High-affinity ouabain binding by a chimeric gastric H^+,K^+-ATPase containing transmembrane hairpins M3-M4 and M5-M6 of the α_1-subunit of rat Na^+,K^+-ATPase

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Na^+,K^+-ATPase and gastric H^+,K^+-ATPase are two related enzymes that are responsible for active cation transport. Na^+,K^+-ATPase activity is inhibited specifically by ouabain, whereas H^+,K^+-ATPase is insensitive to this drug. Because it is not known which parts of the catalytic subunit of Na^+,K^+-ATPase are responsible for ouabain binding, we prepared chimeras in which small parts of the α-subunit of H^+,K^+-ATPase were replaced by their counterparts of the α_1-subunit of rat Na^+,K^+-ATPase. A chimeric enzyme in which transmembrane segments 5 and 6 of H^+,K^+-ATPase were replaced by those of Na^+,K^+-ATPase could form a phosphorylated intermediate, but hardly showed a K^+-stimulated dephosphorylation reaction. When transmembrane segments 3 and 4 of Na^+,K^+-ATPase were also included in this chimeric ATPase, K^+-stimulated dephosphorylation became apparent. This suggests that there is a direct interaction between the hairpins M3-M4 and M5-M6. Remarkably, this chimeric enzyme, HN34/56, had obtained a high-affinity ouabain-binding site, whereas the rat Na^+,K^+-ATPase, from which the hairpins originate, has a low affinity for ouabain. The low affinity of the rat Na^+,K^+-ATPase previously had been attributed to the presence of two charged amino acids in the extracellular domain between M1 and M2. In the HN34/56 chimera, the M1/M2 loop, however, originates from H^+,K^+-ATPase, which has two polar uncharged amino acids on this position. Placement of two charged amino acids in the M1/M2 loop of chimera HN34/56 results in a decreased ouabain affinity. This indicates that although the M1/M2 loop affects the ouabain affinity, binding occurs when the M3/M4 and M5/M6 hairpins of Na^+,K^+-ATPase are present.

Na^+,K^+-ATPase, the enzyme responsible for active Na^+ and K^+ transport over the plasma membrane, is found in the cells of all higher eukaryotes (1). It is the target molecule for cardiac glycosides, such as ouabain, which inhibit the enzyme activity by binding from the extracellular side of the enzyme. Recently, the presence of endogenous ouabain has been demonstrated in mammalians (2–4), and it is suggested that it may play a role in the pathogenesis of hypertension (5). The interaction between ouabain and Na^+,K^+-ATPase appears to be important for understanding the regulation of Na^+,K^+-ATPase activity. Until now the location of the binding site for ouabain remained to be elucidated.

H^+,K^+-ATPase, like Na^+,K^+-ATPase, belongs to the subfamily of P_2-type ATPases. Both these enzymes hydrolyze ATP and use the liberated energy for transport of cations across the membrane. Their heavily glycosylated β-subunits are structurally similar but only 30% identical and play a modulatory role in cation-dependent reactions (6). This is in contrast to their catalytic α-subunits, which share a higher degree of identity (63%) and are responsible for the cation specificity. Despite these structural similarities, Na^+,K^+-ATPase is specifically inhibited by ouabain, whereas H^+,K^+-ATPase is insensitive to this drug.

It is now generally accepted that amino acids present in the first extracellular loop of Na^+,K^+-ATPase are involved in the binding of ouabain (7–11). The border residues of this loop, Arg113 and Asp124, are responsible for the ouabain-resistant character of the rat enzyme (12, 13). Further studies demonstrated that other residues in this loop are also important for determining the ouabain sensitivity (14, 15). A chimeric sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase with Ala70 until Asp200 of Na^+,K^+-ATPase can bind ouabain with a rather high affinity (16, 17). A chimeric H^+,K^+-ATPase with the first extracellular loop and C815 (present in the extracellular loop between transmembrane helix 5 and 6) replaced by the corresponding amino acids of Na^+,K^+-ATPase (Thr) also acquired ouabain sensitivity, although the affinity was very low (11). It has been demonstrated previously, on the other hand, that the ouabain sensitivity is not confined to the amino-terminal half of Na^+,K^+-ATPase (18, 19). Indeed, many amino acid substitutions throughout the α-subunit increase the ouabain resistance (reviewed in ref. 8).

For a number of years the localization of the ouabain-binding site on Na^+,K^+-ATPase has been the subject of many studies, but despite attempts to unravel the precise location of ouabain binding, the amino acids involved in direct binding are still unknown. The K^+-ouabain antagonism suggests that the K^+-binding site, which probably includes M4, M5, and M6, also may be involved in ouabain binding (20). In the present study we have investigated the role of transmembrane hairpins M1-M2, M3-M4, and M5-M6 in the binding of ouabain. In a chimera (HN34/56) the transmembrane regions M3-M4 and M5-M6 of H^+,K^+-ATPase were replaced by those of the α_1-subunit of rat Na^+,K^+-ATPase, an isotype with a low affinity for ouabain. Surprisingly, chimera HN34/56 did bind ouabain with a very high affinity. We demonstrated that the presence of the M3/M4 and M5/M6 hairpins is crucial for ouabain binding and that the observed high affinity for ouabain binding is due to the presence of two neutral residues in H^+,K^+-ATPase at the position of Arg113 and Asp124 of rat Na^+,K^+-ATPase.

Experimental Procedures

Expression Constructs. The rat gastric H^+,K^+-ATPase α- and β-subunits and the rat Na^+,K^+-ATPase α_1- and β_1-subunits were cloned into the pFastbacdual vector (Life Technologies, Grand Island, NY) as described previously (6). We used the Altered

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Abbreviations: Sf, *Spodoptera frugiperda*; M, transmembrane helix.

