RbCl after denaturation in the buffers supplied with the PNGase. The digestion was arrested by addition of concentrated gel sample buffer.
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Chymotryptic Cleavages

Purified kidney enzyme or NHS-ASA-cross-linked membranes were suspended in 10 mM HEPES, pH 7.4, containing either 20 mM RbCl or 20 mM NaCl (0.1 mg/ml protein) and incubated with 1:20 chymotrypsin (mg/mg of protein) for 15 min (see Refs. 29 and 30). The enzymatic digestion was stopped by 10 volumes of ice-cold HEPES buffer containing 1 mM phenylmethylsulfonyl fluoride and 150 mM RbCl.

Fe²⁺-catalyzed Oxidative Cleavage

Purified kidney enzyme or NHS-ASA-cross-linked membranes were suspended in 10 mM HEPES, pH 7.4, in the presence of either 130 mM Na⁺, 50 mM NaCl or 40 μM AMPPNP. The enzyme in F₆Na conformation was incubated with 5 mM ascorbic acid, 5 mM H₂O₂, 5 μM Fe²⁺ for 15–20 min, and the cleavage was stopped by addition of sample buffer containing 5 mM Desferal (see Refs. 31–33).

SDS-PAGE and Western Blots

Proteins were separated on 10% SDS-Tricine gels (34) and blotted to polyvinylidene difluoride membranes. Western blots utilized the following antibodies as indicated: affinity-purified anti-γ, polyclonal antibody raised against the peptide KHRQVNEDEL at the C terminus of the α subunit (dilution, 1:4000); a monoclonal antibody (6H) recognizing a peptide sequence corresponding to pig kidney RNA was reverse transcribed from the poly(A) tail and then sequenced from both ends and found to correspond to TGCGATGGGGGCACAGCCGA (35/3/H11032). To verify the amino acid sequence alignments. The sequence alignment was converted to a structure using the molecular dynamics package Gromacs (45, 46) and the Gromos96 vacuum force field (47). To relieve steric strain, 5000 steps of steepest descent minimization were performed followed by 100 ps of molecular dynamics at 300 K with a restrained backbone followed by another 100 ps of free molecular dynamics without any restraints. In a final step, 5000 steps of conjugate gradient minimization were performed. Although the complex membrane-water interface is not well reproduced by a simulation in vacuo, the emphasis of our calculation was less on observing the dynamics of the system and more on relieving local stress induced by the interactive modeling. After the short run, the root mean square deviation between the initial and final γ conformation was less than 2 Å.

RESULTS

Fig. 1 shows structures of the three cross-linkers used in this study. NHS-ASA is a heterobifunctional, S-A spacer, photoactivated reagent. The NHS ester reacts with lysine residues, whereas the arylitrine can react with double bonds, insert into C–H and N–H bonds, or undergo subsequent ring expansion to react with a nucleophile (e.g. primary amines and

domly rotating the helix 0–360° around the long axis; translating it –3 to +5 Å toward or away from the center of the bundle, respectively; translating the helix ±10 Å along its long axis; and tilting the helix relative to the fixed angles of the TM helices of the α subunit by ±20°. The coordinate transformations were done using the program package CNS (39) with the OPLS (40) parameter set. Each such generated conformation was subjected to 50 steps of Powell minimization, and the Van der Waals interaction energy of the γ-helix with the α subunit was calculated. All degrees of freedom were sampled in 20 bins per parameter. The average energy of each bin was evaluated independently by using Boltzmann averaging of all conformations within this bin. After Boltzmann averaging the minimal average energy of the parameter in question was taken as that at the center of the bin of lowest energy. A related procedure has been shown to successfully reproduce structures of four-helix bundles (41, 42).

Interactive Modeling of the Cytoplasmic Segment of the γ Subunit—A secondary structure prediction by Jpred2 (43) revealed that the region starting from residue Ser⁴¹³ has no preference for any secondary structure. Therefore the sequence SKRLRCGGKKHR (pig γa sequence) was modeled interactively as a random coil using the DeepView modeling package (44) with the following constraints. 1) The Ramachandran plot should be fulfilled. 2) Neither backbone nor side chains of γ should clash with the α subunit. 3) The positive charges of Lys⁴⁴ and Lys⁶⁴ and of His⁵⁹ and Arg⁷⁷ should interact with negatively charged residues of the α subunit. In a first step the φ/ψ angles of the backbone were adjusted so that the segment came into contact with the α subunit without backbone clashes and so that side chains of acidic residues of the α subunit came into proximity with the side chains of Lys⁴⁴, Lys⁶⁴, His⁵⁹, and Arg⁷⁷ should interact with negatively charged residues of the α subunit. In a second step, a rotameric search of each individual contact side chain for both α and γ subunits was performed to optimize the side-chain packing. The two steps were iterated until no clashes occurred.

