THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 37, pp. 32349–32355, September 16, 2005

Reconstruction of the Complete Ouabain-binding Pocket of Na,K-ATPase in Gastric H,K-ATPase by Substitution of Only Seven Amino Acids*

Received for publication, May 11, 2005, and in revised form, July 19, 2005 Published, JBC Papers in Press, July 28, 2005, DOI 10.1074/jbc.M505168200

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Although cardiac glycosides have been used as drugs for more than 2 centuries and their primary target, the sodium pump (Na,K-ATPase), has already been known for 4 decades, their exact binding site is still elusive. In our efforts to define the molecular basis of digitalis glycosides binding we started from the fact that a closely related enzyme, the gastric H,K-ATPase, does not bind glycosides like ouabain. Previously, we showed that a chimera of these two enzymes, in which only the M3-M4 and M5-M6 hairpins were of Na,K-ATPase, bound ouabain with high affinity (Koenderink, J. B., Hermsen, H. P. H., Swarts, H. G. P., Willems, P. H. G. M., and De Pont, J. J. H. H. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 11209–11214). We also demonstrated that only three amino acids (Phe^{783}, Thr^{797}, and Asp^{804}) present in the M5-M6 hairpin of Na,K-ATPase were sufficient to confer high affinity ouabain binding to a chimera which contained in addition the M3-M4 hairpin of Na,K-ATPase (Qiu, L. Y., Koenderink, J. B., Swarts, H. G., Willems, P. H., and De Pont, J. J. H. H. M. (2003) *J. Biol. Chem.* 278, 47240–47244). To further pinpoint the ouabain-binding site here we used a chimera-based loss-of-function strategy and identified four amino acids (Glu^{312}, Val^{314}, Ile^{315}, Gly^{319}), all present in M4, as being important for ouabain binding. In a final gain-of-function study we showed that a gastric H,K-ATPase that contained Glu^{312}, Val^{314}, Ile^{315}, Gly^{319}, Phe^{783}, Thr^{797}, and Asp^{804} of Na,K-ATPase bound ouabain with the same affinity as the native enzyme. Based on the E_2P crystal structure of Ca^{2+}-ATPase we constructed a homology model for the ouabain-binding site of Na,K-ATPase involving all seven amino acids as well as several earlier postulated amino acids.

Cardiac glycosides, like ouabain, were already used in medicine in the 18th century. Nowadays, these drugs are mainly used in the treatment of congestive heart failure and arrhythmias. In 1953 Schatzmann (1) found that these cardiac glycosides inhibit the active transport of Na^+ and K^+. A few years later Skou (2) found that this cation transport was caused by the Na,K-ATPase. This enzyme (also called the sodium pump) is an integral membrane protein found in the cells of all higher eukaryotes and is responsible for translocating sodium and potassium ions across the cell membrane utilizing ATP as the energy source (3). Since 1960 a large number of studies have established that ouabain inhibits Na,K-ATPase by binding to a hitherto undefined binding pocket that is accessed from the extracellular side. The sodium pump alternates between two conformational states (E_1 and E_2) during the catalytic cycle. Ouabain binds to the phosphorylated E_2 form (E_2P) of the enzyme with high affinity (4). Identifying the amino acid residues involved in ouabain binding is important for understanding the molecular mechanism by which this drug interacts with and inhibits the activity of Na,K-ATPase. The localization of the ouabain-binding site on Na,K-ATPase has been studied for many years, but the amino acids involved in direct binding are still unknown.

Since ouabain binds to Na,K-ATPase from the extracellular side, most previous studies focused on substituting amino acids in the extracellular loops of the enzyme. Price and Lingrel (5, 6) found that residues Arg^{111} and Asp^{122}, which lie on the border of the first extracellular loop of the rat α-subunit, were responsible for the ouabain-resistant nature of mouse and rat enzymes. Other studies demonstrated that mutation of Cys^{104}, Tyr^{108}, Pro^{118}, Asp^{121}, Tyr^{124}, Tyr^{308}, Leu^{330}, Ala^{331}, Thr^{338}, Cys^{367}, Cys^{656}, Phe^{786}, Leu^{793}, Thr^{797}, Phe^{863}, Arg^{880}, and Phe^{982} had negative effects on the binding of ouabain to Na,K-ATPase (7, 8). The difficulty with most of these studies is that the observed loss of ouabain binding can equally well be the result of indirect effects. To address this problem, one can perform a gain-of-ouabain binding experiment by introducing the identified amino acids in a closely related enzyme that lacks the capacity to bind ouabain. The gastric H,K-ATPase is unable to bind ouabain but shows a high degree of homology to Na,K-ATPase. The catalytic α-subunits of these two P-type ATPase are 63% identical in amino acid composition (9).