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Sites II *in vitro* mutagenesis system (Promega) to introduce silent mutations to generate new *Pvu*I, *Nhe*I, *Vsp*I, and *Sst*II sites in the cDNA of the H^+,K^+-ATPase and Na^+,K^+-ATPase α-subunits. Thereafter, the N terminus until *Pvu*I, the *Pvu*I-*Sst*II fragment, the *Pvu*I-*Nhe*I fragment, the *Vsp*I-*Sst*II fragment, and the combination of the last two fragments of H^+,K^+-ATPase were replaced by those of Na^+,K^+-ATPase, resulting in the chimeras HNN3, HN36, HN34, HN56, and HN34/56, respectively (Fig. 1). We introduced in the rat Na^+,K^+-ATPase and chimera HNN3 α-subunits the R113Q and D124N mutations. In the rat H^+,K^+-ATPase and chimera HN34/56 α-subunits, the opposite mutations (Q127R and N138D) were introduced. The sequence of all mutants was verified.

Production of Recombinant ATPases. Recombinant baculoviruses were produced and the membrane fraction containing the recombinant ATPases was obtained as described previously (6). As a mock, a baculovirus not expressing the α- and β-subunits of H^+,K^+-ATPase and Na^+,K^+-ATPase was prepared.

Protein Determination. The amount of protein was determined with the modified Lowry method described by Peterson (21) by using BSA as a standard.

ATPase Activity Assay. The ATPase activity was determined with a radiochemical method (6). For this purpose, *Spodoptera frugiperda* (Sf)9 membranes were added to 100 μl of medium, which contained 50 mM Tris-acetic acid (pH 6.0), 0.2 mM EDTA, 0.1 mM EGTA, 1 mM Tris-N_3, 1.2 mM $MgCl_2$, 3.0 mM KCl, and 10 μM [γ-^{32}P]ATP. Na^+,K^+-ATPase activity was measured in the presence of 50 mM Tris-acetic acid, pH 7.0/0.2 mM EDTA/0.1 mM EGTA/1 mM Tris-N_3/1.2 mM $MgCl_2$/10 mM KCl/100 mM NaCl/100 μM [γ-^{32}P]ATP. After incubation at 37°C, the reaction was stopped by the addition of 500 μl of 10% (wt/vol) charcoal in 6% (wt/vol) trichloroacetic acid, and after incubation at 0°C, the mixture was centrifuged for 30 s (10,000 $\times$ *g*). To 200 μl of the clear supernatant containing the liberated inorganic phosphate ($^{32}P_i$), 4 ml of OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added, and the mixture was analyzed by liquid scintillation analysis. Blanks were prepared by incubating in the absence of enzyme. The specific activity is presented as the difference between that of the expressed enzyme and the mock.

ATP Phosphorylation and Dephosphorylation Assay. ATP phosphorylation was determined as described before (6). Sf9 membranes were incubated at 21°C in 50 mM Tris-acetic acid, pH 6.0/0.2 mM EDTA/1.2 mM $MgCl_2$ with or without ouabain in a volume of 50 μl. After 30–60 min of preincubation, 10 μl of 0.6 μM [γ-^{32}P]ATP was added and incubated for 10 s at 21°C. For dephosphorylation studies, the reaction mixture was diluted 8.3 times with nonradioactive ATP (100 μM, to prevent rephosphorylation with radioactive ATP), with or without ouabain, and was incubated further for 3 s at 21°C. The reaction was stopped by adding 5% trichloroacetic acid in 0.1 M phosphoric acid, and the phosphorylated protein was collected by filtration over a 0.8-μm membrane filter (Schleicher & Schüll). After repeated washing, the filters were analyzed by liquid scintillation analysis. The specific phosphorylation is presented as the phosphorylation level obtained with the expressed enzyme minus that of the mock.

Ouabain Binding. Sf9 membranes ($\approx$100 μg of protein) were incubated at 21°C in 50 mM Tris-acetic acid, pH 7.0/5.0 mM $MgCl_2$/1.0 mM H_3PO_4 or 1.0 mM ATP (with or without 100 mM NaCl) and 10 nM [^{3}H]ouabain with and without 1 mM nonradioactive ouabain in a volume of 200 μl. After 45–60 min of incubation, the reaction mixture was incubated for 15 min at 0°C.

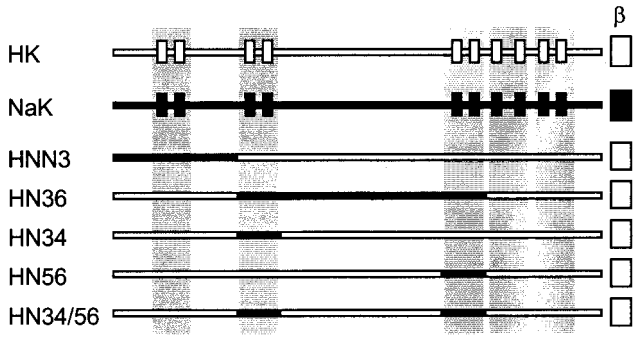

Fig. 1. Schematic representation of the produced chimeras and wild-type H^+,K^+-ATPase. The open bars represent H^+,K^+-ATPase sequences, and the solid bars represent Na^+,K^+-ATPase sequences. HK, H^+,K^+-ATPase; NaK, Na^+,K^+-ATPase; HNN3, H^+,K^+-ATPase with amino acids Met1-Ile293 replaced by those of Na^+,K^+-ATPase (Met1-Ile279); HN36, H^+,K^+-ATPase with amino acids Ile293-Arg846 replaced by those of Na^+,K^+-ATPase (Ile279-Arg832); HN34, H^+,K^+-ATPase with amino acids Ile293-Leu346 replaced by those of Na^+,K^+-ATPase (Ile279-Leu332); HN56, H^+,K^+-ATPase with amino acids Leu776-Arg846 replaced by those of Na^+,K^+-ATPase (Leu762-Arg832); HN34/56, H^+,K^+-ATPase with amino acids Ile293-Leu346 and Leu776-Arg846 replaced by those of Na^+,K^+-ATPase (Ile279-Leu332 and Leu762-Arg832).