Refinement—The initial model was refined with a short molecular dynamics protocol in vacuo using the molecular dynamics package Gromacs (45, 46) and the Gromos96 vacuum force field (47). To relieve steric strain, 5000 steps of steepest descent minimization were performed followed by 100 ps of molecular dynamics at 300 K with a restrained backbone followed by another 100 ps of free molecular dynamics without any restraints. In a final step, 5000 steps of conjugate gradient minimization were performed. Although the complex membrane-water interface is not well reproduced by a simulation in vacuo, the emphasis of our calculation was less on observing the dynamics of the system and more on relieving local stress induced by the interactive modeling. After the short run, the root mean square deviation between the initial and final γ conformation was less than 2 Å.

Homology Modeling of Pig γ1 Subunit—The alignment used by Li and co-workers (22) for Ca-ATPase and Bufo Na,K α subunits was modified to the appropriate Ca-ATPase and pig Na,K α subunit alignments. The sequence alignment was converted to a structure using the homology-modeling program package Modeller v. 2 with default settings (35, 36) and the crystal structure of Ca-ATPase in the ATP-bound form (37, 38) as template. 50 structures were generated using different initial random velocities for all atoms. The best model was chosen based on the in-built objective function, which uses secondary structure prediction by Jpred2 (43) revealed that the region starting from residue Ser⁴¹³ has no preference for any secondary structure. Therefore the sequence SKRLRCGGKKHR (pig γa sequence) was modeled interactively as a random coil using the DeepView modeling package (44) with the following constraints. 1) The Ramachandran plot should be fulfilled. 2) Neither backbone nor side chains of γ should clash with the α subunit. 3) The positive charges of Lys⁴⁴ and Lys⁶⁴ and of His⁵⁹ and Arg⁷⁷ should interact with negatively charged residues of the α subunit. In a first step the φ/ψ angles of the backbone were adjusted so that the segment came into contact with the α subunit without backbone clashes and so that side chains of acidic residues of the α subunit came into proximity with the side chains of Lys⁴⁴, Lys⁶⁴, His⁵⁹, and Arg⁷⁷ should interact with negatively charged residues of the α subunit. In a second step, a rotameric search of each individual contact side chain for both α and γ subunits was performed to optimize the side-chain packing. The two steps were iterated until no clashes occurred.

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Fig. 1 shows structures of the three cross-linkers used in this study. NHS-ASA is a heterobifunctional, S-A spacer, photoactivated reagent. The NHS ester reacts with lysine residues, whereas the arylitrine can react with double bonds, insert into C–H and N–H bonds, or undergo subsequent ring expansion to react with a nucleophile (e.g. primary amines and

![Image](http://www.jbc.org/Downloaded from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5733264/figure/f1.png)
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![Alignment of pig \( \gamma a \) and \( \gamma b \) sequences with human and rat \( \gamma a \).](http://www.jbc.org/)

The pig \( \gamma a \) amino acid sequence was deduced by sequencing a reverse transcription-PCR product. Underlined segments were determined by full sequencing of tryptic peptides using mass spectrometry (see Ref. 5). Mass spectrometry also provided the unique \( \gamma b \) N-terminal sequence.