We have made chimeric enzymes between the Na,K-ATPase and H,K-ATPase in an attempt to identify the residues that are crucial for ouabain binding. In a previous study, we demonstrated that replacement of the transmembrane hairpins M3-M4 and M5-M6 in H,K-ATPase by those of Na,K-ATPase results in the formation of a high affinity ouabain-binding site (10). In a follow-up study we showed that a chimera, in which only the M3-M4 hairpin and three amino acids of M5-M6 (Phe^{783}, Thr^{797}, and Asp^{804}) originate from Na,K-ATPase, binds ouabain with high affinity (11). However, the M3-M4 hairpin in the latter chimera still contains 27 amino acids that are unique for Na,K-ATPase. Here we report a gastric H,K-ATPase that gained a high affinity ouabain-binding site after replacing only seven amino acids by their Na,K-ATPase counterparts. By homology modeling starting from the E_2P crystal structure of Ca^{2+}-ATPase (12) we constructed a model for

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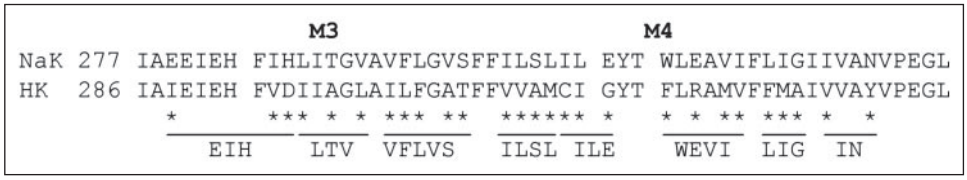

FIGURE 1. **Alignment of amino acid sequence between the M3-M4 region of rat Na,K-ATPase and rat gastric H,K-ATPase α_1-subunits.** Amino acid numbers are indicated on the *left side*. The amino acids that differ between the two enzymes are marked with *asterisks*. The eight mutants that incorporate two to five amino acids of gastric H,K-ATPase into the HN34/56 chimera (10) are indicated by the Na,K-ATPase amino acids they replace: EIH, LTV, VFLVS, ILSL, ILE, WEVI, LIG, and IN.

the ouabain-binding pocket in Na,K-ATPase in which several of the amino acids, of which a role in ouabain binding has been postulated, are shown to play a crucial role in the binding of ouabain.

EXPERIMENTAL PROCEDURES

Construction of Chimeras and Mutants—The chimeras and mutants presented in this paper were constructed from the rat Na,K-ATPase α_1-subunit containing the R111Q and D122N mutations (5, 10) and the rat gastric H,K-ATPase α-subunit (9). The rat Na,K-ATPase α_1- and β_1-subunits, the rat gastric H,K-ATPase α- and β-subunits, and the chimeras were subcloned into the pTLN vector, which is suitable for the *Xenopus laevis* oocyte expression system (14). Site-directed mutagenesis was used to generate the mutants described in this paper. All introduced mutations were verified by sequencing. For clarity reasons we used the numbering of the pig Na,K-ATPase, also for residues in parts originating from H,K-ATPase.

Expression, [^{3}H]Ouabain Binding, and ATP Phosphorylation—Oocytes were injected with cRNAs, and after 3 days the membranes were isolated as described previously (11). For immunoblotting, the α-subunits of H,K-ATPase and the chimeras were detected with the polyclonal antibody HKB (15). [^{3}H]Ouabain binding and ATP phosphorylation were carried out as described previously (11).

In previous studies using the baculovirus expression system we determined that the maximal ATPase activity at infinite ATP concentrations of Na,K-ATPase was 1.8 ± 0.6 μmol/mg/h and that of H,K-ATPase was 1.4 ± 0.1 μmol/mg/h (16). The ATPase activity of chimera HN34/56 was 49% of that of the wild type H,K-ATPase at 10 μM ATP, 3 mM KCl, and pH 6.0 (10). The ouabain binding level of Na,K-ATPase (QN) was 0.40 ± 0.04 pmol/mg when 1 mM P_i was present, whereas that of HN34/56 was 0.20 ± 0.01 pmol/mg when 1 mM ATP was present (10). The ouabain binding level of H,K-ATPase was only 0.04 ± 0.01 pmol/mg in the presence of 1 mM P_i or ATP.

Molecular Modeling of Ouabain-binding ATPases—Sequence alignment of Na,K-ATPase (Swiss-Prot accession number P05023) to the newly released crystal structure of Ca^{2+}-ATPase in the E_2P state (12) (Protein Data Bank ID code 1WPG) was obtained as described previously and shown in Fig. 7 (17). The E_2P state was chosen because of its higher affinity to ouabain. The homology model was built with SCWRL (18), while structurally divergent loop regions were copied from other Protein Data Bank structures with similar local sequence and geometry. Then, molecular dynamics simulations were run in explicit solvent with the Yamber2 force field and the associated protocol (19) until the WHAT IF (20) quality indicators, Ramachandran plot, backbone conformation, and three-dimensional packing quality (21), converged. During these simulations, the backbone atoms of aligned residues were kept fixed.

Docking of Ouabain—The homology model of Na,K-ATPase was used for *in silico* docking with the FlexX program (22), Version 1.13.5 L. FlexX predicts the conformations of a set of energetically favorable molecular complexes consisting of the ligand bound to the active site of the protein. The complexes are labeled with a score that approximates binding energy. Because of the huge size of the ATPase model, placement of the ligand was restricted to the previously postulated binding pocket on the extracellular side of the membrane (11). Besides an overall pocket, a subpocket was defined to include residues known from mutation experiments to be important for ligand binding. The use of a subpocket lets FlexX place the base fragment (the first placed fragment of the ligand) near one of the residues in the subpocket. The rest of the ligand still has the freedom to completely move away from these residues as long as the ligand remains within the overall pocket.