The ouabain–protein complex was collected by filtration over a 0.8-μm membrane filter (Schleicher & Schüll). After repeated washing with ice-cold water, the filters were analyzed by liquid scintillation analysis. The specific ouabain binding is presented as the difference between [^{3}H]ouabain binding in the absence and presence of 1 mM nonradioactive ouabain.

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

Materials. The rat cDNA clones of the H^+,K^+-ATPase α- and β-subunits and the rat cDNA clone of the Na^+,K^+-ATPase α_1-subunit were provided by G. E. Shull and J. B. Lingrel, respectively. [^{3}H]Ouabain (45 Ci mmol^{-1}) and [γ-^{32}P]ATP (3,000 Ci mmol^{-1}) were purchased from Amersham Pharmacia.

Results

Five chimeric constructs were produced after the introduction of four unique restriction sites in both the cDNAs of the rat gastric H^+,K^+-ATPase α-subunit and the rat Na^+K^+-ATPase α_1-subunit. The N terminus until transmembrane segment 3, transmembrane segments 3–4, transmembrane segments 5–6, the combination of 3–4 and 5–6, as well as the complete region between and including transmembrane domains 3 and 6 of H^+,K^+-ATPase were exchanged for the similar parts of Na^+K^+-ATPase, generating the chimeric ATPases HNN3, HN34, HN56, HN34/56, and HN36, respectively (Fig. 1). These chimeric α-subunits and the wild-type H^+,K^+-ATPase α-subunit (HK) were introduced in the genome of a baculovirus together with the H^+,K^+-ATPase β-subunit. The recombinant baculoviruses were used to infect Sf9 insect cells, and the membrane fractions of these cells expressing the recombinant ATPase proteins were isolated. Western blot analysis revealed similar expression levels for the wild-type H^+,K^+-ATPase and the chimeras HNN3, HN36, HN34, and HN34/56. The expression level of chimera HN56, however, was slightly lower than that of the wild-type H^+,K^+-ATPase (data not shown).

To measure ATPase activity in the presence of 10 μM ATP, we used optimal reaction conditions for the ATP hydrolyzing chimeras HN34 and HN34/56 (pH 6.0 and 3 mM KCl). Under these conditions the wild-type H^+,K^+-ATPase activity was about

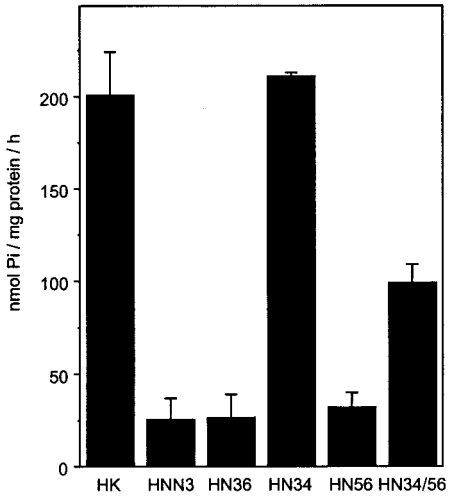

Fig. 2. ATPase activity of the chimeras and wild-type H^+,K^+-ATPase. The assay was performed at 37°C with ≈1 μg of protein in the presence of 0.2 mM EDTA/0.1 mM EGTA/1.2 mM $MgCl_2$/50 mM Tris-acetic acid, pH 6.0/3.0 mM KCl/10 μM ATP. The ATPase activity determined was corrected for that of the mock. Shown are mean values ± SE of two to four enzyme preparations.

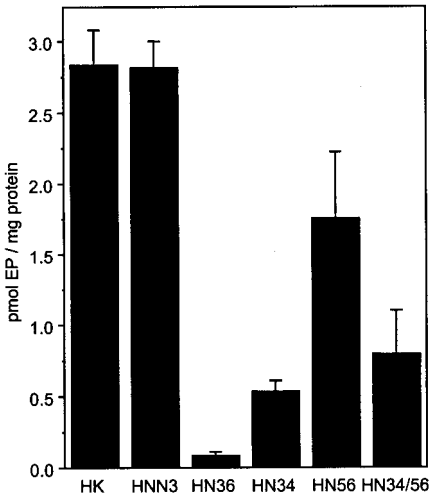

Fig. 4. Phosphorylation capacity of the chimeras and wild-type H^+,K^+-ATPase. Sf9 membranes (≈5 μg) were preincubated at 21°C with 0.2 mM EDTA/1.2 mM $MgCl_2$/50 mM Tris-acetic acid, pH 6.0. After phosphorylation at 21°C for 10 s with 0.1 μM [γ-^{32}P]ATP, the phosphorylation level (EP) was determined and corrected for that of the mock. Shown are mean values ± SE of two to four enzyme preparations.