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The error of the mass measurement of the intact protein is about 0.05–0.1% (or 3–7 Da).
two well characterized chymotryptic fragments, Val\textsuperscript{440}–Tyr\textsuperscript{1016} and Ala\textsuperscript{589}–Tyr\textsuperscript{1016}, were detected with anti-KETYY, which recognizes the C terminus of the $\gamma$ subunit. Neither of these fragments was recognized by the anti-$\gamma$ subunit. The fragments that were recognized by the anti-$\gamma$ subunit are identifiable as (a) the complementary fragment Gly\textsuperscript{1}–Val\textsuperscript{440} and (b) probably a secondary cleavage fragment of the complement to Gly\textsuperscript{1}–Ala\textsuperscript{589} (Fig. 6, asterisk). The experiment shows that the cross-link must be located upstream of Val\textsuperscript{440}. In an $\alpha$ subunit conformation, Fe\textsuperscript{2+}/ascorbate/H\textsubscript{2}O\textsubscript{2} treatment produced two fragments of the $\alpha$ subunit recognized by anti-KETYY, Glu\textsuperscript{60}–Tyr\textsuperscript{1016} and His\textsuperscript{283}–Tyr\textsuperscript{1016} (32, 50). Cleavage of the cross-linked product produced the same fragments, including a broader band, presumably a doublet corresponding to the His\textsuperscript{283}–Tyr\textsuperscript{1016} fragment of un-cross-linked and cross-linked $\alpha$ subunit, respectively. The upper His\textsuperscript{283}–Tyr\textsuperscript{1016} fragment was also recognized by anti-$\gamma$. The complementary fragment Gly\textsuperscript{1}–His\textsuperscript{283} was recognized by antibody 6H, which has an epitope near the N terminus of the $\alpha$ subunit, but was not recognized by anti-$\gamma$. The experiment indicates the presence of the cross-link in the His\textsuperscript{283}–Tyr\textsuperscript{1016} fragment and lo-
cates it downstream of His \(^{283}\). Fig. 7B shows results of incubation with AMPPNP-Fe/ascorbate/H\(_2\)O\(_2\), which produced two well characterized fragments recognized by anti-KETYY, Val\(^{140}\)–Tyr\(^{1016}\) and Val\(^{712}\)–Tyr\(^{1016}\) (33, 50), in both the control and cross-linked preparations. Neither of these fragments was recognized by anti-\(\gamma\)C. A fragment that was not recognized by anti-\(\gamma\)C, but was recognized by the anti-N-terminal antibody, was identified as Gly\(^1\)–Val\(^{712}\). This experiment confirms that DST and NHS-ASA can both be used to study the Na\(_{\text{K}}\)-ATPase cross-link. The figure also presents the same cross-links by their mobility on the gels just above uncross-linked subunits and in the case of the \(\beta\)-\(\gamma\) cross-link by the change of mobility after deglycosylation (seen in Figs. 9 and 10). The \(\alpha\)-\(\gamma\) cross-link was amplified in the Na\(_{\text{K}}\)-containing compared with the Na\(_{\text{Rb}}\)-containing medium as found also for NHS-ASA. The \(\beta\)-\(\gamma\) cross-link was usually unaffected by the ionic composition of the medium (although it appears to be amplified in Fig. 8, lane 4). The \(\alpha\)-\(\gamma\) cross-link was more efficient at pH 9 compared with pH 8 (Fig. 8, right-hand lanes) consistent with reactivity of DST with lysine residues. The anti-\(\gamma\)C did not block the DST-induced \(\alpha\)-\(\gamma\) cross-link unlike the result with NHS-ASA, suggesting that the sites of NHS-ASA- and DST-induced cross-linking are not identical (see the “Discussion”).

DST-induced \(\alpha\)-\(\gamma\) and \(\beta\)-\(\gamma\) cross-links on Pig Kidney Na\(_{\text{K}}\)-ATPase—The immunoblot, probed with anti-\(\gamma\)C, in Fig. 8 shows that DST induced a prominent \(\alpha\)-\(\gamma\) cross-link and a less prominent \(\beta\)-\(\gamma\) cross-link. The figure also presents the same controls for specificity as discussed in relation to NHS-ASA. The bands were identified as \(\alpha\)-\(\gamma\) and \(\beta\)-\(\gamma\) cross-links by their mobility on the gels just above uncross-linked \(\alpha\) or \(\beta\) subunits and in the case of the \(\beta\)-\(\gamma\) cross-link by the change of mobility after deglycosylation (seen in Figs. 9 and 10). The \(\alpha\)-\(\gamma\) cross-link was amplified in the Na\(_{\text{K}}\)-containing compared with the Na\(_{\text{Rb}}\)-containing medium as described under “Materials and Methods.” Aliquots of the untreated (C) and NHS-ASA-treated enzyme were digested with \(\alpha\)-chymotrypsin (CHY) in Rb\(^{-}\)-containing media (A) or Na\(^{-}\)-containing media (B). Samples were applied to gels, and immunoblots were developed either with anti-KETYY or anti-\(\gamma\)C. *\(\gamma\), unknown chymotryptic fragment.
was recognized by anti-\(\gamma\)C. The chymotryptic cleavage in the E\(_N\)a conformation produced the expected bands Ile\(^{263}\)–Tyr\(^{1016}\), and this band was recognized by the anti-\(\gamma\)C. An aliquot was also treated overnight with PNGase prior to application to the gel. Degly., deglycosylated.