The starting structure for ouabain was obtained from the Cambridge Structural Data Base (23). Hydrogens were added interactively with the Molden package (24). Molden was also used to assign the atom types of the Sybyl force field. This assignment was checked by visual inspection. Bond lengths and bond angles were optimized with the Sybyl force field.

FlexX produced a number of protein-ligand complexes, of which the 10 best scoring ones were inspected visually. These so-called poses showed little variation in geometries. The best scoring pose was used for this work. As FlexX keeps the protein completely fixed, the amino acid side chain rotamers could not be expected to be optimal, as the homology model was built in absence of ouabain. Consequently, the best scoring pose was subjected to an additional energy minimization with the NOVA force field (25), which resulted in improved hydrogen bonding patterns shown in Fig. 8.

Calculations—Data are presented as mean values with standard error of the mean. Differences were tested for significance by means of Student's *t* test.

RESULTS

Identification of Amino Acids in M3-M4 That Are Involved in Ouabain Binding—To identify the amino acids that are essential for ouabain binding in the M3-M4 region, the 27 unique amino acids were replaced with those of H,K-ATPase after which ouabain binding was measured. In first instance eight mutants (see Fig. 1) of chimera HN34/56 were generated and co-expressed with the β-subunit of H,K-ATPase in *X. laevis* oocytes. Western blot analysis of total membrane protein using the polyclonal antibody HKB that recognizes the loop between M4 and M5 of the gastric H,K-ATPase α-subunit (15) showed that the expression levels of the mutants and chimera HN34/56 were virtually the same (Fig. 2*A*). Ouabain binding experiments were carried out in the presence of 1 mM ATP and 250 nM ouabain (Fig. 2*B*). The [^{3}H]ouabain binding levels of the mutants WEVI, LIG, and IN were significantly lower than that of chimera HN34/56 ($p < 0.05$), and not significantly different from that of non-injected oocytes ($p > 0.05$), indicating that these mutants had lost their ability to bind [^{3}H]ouabain. However, the [^{3}H]ouabain-binding capacity of the mutants EIH, LTV, VFLVS, ILSL, and ILE was rather similar to that of chimera HN34/56 ($p > 0.05$). From these findings it can be concluded that three to nine amino acids present in transmembrane segment M4 of Na,K-ATPase are important for ouabain binding and that residues in the M3 segment do not play a crucial role.

To assess which of these nine amino acids are required for high affinity ouabain binding, we generated individual mutants of chimera HN34/

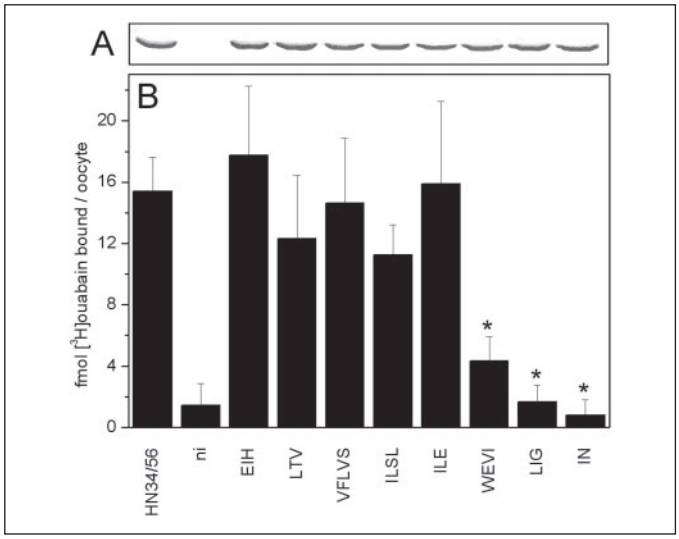

FIGURE 2. **[^{3}H]Ouabain binding of HN34/56 mutants.** *A*, total membrane proteins from oocytes were probed with the antibody, HKB, which specifically reacts with the large intracellular loop of the H,K-ATPase α-subunit. *B*, [^{3}H]ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, and 250 nM [^{3}H]ouabain. Mean values ± S.E. of three enzyme preparations are shown. *, significantly different from HN34/56 ($p < 0.05$). The eight mutants (EIH, LTV, VFLVS, ILSL, ILE, WEVI, LIG, and IN) are described in the legend to Fig. 1.