60% of that measured previously (pH 7.0 and 1 mM KCl) (6). HNN3, HN36, and HN56 possessed an ATPase activity of less than 17% of that of H^+,K^+-ATPase (Fig. 2) that hardly could be stimulated by K^+ or by changing the pH or ATP concentration (data not shown). HN34 and HN34/56, however, possessed a K^+-stimulated ATPase activity that was 105% and 49% of that of the wild-type enzyme, respectively (Fig. 2). The addition of Na^+, in the presence of K^+ (with either 10 μM or 1 mM ATP), could not activate the ATPase activity of any of these chimeras (data not shown).

The effect of ouabain on the ATPase activity was studied to determine the binding site of this specific Na^+,K^+-ATPase inhibitor (Fig. 3*A*). Ouabain did not affect the ATPase activity of the wild-type H^+,K^+-ATPase and the chimeras HNN3, HN36, HN34, and HN56. Chimera HN34/56, however, was inhibited by ouabain with a very high sensitivity (IC_{50} = 2 μM). Because it

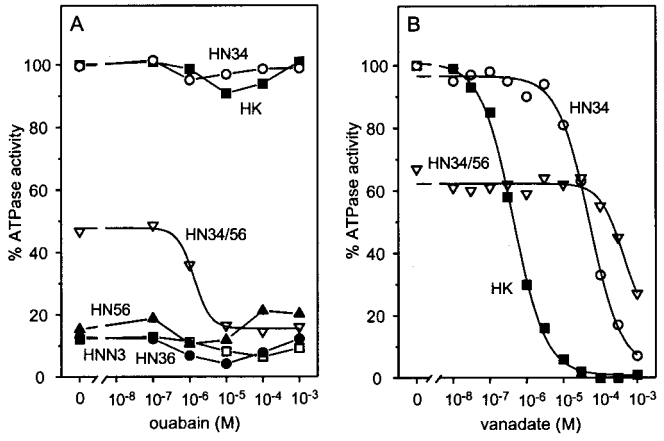

Fig. 3. Effects of ouabain and vanadate on the ATPase activity of the chimeras and wild-type H^+,K^+-ATPase. The assay was performed as in Fig. 2, using varying concentrations of ouabain and vanadate. The ATPase activity determined was corrected for that of the mock. The H^+,K^+-ATPase activity in the absence of inhibitors was set at 100%. ■, H^+,K^+-ATPase; □, HNN3; ●, HN36; ○, HN34; ▲, HN56; ▽, HN34/56. Shown is the average of two enzyme preparations.

is known that ouabain reacts with either the E_2 or the E_2-P form of Na^+,K^+-ATPase, it is important to know the conformational equilibria of the chimeras. Vanadate reacts with the E_2 conformation of the enzyme and forms a stable intermediate that inhibits enzyme activity. The longer the enzyme is in this form during the reaction cycle, the lower the vanadate concentration needed for 50% inhibition (22). Fig. 3*B* shows that the ouabain-sensitive mutant has a very low affinity for vanadate, indicating that the observed inhibition by ouabain is not due to an equilibrium shift toward E_2.

The formation of an acid-stable phosphorylated intermediate during the catalytic cycle is a characteristic property of P-type ATPases. To investigate this feature for the chimeric enzymes, we performed phosphorylation experiments at 21°C for 10 s. H^+,K^+-ATPase and chimera HNN3 were both phosphorylated to a level of 2.8 pmol EP mg^{-1} protein, whereas the phosphorylation level of chimera HN36 was not different from that of the mock (Fig. 4). The amounts of phosphoenzyme of HN34 and HN34/56 were only 0.5 and 0.8 pmol EP mg^{-1} protein, respectively. The amount of phosphorylated intermediate of chimera HN56 was 1.8 pmol EP mg^{-1} protein, which is 64% of that of the wild-type enzyme. Na^+ could not enhance the formation of a phosphorylated intermediate in any of the chimeras (data not shown).

Next, we preincubated the preparations with increasing concentrations of the specific Na^+,K^+-ATPase inhibitor ouabain (Fig. 5). The steady-state phosphorylation levels of the wild-type H^+,K^+-ATPase and the chimeras HNN3, HN36, HN34, and HN56 were not affected. The addition of ouabain to chimera HN34/56, however, increased the phosphorylation level.

To study the effect of ouabain on the phosphorylated intermediate of HN34/56 more closely, we measured the spontaneous dephosphorylation rates of this intermediate in the presence and absence of ouabain. Table 1 shows that the spontaneous dephosphorylation rate of the chimeras HN34 and HN34/56 is enhanced significantly as compared with that of the wild-type H^+,K^+-ATPase. In the presence of ouabain, the spontaneous dephosphorylation rate of HN34/56 decreased, indicating that the phosphorylated intermediate was stabilized by this inhibitor.

The ouabain affinity of chimera HN34/56 seems to be much higher than that reported for the rat Na^+,K^+-ATPase (12). The

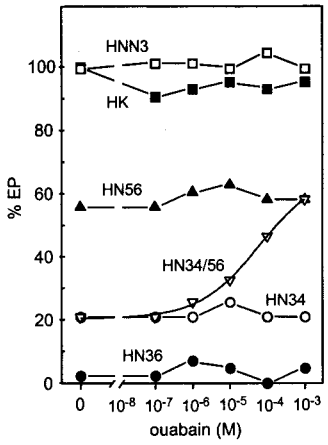

Fig. 5. Effect of ouabain on the phosphorylation capacity of the chimeras and wild-type H^+,K^+-ATPase. Sf9 membranes were preincubated as in Fig. 4, using varying concentrations of ouabain. After phosphorylation at 21°C for 10 s with 0.1 μM [γ-^{32}P]ATP, the phosphorylation level (EP) was determined and corrected for that of the mock. The phosphorylation level of H^+,K^+-ATPase in the absence of ouabain was set at 100%. ■, H^+,K^+-ATPase; □, HNN3; ●, HN36; ○, HN34; ▲, HN56; ▽, HN34/56. Shown is the average of two enzyme preparations.