DST-induced \(\beta\)-\(\gamma\) cross-links in 19-kDa membranes. 19-kDa membranes suspended in a Rb\(^-\)-containing medium at pH 7.4 were treated with 2 mM DST for 30 min at room temperature. Controls (C) refer to 19-kDa membranes pretreated with 2% SDS and then with DST. The blots were probed with anti-\(\gamma\), anti-\(\beta\), or anti-\(\beta\)-\(\gamma\) antibodies. An aliquot was also treated overnight with PNGase prior to application to the gel. Degly., deglycosylated.

**Fig. 9.** Specific chymotryptic and Fe\(^{3+}\)-catalyzed cleavage of DST-induced \(\alpha\)-\(\gamma\) cross-link. Membrane-bound Na,K-ATPase was treated with DST as described under “Materials and Methods.” Aliquots of the untreated (C) and DST-treated enzyme were digested with \(\alpha\)-chymotrypsin (Chy) in Rb\(^-\)-containing or Na\(^+\)-containing media (A) or cleaved with Fe\(^{3+}\)-ascorbate/H\(_2\)O\(_2\) in a Na\(^+\)-containing medium (B). A sample of the enzyme digested with chymotrypsin was also treated with PNGase. Samples were applied to gels, and immunoblots were developed either with anti-KETY or anti-\(\gamma\)C. Degly., deglycosylated. * unknown chymotryptic fragment.

**Fig. 10.** DST-induced \(\beta\)-\(\gamma\) cross-links in 19-kDa membranes. 19-kDa membranes suspended in a Rb\(^-\)-containing medium at pH 7.4 were treated with 2 mM DST for 30 min at room temperature. Controls (C) refer to 19-kDa membranes pretreated with 2% SDS and then with DST. The blots were probed with anti-\(\gamma\), anti-\(\beta\), or anti-\(\beta\)-\(\gamma\) antibodies. An aliquot was also treated overnight with PNGase prior to application to the gel. Degly., deglycosylated.

**Fig. 11.** Mutational analysis of DST-induced cross-link on \(\gamma\) expressed in HeLa cells. Membranes were solubilized and treated with DST or NHS-ASA as described under “Materials and Methods.” The amounts of membranes applied to each lane were corrected for the level of expression of the \(\gamma\) subunit in each condition to allow comparisons of the effects of the different mutations. Blots were developed with anti-\(\gamma\), sol., solubilized; C, control.
Cross-linking γ to α and β Subunits of Na,K-ATPase

**Fig. 12.** EDC-induced β-γ cross-links in intact renal Na,K-ATPase and 19-kDa membranes. A, membrane-bound or C12,E6- solubilized Na,K-ATPase was treated with 1% EDC plus 5 mM NHS at pH 6 for 2 h at room temperature (see “Materials and Methods”). Controls (C) refer to enzyme pretreated with 2% SDS and then with EDC. The immunoblot was developed with anti-γ antibodies. The EDC induced cross-links in either membrane-bound or detergent-soluble pig kidney Na,K-ATPase with only minor α-γ cross-linking and little or no difference in Rb⁺-containing medium at pH 6 were treated with 1% EDC plus 5 mM NHS for 2 h at room temperature. Controls (C) refer to enzyme pretreated with 2% SDS and then with EDC. Aliquots were treated overnight with PNGase prior to application to the gel. The blots were probed with anti-γ, anti-β50, or anti-β16 antibodies.

and Lys56 of rat γ and a proximal lysine residue in the α subunit.

**EDC-induced β-γ Cross-link on Pig Kidney Na,K-ATPase**—Finally Fig. 12A shows that EDC induced primarily β-γ cross-links in either membrane-bound or detergent-soluble pig kidney Na,K-ATPase with only minor α-γ cross-linking and little or no difference in Rb⁺-containing medium at pH 6. The experiment with 19-kDa membranes in Fig. 12B shows essentially the same features as found for DST-induced cross-linking, namely all bands recognized by the anti-γC were also recognized by anti-β antibodies. The EDC induced cross-links to both the 50-kDa fragment and 16-kDa fragments, confirming the existence of two separate β-γ cross-linking positions.