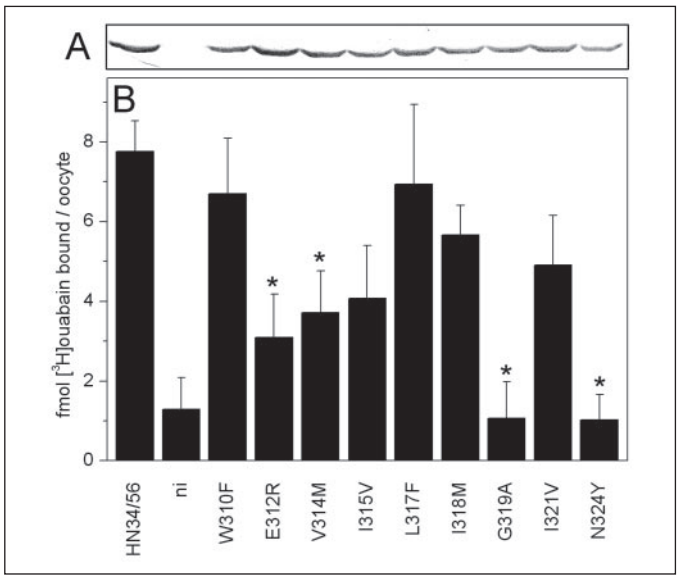

FIGURE 3. **[^{3}H]Ouabain binding of HN34/56 mutants.** *A*, total membrane proteins from oocytes were probed with the antibody, HKB, which specifically reacts with the large intracellular loop of the H,K-ATPase α-subunit. *B*, [^{3}H]ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, and 250 nM [^{3}H]ouabain. Mean values ± S.E. of four enzyme preparations are shown. *, significantly different from HN34/56 ($p < 0.05$).

56, in which each of the nine amino acids was replaced by the corresponding residue from gastric H,K-ATPase. No major differences in expression levels between the mutants and chimera HN34/56 were observed (Fig. 3*A*). Fig. 3*B* shows that the [^{3}H]ouabain binding levels of mutants G319A and N324Y were significantly lower than that of chimera HN34/56 ($p < 0.05$) and not different from that of non-injected oocytes ($p > 0.05$), indicating that these two mutants had lost their ability to bind ouabain. All mutants could be phosphorylated by ATP, except mutant N324Y (data not shown). This lack of phosphorylation is in agreement with previous reports showing the importance of this residue in determining the cation selectivity and E_1/E_2 conformational equilibrium (26, 27). Because formation of the phosphorylated intermediate is required for high affinity ouabain binding, we conclude that loss of ouabain binding by N324Y is most likely caused by its inability to become phosphorylated. The [^{3}H]ouabain binding levels of mutants E312R, V314M, and I315V were higher than that of non-injected oocytes ($p < 0.05$), whereas they were lower (E312R and V314M; $p < 0.05$) or tended to be lower (I315V; $p < 0.08$) than that of chimera HN34/56. This indicates that these three mutants probably have a reduced ouabain affinity. Altogether, these findings suggest that four amino acids Glu^{312}, Val^{314}, Ile^{315}, and Gly^{319}, all present in M4 of Na,K-ATPase, are essential for ouabain binding.

Ouabain Binding by a Mutated Gastric H,K-ATPase—The disadvantage of "loss-of-function" experiments is that mutation of a certain amino acid can lead to a loss of ouabain binding, by an indirect mechanism. In "gain-of-function" experiments these faulty identified amino acids will not be essential for ouabain binding and will be deleted from the amino acids identified as essential for ouabain binding. In addition, the affinity of the constructs that gained ouabain binding indicates whether the complete binding site is present. Therefore, we used a gain-of-function experiment in which we introduced the above four amino acids together with Asn^{324} into HN-FTD (HN-EVIGN-FTD, see TABLE ONE). The importance of Asn^{324} was tested by including HN-EVIG-FTD in the same experiment. HN-FTD itself contains only three amino acids (Phe^{783}, Thr^{797}, and Asp^{804}) from Na,K-ATPase, of which we previously demonstrated that they were sufficient to confer high affinity ouabain binding to HN34 (11). The Western blot presented in Fig. 4*A* shows equal expression of HN34/56, HN-FTD, HN-EVIGN-FTD, and HN-EVIG-FTD. Ouabain bound similarly to both HN-EVIGN-FTD and HN-EVIG-FTD, indicating that Asn^{324} is not required for ouabain binding and confirming the importance of the other four amino acids identified in the loss-of-function analysis. Although replacement of Gly^{319} in HN34/56 (G319A) caused complete loss of ouabain binding (Fig. 3*B*), its introduction in HN-FTD (HN-G-FTD) did not result in any increase in ouabain binding (Fig. 4*B*). Similarly, introduction in HN-FTD of the three amino acids, which upon individual replacement in HN34/56 caused only partial reduction in ouabain binding (Fig. 3*B*; HN-EVI-FTD) did not confer ouabain-binding capacity to this chimera. Based on these findings it can be concluded that a combination of Gly^{319} and one or more of the three other amino acids (Glu^{312}, Val^{314}, Ile^{315}) is required for ouabain binding. To test this idea we generated HN-VIG-FTD, HN-EIG-FTD, and HN-EVG-FTD and determined their ouabain-binding capacity (Fig. 5). All three mutants were readily expressed (Fig. 5*A*), but none of them showed significant ouabain binding (Fig. 5*B*). This result clearly demonstrates that Glu^{312}, Val^{314}, Ile^{315}, and Gly^{319} are all four required to confer ouabain-binding capacity to HN-FTD. Taken together our work shows that replacement of only seven amino acids in gastric H,K-ATPase by their Na,K-ATPase counterparts lends ouabain sensitivity to the enzyme.