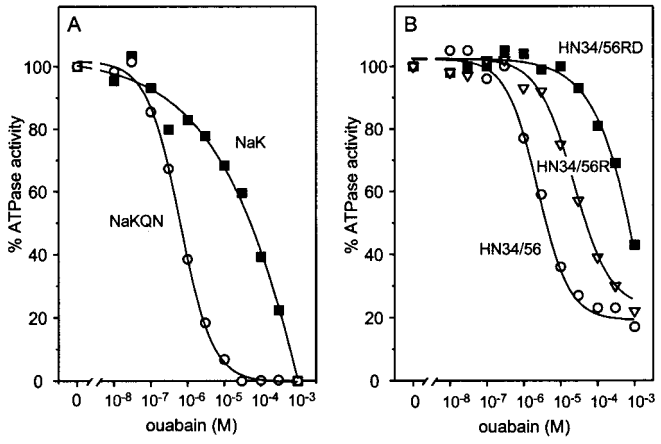

Fig. 6. Effect of ouabain on the ATPase activity of the rat Na^+,K^+-ATPase, mutant Na^+,K^+-ATPase R113Q/D124N, chimera HN34/56, mutant HN34/56 Q127R, and mutant HN34/56 Q127R/N138D. The assay shown in *A* was performed in the presence of 0.2 mM EDTA/0.1 mM EGTA/1.2 mM $MgCl_2$/50 mM Tris-acetic acid, pH 7.0/10 mM KCl/100 mM NaCl/100 μM ATP. The assay conditions used for the assay shown in *B* were similar to those described in Fig. 2. The ATPase activity determined was corrected for that of the mock. The ATPase activity in the absence of ouabain was set at 100%: Na^+,K^+-ATPase, 0.37 μmol Pi mg^{-1}·protein h^{-1} (■); NaKQN, 0.41 μmol Pi mg^{-1}·protein h^{-1} (○); HN34/56, 0.10 μmol Pi mg^{-1}·protein h^{-1} (○); HN34/56R, 0.08 μmol Pi mg^{-1}·protein h^{-1} (▽); HN34/56RD, 0.04 μmol Pi mg^{-1}·protein h^{-1} (■). Results are representative of two enzyme preparations.

low affinity of rat Na^+,K^+-ATPase has been attributed to the presence of two charged amino acids (Arg 113 and Asp 124) in the M1/M2 loop (12). In the chimera HN34/56, the M1/M2 loop originates from H^+,K^+-ATPase. Although the overall amino acid composition of this loop is rather different from that of Na^+,K^+-ATPase, the amino acids corresponding to Arg113 and Asp124 of the rat Na^+,K^+-ATPase are Gln and Asn, respectively, as is the case in Na^+,K^+-ATPase of species with a high affinity for ouabain (12). We therefore prepared the Na^+,K^+-ATPase R113Q/D124N mutant as well as the HN34/56-R127Q and the HN34/56-R127Q/D138N mutants and measured their sensitivities for ouabain. Fig. 6*A* demonstrates that the rat Na^+,K^+-ATPase (IC_{50} = ≈60 μM) has a 100 times lower ouabain affinity than the mutated Na^+,K^+-ATPase R113Q/D124N (IC_{50} = 0.6 μM). The ouabain affinity of chimera HN34/56-Q127R/N138D (IC_{50} = ≈400 μM) was about 200 times lower than that of HN34/56 (IC_{50} = 2 μM, Fig. 6*B*).

Table 1. Dephosphorylation of the phosphorylated enzymes

Enzyme	Dephosphorylation, %	Dephosphorylation, % + ouabain
HK	36 ± 2	34 ± 4
HN13	34 ± 1	29 ± 4
HN34	55 ± 4*	47 ± 3
HN56	31 ± 5	29 ± 2
HN34/56	102 ± 4*	28 ± 4†

Sf9 membranes (5 μg) of cells infected with viruses expressing H^+,K^+-ATPase (HK) or the chimeras were preincubated at 21°C with 0.2 mM EDTA/1.2 mM $MgCl_2$/50 mM Tris-acetic acid, pH 6.0, with or without 1 mM ouabain. After 10 s of phosphorylation at 21°C with 0.1 μM [γ-^{32}P]ATP, the enzymes were dephosphorylated in 50 mM Tris-acetic acid, pH 6.0/100 μM ATP, with or without 1 mM ouabain. The phosphorylation level was determined and corrected for that of the mock. Dephosphorylation is expressed as the percentage decrease of the phosphorylation level in 3 s. The data presented are the mean values ± SE of three samples.

*Comparison of the dephosphorylation levels of the chimeras and the wild-type enzyme without ouabain ($P < 0.05$).

†Comparison of the dephosphorylation levels with and without ouabain ($P < 0.05$).

Chimera HN34/56-Q127R (IC_{50} = 26 μM) had intermediate ouabain sensitivity.