**DISCUSSION**

The first point to make is that NHS-ASA and DST cross-link the same regions of the γ and α subunits at the cytoplasmic side, whereas DST and EDC cross-link the same regions of γ and β subunits at the extracellular side. The fact that bifunctional reagents with different chemical specificities cross-link the γ subunit with α or β subunits in both pig and rat and the same regions of cross-linking are involved in the different α-γ and β-γ cross-links provides a strong indication that they are specific intramolecular cross-links and that they occur in regions of subunit interactions. Although NHS-ASA is an efficient cross-linker and was very useful for establishing criteria for specific cross-links and the initial analysis of the site of the cross-link, a detailed analysis was complicated by the lack of knowledge of which chain, α or γ, contains the reacted lysine. Use of DST, which creates specific lysine-lysine cross-links, avoids this ambiguity and eventually provided more detailed information.

**Locating the α-γ Cross-link**—The mutation work with HeLa cells showed that the DST-induced cross-link involved Lys55 and Lys56 in the cytoplasmic segment. Conversely the analysis showed that NHS-ASA cross-link does not involve lysines on the γ subunit, and thus the lysine modified in the dark reaction must be located on the α subunit. Because both DST and NHS-ASA are N-hydroxysuccinimide esters, the same lysine on the α subunit could be involved in the DST-induced cross-link and the dark reaction of NHS-ASA. We have not attempted to identify the residue of γ involved in the NHS-ASA light reaction by mutational analysis because the nitrene is not specific and could react with more than one proximal residue.

Both DST- and NHS-ASA-induced α-γ cross-links were amplified in Na⁺-containing media, which stabilizes the E₁,Na conformation. Presumably the α-γ interaction is responsible for the functional effect of γ on the apparent ATP affinity for Na,K-ATPase, which has been shown to be an indirect result of stabilization of the E₁ conformation (10). Amplification of the cross-link in E₁ is consistent with this interpretation for if γ interacts with α to stabilize E₁, the principle of linked equilibria requires that stabilization of E₁ by a different ligand (Na⁺ ions) should strengthen the α-γ interaction. The functional effect of γ on the apparent ATP affinity, like the NHS-ASA-mediated cross-link, was abrogated by the anti-γC. Inhibition of the NHS-ASA-induced cross-link by the anti-γC is suggestive of a position nearer the C terminus than Lys54 or Lys55 within the epitope His56-Lys64.

The combination of extensive and controlled cleavages of the DST-induced and NHS-ASA-induced cross-linked products showed that in both cases the cross-link on the α subunit lies at the cytoplasmic side, downstream of His283 and upstream of Val440. In reality, however, there are more stringent constraints than revealed directly by the experiments. First, His283 is itself located at the cytoplasmic entrance of M3 (see Fig. 5), and because neither NHS-ASA- nor DST-mediated cross-links can be in M3, the extracellular L3-4, or M4, one can infer that they are, in fact, located after the cross-link of the cytoplasmic exit of M4 and before Val440. Second, because the DST-mediated cross-link on the γ subunit is located on Lys54 or Lys55, 10 or 11 residues from the cytoplasmic exit of the transmembrane segment (L4–5) (pig numbering, see Fig. 2), the cross-linked residues in the α subunit are unlikely to be much further away from the cytoplasmic exit of M4, i.e. they are likely to be located within the S4 cytoplasmic stalk segment (Thr328-Glu358). Third, there are only three candidate lysines within 15 residues of the exit of M4, Lys342, Lys347, and Lys352, and the only other lysines upstream of Val440, Lys370, Lys406, and Lys428, are 33, 69, and 101 residues distant from the end of M4, respectively. Fourth, of the three most likely candidates, Lys342, Lys347, and Lys352, Lys342 can be excluded. This arises from the fact that Lys342 is the first tryptic cleavage site after M4. The C-terminal residue of the M3–M4 fragment of 19-kDa membranes is not exactly known, although Arg346 or Arg343 are likely possibilities; but in any case, the M3–M4 fragment must include Lys342. Since no DST- or NHS-ASA-induced α-γ cross-link was detected in 19-kDa membranes, Lys342 cannot be involved in the cross-link of the native enzyme. Thus, we are left with the conclusion that the most likely lysine residue for DST-mediated cross-linking on the α subunit is Lys347 or Lys352. Similarly the lysine residue modified by NHS-ASA cross-link is predicted to be Lys347 or Lys352.