Finally, the ouabain affinity of this modified H,K-ATPase (HN-EVIG-FTD) was compared with that of chimera HN34/56 and Na,K-ATPase (Fig. 6). In this assay, which was performed in the presence of 1 mM ATP and 100 mM NaCl, the apparent ouabain affinities for Na,K-ATPase (0.2 ± 0.1 μM), HN34/56 (0.5 ± 0.1 μM), and HN-EVIG-FTD (0.5 ± 0.1 μM) were not significantly different ($p > 0.05$), confirming that only seven amino acids of Na,K-ATPase can confer high affinity ouabain binding to gastric H,K-ATPase.

Homology Model of the Ouabain-binding Site—We used two homology models of the α_1-subunit of Na,K-ATPase to dock ouabain into its putative binding site using FlexX (22). The first model, built from Ca^{2+}-ATPase in the E_2 conformation (Protein Data Bank ID code 1IWO), did not yield a high scoring solution, most likely because the binding cleft was too narrow. The 1IWO structure is a Ca^{2+}-unbound form that is

TABLE ONE

Description of the used mutants

All mutants used in this study have the rat gastric H,K-ATPase α-subunit as a backbone and contain the β-subunit of this enzyme. On positions indicated with an *X* the amino acids of H,K-ATPase (second line) are replaced with those of rat Na,K-ATPase (first line).

Na,K-ATPase	Glu^{312}	Val^{314}	Ile^{315}	Gly^{319}	Asn^{324}	Phe^{783}	Thr^{797}	Asp^{804}
H,K-ATPase	Arg^{328}	Met^{330}	Val^{331}	Ala^{335}	Tyr^{340}	Tyr^{799}	Cys^{813}	Glu^{820}
HN-FTD	—	—	—	—	—	*X*	*X*	*X*
HN-G-FTD	—	—	—	*X*	—	*X*	*X*	*X*
HN-GN-FTD	—	—	—	*X*	*X*	*X*	*X*	*X*
HN-EVIG-FTD	*X*	*X*	*X*	*X*	—	*X*	*X*	*X*
HN-EVIGN-FTD	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
HN-EVI-FTD	*X*	*X*	*X*	—	—	*X*	*X*	*X*
HN-VIG-FTD		*X*	*X*	*X*	—	*X*	*X*	*X*
HN-EIG-FTD	*X*		*X*	*X*	—	*X*	*X*	*X*
HN-EVG-FTD	*X*	*X*	—	*X*	—	*X*	*X*	*X*

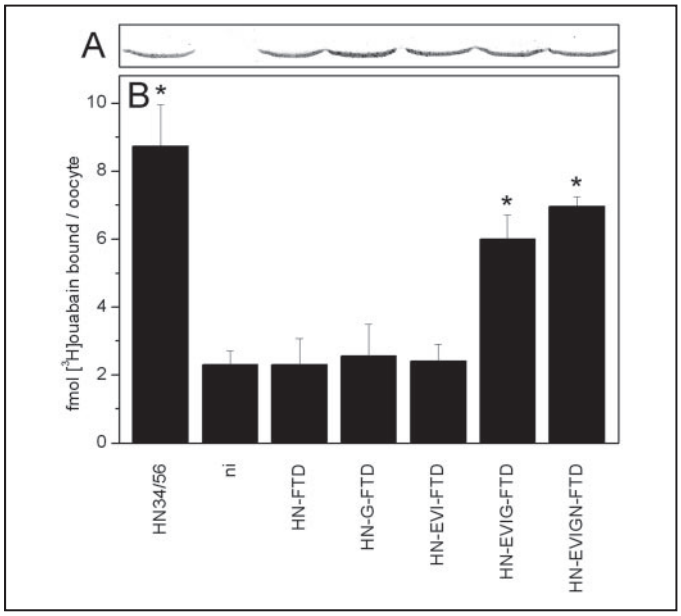

FIGURE 4. **[^{3}H]Ouabain binding of HN-FTD mutants.** *A*, total membrane proteins from oocytes were probed with the antibody, HKB, which specifically reacts with the large intracellular loop of the H,K-ATPase α-subunit. *B*, [^{3}H]ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, and 250 nM [^{3}H]ouabain. Mean values ± S.E. of three to four enzyme preparations are shown. *, significantly different from non-injected oocytes ($p < 0.05$). The mutants are described in TABLE ONE.