Because the chimera HN34/56 probably has acquired the binding site for ouabain, we investigated the direct binding of [^{3}H]ouabain to the chimeras, H^+,K^+-ATPase and Na^+,K^+-ATPase. Ouabain hardly bound to rat Na^+,K^+-ATPase, H^+,K^+-ATPase, HNN3, HNN3–R113Q/D124N, HN36, HN34, HN56, HN34/56-Q127R, and HN34/56-Q127R/N138D (Fig. 7). In the presence of inorganic phosphate, ouabain binds to Na^+,K^+-ATPase R113Q/D124N, whereas in the presence of ATP it did bind to chimera HN34/56 (Fig. 7). The replacement of ATP by inorganic phosphate in the binding assay abolished the binding of [^{3}H]ouabain to chimera HN34/56, which is probably due to the preference of this chimera for the E_1 conformation (Fig. 3*B*). To compare the apparent affinity for ouabain of chimera HN34/56 and Na^+,K^+-ATPase, the concentration of unlabeled ouabain required to remove the bound [^{3}H]ouabain was determined. The ouabain-binding buffer contained 1 mM ATP and 100 mM NaCl, which permitted suboptimal ouabain binding to both chimera HN34/56 and Na^+,K^+-ATPase R113Q/D124N (Fig. 8). The apparent affinity for ouabain binding of HN34/56 was 0.4 μM, which is similar to that of Na^+,K^+-ATPase R113Q/D124N (0.3 μM).

Discussion

Na^+,K^+-ATPase is inhibited specifically by ouabain. The goal of this study was to perceive the region responsible for the specific interaction of this enzyme with this drug. Therefore, five chimeric ATPases were prepared in which small parts of the α-subunit of Na^+,K^+-ATPase were inserted into that of gastric H^+,K^+-ATPase. Next, we expressed them in Sf9 cells and analyzed their properties. Surprisingly, we revealed two distinct regions in Na^+,K^+-ATPase, which, only in concert, could donate ouabain sensitivity to recombinant H^+,K^+-ATPase. These two regions, M3-M4 and M5-M6, generally are not associated with ouabain binding but are thought to play a role in cation occlusion (1).

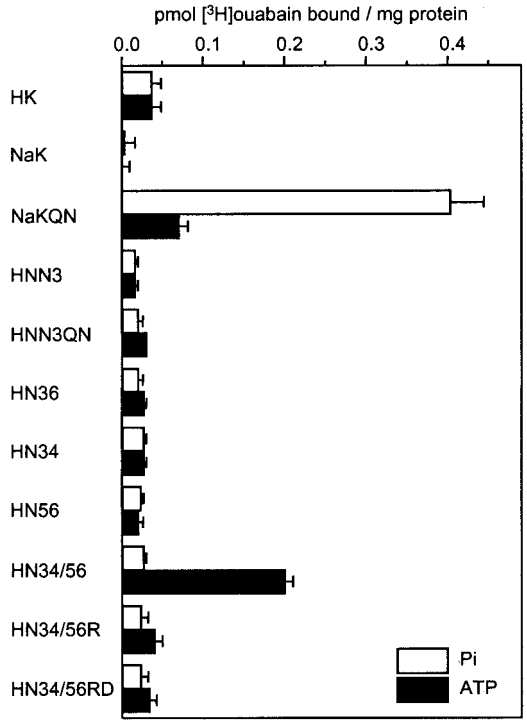

Fig. 7. [3H]Ouabain binding to the chimeras, mutants, and wild-type ATPases. Approximately 100 μg of protein was incubated at 21°C in the presence of 5.0 mM $MgCl_2$/50 mM Tris-acetic acid, pH 7.0/1.0 mM H_3PO_4 or 1.0 mM ATP and 10 nM [3H]ouabain. The determined level of bound ouabain was corrected for that of the mock. Shown are mean values ± SE of three enzyme preparations.

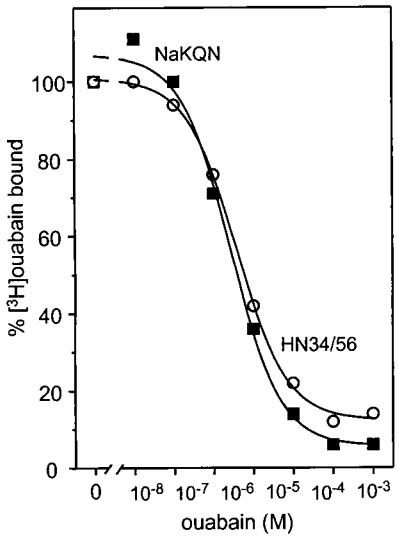

Fig. 8. [3H]Ouabain binding to chimera HN34/56 and rat Na^+,K^+-ATPase R113Q/D124N. The enzymes were incubated at 21°C in the presence of 5.0 mM $MgCl_2$/50 mM Tris-acetic acid, pH 7.0/1.0 mM ATP/100 mM NaCl/10 nM [3H]ouabain and varying concentrations of nonradioactive ouabain. The binding of [3H]ouabain in the absence of nonradioactive ouabain was set at 100%: NaKQN, 0.19 ± 0.03 pmol [3H]ouabain mg^{-1} protein (■); HN34/56, 0.14 ± 0.03 pmol [3H]ouabain mg^{-1} protein (○). Results are the average of three enzyme preparations.

The β-subunit of H^+,K^+-ATPase was used for all chimeras, because the extracellular domain between M7 and M8 originated in all chimeras from H^+,K^+-ATPase. There is strong evidence (23–25) that specific interaction between the α- and β-subunit occurs in this region of the α-subunit. Of the five chimeras prepared in this study, only two (HN34 and HN34/56) showed considerable K^+-activated ATPase activity. Two other chimeras (HNN3 and HN56) had a low K^+-insensitive ATPase activity but could be phosphorylated by ATP. The ATPase activity of all of the chimeras could not be stimulated by the addition of Na^+, indicating that (part of) the Na^+-binding site is absent in all chimeras.