**Modeling α-γ Interactions**—We attempted to model the interaction of the γ subunit and a homology model of pig α1 subunit using information on α-γ proximities and interactions. Fig. 13A (ribbons) and Fig. 13B (surface) shows the proposed general disposition of the γ and α subunits. Fig. 14 shows details of proposed proximities and interactions of transmembrane (A) and cytoplasmic segment (B), respectively. An important consideration is that secondary structure predictions (Jpred, protein predict, npredict, and psipred at the Swiss-Prot web site at www.expasy.org/tools/)
all indicate that, whereas the transmembrane segment of the γ subunit is α-helix, the cytoplasmic sequence is unstructured or random coil. The inference from the current work that either Lys54 or Lys55 (pig numbering) of γ is cross-linked at 6–8 Å distance from Lys347 or Lys352 in S4 of the α subunit strongly supports a location of the transmembrane of γ in the groove between M2, M9, and M6, overlooked by M4, as proposed previously (20, 22) and also seen in Fig. 13A. Therefore the first step was to optimize docking of the transmembrane segment. The next step was the manual interactive modeling of the unstructured tail SKRLRCGGKHKR. In addition to the cross-linking requirements, the modeling utilized two further constraints, namely that 1) the KKHR residues are known to be necessary for the α-γ interaction (12) and 2) the γ subunit in 19-kDa membranes is intact (49) indicating that the KKHR residues are well protected from tryptic digestion, presumably by interacting with the α subunit. The final model, encompassing both the TM segment as well as the tail (Figs. 13 (overview) and 14 (detailed)), was obtained after a short equilibration molecular dynamics calculation in vacuo as described under “Materials and Methods.”

In the process of docking of the transmembrane segment the tilt and translations were well defined with a single energy minimum. However, two nearly indistinguishable minima were observed for the rotation around the helix long axis. Only one of these minima (slightly higher in energy) placed residues Ala33, Ile44, and Ile43 in contact with the transmembrane segments of the α subunits. Because prior observations, based on mutations, showed that Ala33, Ile44, and Ile43 are important for α-γ interactions (23), the minimum with slightly higher energy was chosen to place the helix (seen in Fig. 14A). In the final model, the Phe949, Glu953, Leu957, and Phe960 in M9 are in contact with γ in line with the recent mutational work (22). Additional TM contacts involve M6 and M2. The model in Fig. 14A is, in essence, a four-helix bundle consisting of M2, M6, M9, and TMγ (41). It is similar to the recently published model (22), although the position of the transmembrane segment of γ is somewhat different.

The placement of the transmembrane segment served as an anchor for the manual, interactive modeling of the unstructured tail. One clear implication of the positioning of the TM segment between M2, M9, and M6 is that Lys352 can be excluded as a cross-linking partner for Lys54 or Lys55. Lys352 faces the opposite side of the protein with respect to γ as seen in the ribbon diagram (Fig. 13A). The distance between the C-terminal end of the TM helix and Lys352 is too far. Thus Lys352 is not seen in the surface diagram of Fig. 13B that shows that only Lys247 is at the same surface and thus close enough to be cross-linked to the cytoplasmic segment of the γ subunit.

Two different models for the positioning of the unstructured tail were evaluated. One places the tail as a rather straight extension from the TM segment toward Lys347, whereas the other follows a groove in the protein surface to the right as seen in Fig. 13B.

The straight model appears less desirable based on the following considerations. First, there is no obvious groove, extending straight up from the transmembrane segments, in which the tail can be placed and protected from tryptic digestion in 19-kDa membranes. Second, the high charge density of the unstructured tail, KKHR, cannot be satisfactorily compensated...
for. Only two acidic residues, Asp\textsuperscript{746} and Asp\textsuperscript{746}, are in close proximity, and one of these (Asp\textsuperscript{746}) appears to be involved in a salt bridge with Arg\textsuperscript{589}. Third, only Lys\textsuperscript{54}, and not Lys\textsuperscript{55}, is at suitable distance to allow a cross-link to Lys\textsuperscript{347}.