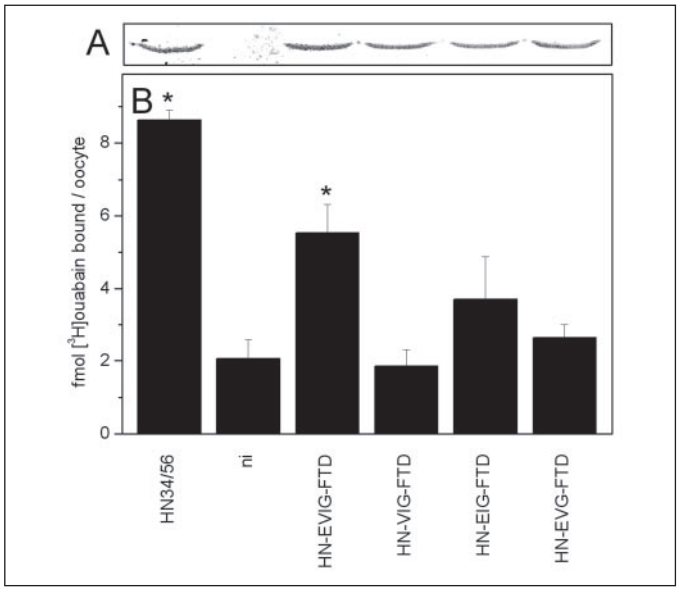

FIGURE 5. **[^{3}H]Ouabain binding of HN-FTD mutants.** *A*, total membrane proteins from oocytes were probed with the antibody, HKB, which specifically reacts with the large intracellular loop of the H,K-ATPase α-subunit. *B*, [^{3}H]ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, and 250 nM [^{3}H]ouabain. Mean values ± S.E. of three to four enzyme preparations are shown. *, significantly different from non-injected oocytes ($p < 0.05$). The mutants are described in TABLE ONE.

stabilized by the inhibitor thapsigargin (E_2). The 1WPG structure is also a Ca^{2+}-unbound form but contains Mg^{2+} next to a bound phosphate analogue, MgF_4^{2-}, and is stabilized with thapsigargin). The structure represents the E_2P_i state, just after the hydrolysis of the aspartyl phosphate but before the release of phosphate from the ATPase. The second model was based on this newly released structure of Ca^{2+}-ATPase (Protein Data Bank ID code 1WPG) (12). In this model, helix M4 was tilted outward leading to a widening of the binding cleft and resulting in a docked complex with a significant FlexX score of 4.053. While docking to homology models is a difficult task, the docked complex fitted well to the experimental data (Figs. 7 and 8) . Five of the seven essential residues (Glu^{312}, Val^{314}, Ile^{315}, Gly^{319}, and Thr^{797}) were in direct proximity of ouabain (closer than 5 Å) forming for example hydrogen bonds (Glu^{312} and Thr^{797}) or hydrophobic contacts (Ile^{315}). The remaining two amino acids (Phe^{783} and Asp^{804}) were providing structural support. The docked complex identified a number of additional residues involved in ouabain binding: Gln^{111} and Asn^{122} between helices M1 and M2 (5), Thr^{309} at the border of M4, and Thr^{799} in M6. Because these amino acids are conserved between Na,K-ATPase (of ouabain-sensitive species) and gastric H,K-ATPase they did not show up in our experiments.

DISCUSSION

Na,K-ATPase and gastric H,K-ATPase belong to the IIC subfamily of the cation transport (P-type) ATPases. Although their α-subunits share 63% homology in amino acid sequence both enzymes differ in ion selectivity, subcellular localization and pharmacological profile. As far as the sensitivity to drugs is concerned, Na,K-ATPase is specifically inhibited by ouabain and related cardiac glycosides. Ouabain binds from the extracellular side but the exact location of the binding site is still unknown. In previous studies, we first narrowed down this site to transmembrane hairpins M3-M4 and M5-M6 (10), after which we identified three residues (Phe^{783}, Thr^{797}, and Asp^{804}) in M5-M6 that play a key role in ouabain binding (11). In the present study, we used our chimera-based loss-of-function approach to identify another four residues (Glu^{312}, Val^{314}, Ile^{315}, Gly^{319}) in the M3-M4 hairpin of Na,K-ATPase. To confirm the importance of the identified amino acids for ouabain binding, we performed a gain-of-function experiment showing that a H,K-ATPase mutant with seven residues from Na,K-ATPase binds ouabain

with the same affinity as the native enzyme. Because ouabain is a highly specific inhibitor of Na,K-ATPase that binds preferentially to the phosphorylated intermediate, in our opinion it is very unlikely that a newly constructed ouabain binding site has been generated that binds ouabain with the same affinity as the parent Na,K-ATPase.

Interactions between M3-M4 and the Sugar Moiety of Ouabain—In our docking model, Thr^{309} and Glu^{312}, which are present in M4, form hydrogen bridges with hydroxyl groups of the sugar moiety of ouabain. Thr^{309} was not identified with our loss-of-function strategy because it is also present in gastric H,K-ATPase. Canessa *et al.* (28) reported that Tyr^{308} played a critical role in ouabain binding. In our model this residue is close to, but not in direct contact with, ouabain. Our docking model is in agreement with previous work pointing to an important role for the second extracellular loop in ouabain binding. Already in 1985 Shull *et al.* (29) reported that only this loop displayed all the requirements for ouabain binding: relatively hydrophobic, shielded from water, and close to a Trp residue (30). A few years later it was shown that the *p*-aminobenzyldiazonio derivative of ouabain, in which the reactive substituent was bound to the rhamnosyl residue of ouabain, labeled Trp^{310} (31). Similarly, it was shown that photosensitive 12-azido-3β-digitoxoside complexed with Trp^{310}–Ala^{313} (32), whereas antibody M45-80, which recognizes the E^{307}YTWLE sequence, was found to enhance the rate of ouabain binding (33, 34).