The chimeric enzyme HN36, which has the same transmembrane domains as chimera HN34/56, showed almost no ATPase activity and phosphorylation capacity. This chimera has intracellular domains originating from two different ATPases. Toyoshima *et al.* (26) showed in SERCA1a Ca^{2+}-ATPase that the N-terminal region and the M2/M3 loop form the so-called A domain that has to interact with the N and P domains that originate from the M4/M5 loop. Goldshleger and Karlish (27) have shown that the intracellular domains of Na^+,K^+-ATPase have a close interaction. It might be that the interaction between these domains is not optimal when they originate from different ATPases. Chimera HNN3, however, also has intracellular domains from different ATPases and still could be phosphorylated.

Ouabain inhibited the ATPase activity and affected the phosphorylation level only when both M3-M4 and M5-M6 were replaced by the similar regions of the α_1-subunit of rat Na^+,K^+-ATPase. Moreover, this chimera was the only one that bound ouabain. Replacement of either the hairpin M3-M4 or M5-M6 alone or of the complete N-terminal part (Met1-Ile293), including hairpin M1-M2, did not result in any ouabain effect. These findings apparently contrast with those of Ishii *et al.* (16, 17). They found that the region between Ala70 and Asp200 of Na^+,K^+-ATPase was sufficient for donating ouabain-binding capacity to Ca^{2+}-ATPase, although the affinity was less than that of the wild-type Na^+,K^+-ATPase (16, 17). We did not observe any ouabain binding by chimera HNN3 and HNN3-R113Q/D124N that contained even a larger part of Na^+,K^+-ATPase. Regarding the large differences in amino acid composition, it is unlikely that the part of Ca^{2+}-ATPase still present in the chimera produced by Ishii *et al.* (16, 17) can take over the role of the M3-M4/M5-M6 hairpins of Na^+,K^+-ATPase, whereas the similar part of H^+,K^+-ATPase cannot.

Surprisingly, the phosphorylation level of chimera HN34/56 increased after the addition of ouabain to the phosphorylation mix. An increase in the steady-state phosphorylation level by an inhibitor had been observed earlier with the H^+,K^+-ATPase mutant E820Q (28). This was explained by the fact that the inhibitor SCH 28080 interacts only with the E_2 forms of the enzyme (29, 30). The E820Q mutant has a strong preference for the E_1 form and did not react with SCH 28080 under resting conditions. Upon phosphorylation, however, the E_2-P intermediate is formed and reacts with SCH 28080. Because the steady-state phosphorylation level of the E820Q mutant was submaximal and the stability of the complex between E_2-P and SCH 28080 was higher than that of E_2-P alone, the increase in the phosphorylation level by SCH 28080 could be explained. A similar explanation is probably true for the increase in the steady-state phosphorylation level of the chimera HN34/56 by ouabain. Indeed, the experiments with vanadate in the ATPase reaction and ATP and inorganic phosphate in the ouabain-binding assay show the preference of HN34/56 for the E_1 conformation. Furthermore, the dephosphorylation experiments demonstrate that the phosphorylated intermediate of the chimera HN34/56 is stabilized in the presence of ouabain.

The high-affinity binding of ouabain by chimera HN34/56 also is indicated by the low ouabain concentration required to replace 50% of bound [3H]ouabain, which is similar to that of purified rabbit kidney Na^+,K^+-ATPase (data not shown). It is also clear

from the IC_{50} value of 2 μM in the ATPase reaction which value is rather similar to that measured for HeLa cells (0.1–2 μM) (8, 10, 12). This high affinity is amazing because the two transmembrane hairpins of Na^+,K^+-ATPase originate from the rat α_1-isoform that is about 1,000 times less sensitive than that of rabbit or human (12). We can explain our finding only by assuming that the M1-M2 hairpin of H^+,K^+-ATPase can take over the role of this hairpin in Na^+,K^+-ATPase. The charged amino acids Arg113 and Asp124, present in rat Na^+,K^+-ATPase α_1, are responsible for the relative ouabain resistance character of this enzyme (12). The corresponding amino acids in H^+,K^+-ATPase are identical to those in Na^+,K^+-ATPase species that have a high affinity for ouabain (Gln and Asn, respectively). We mutated these amino acids in chimera HN34/56 to those present in the rat Na^+,K^+-ATPase enzyme. This resulted in a reduction in the ouabain affinity of the chimera HN34/56-Q127R/N138D that was similar to the reduction in affinity observed when these mutations were performed in Na^+,K^+-ATPase (12). The presence of these polar uncharged amino acids in the M1/M2 loop thus explains the high affinity for ouabain in chimera HN34/56. It has to be mentioned that although the first mutagenesis studies suggested a primary role of the M1-M2 hairpin in ouabain binding to Na^+,K^+-ATPase (7, 12, 13, 31), later studies indicated that other parts of the enzyme are also involved in the interaction with ouabain (9–11, 15, 18–20, 32, 33). It has been suggested that the overall conformation of the first extracellular loop may influence ouabain sensitivity indirectly by altering the stability or structure of the intermediate of the Na^+,K^+-ATPase catalytic cycle that is competent to bind ouabain.