On the other hand, the model, which turns to the right, has features that render it more attractive. First, the tail lies in a groove, can make more extensive interactions, and may be protected from tryptic digestion. Second, positioning the tail in this groove automatically positions Lys\textsuperscript{54} and Lys\textsuperscript{55} in close enough proximity to the presumed cross-linking partner Lys\textsuperscript{347}. Depending on the side-chain positioning and details of the backbone conformations, the distance between the terminal amine moieties is between 4 and 10 Å, and a reasonable conformation puts Lys\textsuperscript{54} at a distance of 7.5 Å and Lys\textsuperscript{55} at a distance of 6.3 Å from Lys\textsuperscript{347}. Third, close to Lys\textsuperscript{347} on S4, there are four acidic residues, namely Glu\textsuperscript{756} and Glu\textsuperscript{757} on S5 as well as Glu\textsuperscript{821} and Asp\textsuperscript{823} on the L6/7 loop, capable of interacting with Lys\textsuperscript{54}, Lys\textsuperscript{55}, His\textsuperscript{56}, and Arg\textsuperscript{57} of γ. Thus, all positive charges are compensated by acidic residues. His\textsuperscript{56} is also in close contact with Asn\textsuperscript{910} of L8/9. Although we have no specific evidence, the sequence of the γ beyond Arg\textsuperscript{57}, PINEDEL up to the C terminus, should interact with the α subunit because truncation of the four residues EDEL abrogates the effect of γ on the \(K_m\) for ATP (17). However, this interaction must be relatively weak because it includes the epitope of the anti-γ antibody, which binds the γ subunit in the native protein (7).

It is important to note that, whereas the model explains economically the available experimental data, the data are not sufficiently detailed to unambiguously place all the residues in context. The main value of this model is that it proposes testable hypotheses. It is nevertheless of interest that phospholamban, the regulator of sarcoplasmic/endoplasmic reticulum Ca\textsubscript{2+}-ATPase, has been shown to interact with the L6/7 cytoplasmic loop (52).

\textbf{β-γ Cross-links}—The major DST- and EDC-induced β-γ cross-link is located in the extracellular domain of the β subunit. There is a single lysine residue, Lys\textsuperscript{11}, in the extracellular segment of γ. Therefore, in the case of the DST, Lys\textsuperscript{11} can be cross-linked to a lysine residue downstream of Arg\textsuperscript{142}, the tryptic cleavage site producing the 50- and 16-kDa fragment in 19-kDa membranes (48). The less prominent DST- and EDC-mediated cross-links of γ-β are located in the 16-kDa fragment, which contains the cytoplasmic N-terminal segment, transmembrane segment, and extracellular segment upstream of Arg\textsuperscript{142}. In principle, these cross-links could be located at either surface of the 16-kDa fragment. However, in the case of DST, it is more likely that a lysine in the extracellular segment upstream of Arg\textsuperscript{142} is the partner of the extracellular Lys\textsuperscript{11} of γ because a cytoplasmic lysine of γ is involved in the α-γ cross-link, and the γ subunit appears to fit into the groove between M2, M6, and M9 at the cytoplasmic side. The β subunit is known to interact strongly with the α subunit in the L7/8 loop (53) within residues 89-\textsuperscript{SYGQ} (54), and Tyr\textsuperscript{701}, Val\textsuperscript{907}, and Cys\textsuperscript{911} are also important (55). Cross-linking shows that TMβ is close to TM8 of α, and in a previous study we showed that the 16-kDa fragment of the β subunit can be covalently cross-linked to the α subunit in L7/8 (within Tyr\textsuperscript{855}-Tyr\textsuperscript{901}) (56), and Cu\textsuperscript{2+}-catalyzed oxidative cleavages indicated that two regions of the β subunit interact with the L7/8 loop (within residues 90-115 and 194-205, respectively) (57). The first of these sequences lies within the sequence span of the 16-kDa fragment (Lys\textsuperscript{54}, Arg\textsuperscript{142}). Thus, one can propose that the extracellular domain of the γ subunit comes into proximity with the β subunit near the site of the α-β interaction.

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Covalent Cross-links between the γ Subunit (FXYD2) and α and β Subunits of Na,K-ATPase: MODELING THE α-γ INTERACTION
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