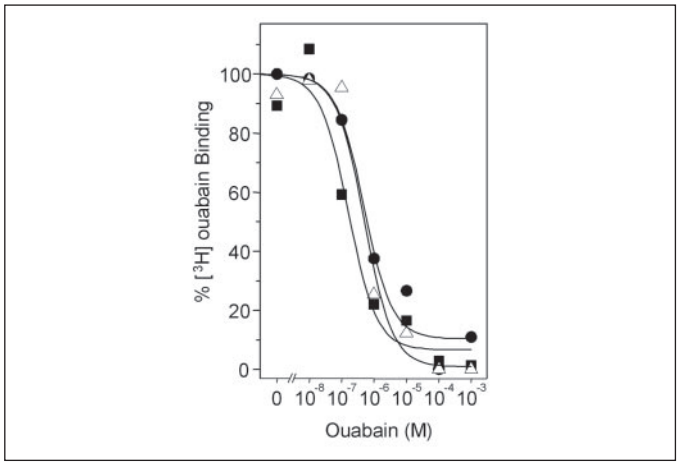

FIGURE 6. **[^{3}H]Ouabain binding to HN34/56, HN-EVIG-FTD, and rat Na,K-ATPase R113Q/D124N.** [^{3}H]Ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, 10 nM [^{3}H]ouabain, and varying concentrations of non-radioactive ouabain. The binding of [^{3}H]ouabain in the absence of nonradioactive ouabain was set at 100%. ■, Na,K-ATPase; ●, HN34/56; △, HN-EVIG-FTD (described in TABLE ONE).

Interactions between M4 and M1-M2 and the Steroid Body of Ouabain—In our docking model, Ile^{315}, which is present in M4, is in close proximity to the steroid body of ouabain and has a hydrophobic interaction. In contrast, Val^{314} is located on the wrong site of the helical wheel and can therefore not have a direct interaction with ouabain. Most probably, replacement of Val^{314} with its H,K-ATPase counterpart Met has a negative structural effect on the ouabain-binding pocket. The same holds true for Gly^{319}, which also has no direct interaction with ouabain in our model. The importance of this residue is also shown by the work of Horisberger *et al.* (35) who found that replacement of this residue with a Cys resulted in a marked decrease in transport activity. Other amino acids present in M4 that have been implicated in ouabain binding are Leu^{330}, Ala^{331}, and Thr^{338} (7). However, these amino acids are located at the inner interface between membrane and cytosol and are therefore not in direct contact with the ouabain molecule. This part of M4 is completely conserved between Na,K-ATPase and H,K-ATPase suggesting an important role in enzyme functioning. Thus, mutations in this part of M4 may affect ouabain binding indirectly through alterations in enzyme activity or conformational equilibrium.

Numerous studies have demonstrated the importance of Gln^{111} and Asn^{122} (first extracellular loop between M1 and M2) for high affinity ouabain binding (5, 6, 10). These residues are also present in rat gastric H,K-ATPase. In our docking complex these residues form hydrogen bridges with the hydroxyl groups on the positions 1 and 11 of the steroid body of ouabain. Our docking model is supported by previous work showing that these two residues do not bind the sugar moiety (36). In helveticoside the hydroxyl groups at positions 1 and 11 are deleted. In addition the hydroxyl group at position 19 is replaced by a keto group, and the rhamnose group is replaced by a single digitoxose group. The binding affinity of this drug is ~40 times lower than that of ouabain (37),

FIGURE 7. **The seed alignment of Na,K-ATPase (Swiss-Prot accession number P05024) and Ca^{2+}-ATPase (Swiss-Prot accession number P04191) used to build the homology model depicted in Fig. 8.** Only the 10 transmembrane helices and connecting loops are shown here, with *circles* marking the seven amino acids transfered to H,K-ATPase. *Minus signs* in the Ca^{2+}-ATPase sequence indicate those loop residues that could not be modeled by homology due to structural divergence. The loop modeling procedure considered secondary structure predictions, which lead to a C-terminal extension of helix 7 and an N-terminal extension of helix 8.

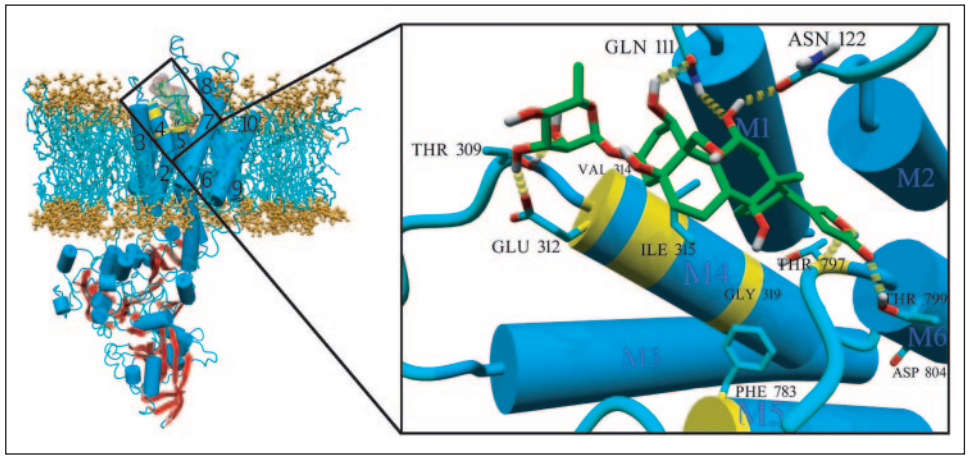

FIGURE 8. **Molecular model of the ouabain binding site in Na,K-ATPase.** Nitrogen is colored *blue*, oxygen *red*, and carbon *cyan* or *green* (in ouabain). Hydrogen bonds are shown as *yellow disks*. Surrounding parts of the structure have been hidden for clarity. Images were created with YASARA (www.yasara.org).

which fits with our model. In addition to Gln^{111} and Asn^{122}, mutagenesis studies have implicated Cys^{104} (M1), which is also present in gastric H,K-ATPase, in ouabain binding (38–40). In agreement with these mutagenesis studies, the digoxigenin derivative *N*-hydroxysuccimidyl digoxigenin-3-*O*-methylcarbonyl-ϵ-aminocaproate was found to form a thioester bond with this residue (41), whereas its replacement had differential effects on the sensitivity of the enzyme to digoxin and digitoxin (42). In our docking model, however, Cys^{104} does not interact directly with ouabain. The same holds true for Tyr^{108}, which is in close proximity to the steroid body of ouabain and has been shown to contribute to the ouabain affinity of the enzyme (38, 40). Other amino acids in the first extracellular loop have also been implicated in ouabain binding. Glu^{116}, Pro^{118}, and Asp^{121} are located between key residues Gln^{111} and Asn^{122}, and it is therefore not surprising that they were shown to influence ouabain binding (7, 8). In the ouabain-binding chimera HNEVIG-FTD, however, Glu^{116} and Pro^{118} are not present. Recently, Crambert *et al.* (43) found that Thr^{114}, Gln^{119}, Ile^{874}, and Gln^{898} were involved in the access and release of ouabain to and from its binding site. Our docking model is compatible with this putative role of Thr^{114} and Gln^{119} in the process of ouabain binding. Ile^{874} and Gln^{898} are located in the fourth extracellular loop between M7 and M8 that interacts with the β-subunit. Our homology model does not include a β-subunit and is therefore not suitable to predict the location of these two amino acids.

Interactions between M5-M6 and the Lactone Ring of Ouabain—The carbonyl oxygen of the lactone ring forms a hydrogen bridge with the hydroxyl group of Thr^{799}, which is present in the third extracellular loop between M5 and M6. Such a bridge is also formed between the oxygen of the lactone ring and the backbone nitrogens of residues Thr^{797} and Val^{798}. In agreement with the present study, previous work demonstrated the importance of Thr^{797} for ouabain binding (44, 45). Similarly, Leu^{793}, which in our model is very close to ouabain, has been found to influence ouabain binding (13, 46). Phe^{783} points toward ouabain but is too far away to interact directly with this molecule. Most probably its replacement with a Tyr indirectly disturbs the ouabain-binding site. As discussed in our previous paper (11) Asp^{804} (M6) affects ouabain binding indirectly through its effects on cation binding. Phe^{786}, whose replacement with an Asn or an Ile was also found to result in a decrease in ouabain affinity (46), is located in the third extracellular loop just outside M5. However, in our docking model this residue does not interact with ouabain.

Role of C-terminal Amino Acids in Ouabain Binding—Several amino acids at the C-terminal end of the α-subunit have been implicated in ouabain binding. Replacement of Phe^{863} with a Leu (46), Arg^{880} with a Pro or Leu (38, 40), and Phe^{982} with a Ser (7) all resulted in a decrease in ouabain affinity. Phe^{863} and Phe^{982} are located in M7 and M10, respectively, and according to our docking model these amino acids are far away from the actual ouabain-binding site. Although Arg^{880} is also not very close to the ouabain-binding site, it may interact with ouabain because the position of the fourth extracellular loop that interacts with the β-subunit is still very uncertain.

Mechanism of Ouabain Inhibition—Paula *et al.* (37) found that substitution of the five-membered lactone by a six-membered lactone caused a decrease in binding affinity and an increase in inhibitory potency, whereas removal of the sugar moiety of ouabain caused a dramatic decrease in binding affinity but had little effect on inhibitory potency (37). These findings are in agreement with our docking model, in which the lactone ring is buried deep inside the lipid bilayer, whereas the rhamnose is located more superficially. A plausible mechanism for Na,K-ATPase inhibition is digitalis-mediated paralysis of M5-M6 loop movement (46). After binding of ouabain to the first extracellular loop and M4, it exerts its inhibitory activity by interacting with the M5-M6 loop that is involved in cation binding, thus restricting its flexibility. This mechanism might also be the basis for the K^+-ouabain antagonism.

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