Transmembrane segments M4, M5, and M6 are thought to establish the cation-binding pocket, in which movement of hairpin M5-M6 may play a role in active transport (34, 35). It has been proposed that binding of ouabain to this hairpin inhibits cation transport by immobilizing these transmembrane domains (20). Our results support this hypothesis and further demonstrate that when in H^+,K^+-ATPase M5-M6 is replaced by the similar region of Na^+,K^+-ATPase, the resulting chimera (HN56) hardly showed a K^+-stimulated dephosphorylation activity. When M3-M4 also was replaced (HN34/56), the dephosphorylation reaction became stimulated by K^+. These results indicate that M3-M4 and M5-M6 are in direct interaction with each other and that they fulfill an essential function in H^+,K^+-ATPase and Na^+,K^+-ATPase. This direct interaction is supported by the 2.6 Å three-dimensional structure of SERCA1a Ca^{2+}-ATPase (26).

In the last decade many amino acid substitutions in Na^+,K^+-ATPase have been shown to decrease the ouabain sensitivity (8). The interpretation of this loss of function is fraught with difficulties. The major reason for this problem is the question of whether the substitution is really directly affecting the ouabain binding. In this study we observed a gain of function that is likely to be due to a direct effect. We can conclude that introduction of transmembrane hairpins M3-M4 and M5-M6 of Na^+,K^+-ATPase into H^+,K^+-ATPase results in the formation of a high-affinity ouabain-binding site. Future studies in which amino acids in these regions will be substituted can more clearly localize the ouabain-binding site.

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1. Møller, J. V., Juul, B. & Le Maire, M. (1996) *Biochim. Biophys. Acta* **1286,** 1–51.
2. Hamlyn, J. M., Blaustein, M. P., Bova, S., Ducharme, D. W., Harris, D. W., Mandel, F., Mathews, W. R. & Ludens, J. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6259–6263.
3. Kölbel, F. & Schreiber, V. (1996) *Mol. Cell. Biochem.* **160/161,** 111–115.
4. Kawamura, A., Guo, J. S., Itagaki, Y., Bell, C., Wang, Y., Haupert, G. T., Magil, S., Gallagher, R. T., Berova, N. & Nakanishi, K. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6654–6659.
5. Ferrandi, M., Manunta, P., Rivera, R., Bianchi, G. & Ferrari, P. (1998) *Clin. Exp. Hypertens. [A]* **20,** 629–639.
6. Koenderink, J. B., Swarts, H. G. P., Hermsen, H. P. H. & De Pont, J. J. H. H. M. (1999) *J. Biol. Chem.* **274,** 11604–11610.
7. Emanuel, J. R., Graw, S., Housman, D. & Levenson, R. (1989) *Mol. Cell. Biol.* **9,** 3744–3749.
8. Croyle, M. L., Woo, A. L. & Lingrel, J. B. (1997) *Eur. J. Biochem.* **248,** 488–495.
9. Canfield, V. A. & Levenson, R. (1998) *Biochemistry* **37,** 7509–7516.
10. Coppi, M. V., Compton, L. A. & Guidotti, G. (1999) *Biochemistry* **38,** 2494–2505.
11. Asano, S., Matsuda, S., Hoshina, S., Sakamoto, S. & Takeguchi, N. (1999) *J. Biol. Chem.* **274,** 6848–6854.
12. Price, E. M. & Lingrel, J. B. (1988) *Biochemistry* **27,** 8400–8408.
13. Price, E. M., Rice, D. A. & Lingrel, J. B. (1990) *J. Biol. Chem.* **265,** 6638–6641.
14. Schultheis, P. J. & Lingrel, J. B. (1993) *Biochemistry* **32,** 544–550.
15. Schultheis, P. J., Wallick, E. T. & Lingrel, J. B. (1993) *J. Biol. Chem.* **268,** 22686–22694.
16. Ishii, T. & Takeyasu, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8881–8885.
17. Ishii, T., Lemas, M. V. & Takeyasu, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6103–6107.
18. Blostein, R., Zhang, R. P., Gottardi, C. J. & Caplan, M. J. (1993) *J. Biol. Chem.* **268,** 10654–10658.
19. Blostein, R., Dunbar, L., Mense, M., Scanzano, R., Wilczynska, A. & Caplan, M. J. (1999) *J. Biol. Chem.* **274,** 18374–18381.
20. Palasis, M., Kuntzweiler, T. A., Arguello, J. M. & Lingrel, J. B. (1996) *J. Biol. Chem.* **271,** 14176–14182.
21. Peterson, G. L. (1983) *Methods Enzymol.* **91,** 95–106.
22. Cantley, L. C., Cantley, L. G. & Josephson, L. (1978) *J. Biol. Chem.* **253,** 7361–7368.
23. Lemas, M. V., Hamrick, M., Takeyasu, K. & Fambrough, D. M. (1994) *J. Biol. Chem.* **269,** 8255–8259.
24. Lemas, M. V., Yu, H. Y., Takeyasu, K., Kone, B. & Fambrough, D. M. (1994) *J. Biol. Chem.* **269,** 18651–18655.
25. Melle-Milovanovic, D., Milovanovic, M., Nagpal, S., Sachs, G. & Shin, J. M. (1998) *J. Biol. Chem.* **273,** 11075–11081.
26. Toyoshima, C., Nakasako, M., Nomura, H. & Ogawa, H. (2000) *Nature (London)* **405,** 647–655.
27. Goldshleger, R. & Karlish, S. J. D. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9596–9601.
28. Swarts, H. G. P., Hermsen, H. P. H., Koenderink, J. B., Willems, P. H. G. M. & De Pont, J. J. H. H. M. (1999) *Mol. Pharmacol.* **55,** 541–547.
29. Keeling, D. J., Taylor, A. G. & Schudt, C. (1989) *J. Biol. Chem.* **264,** 5545–5551.
30. Van der Hijden, H. T. W. M., Koster, H. P. G., Swarts, H. G. P. & De Pont, J. J. H. H. M. (1991) *Biochim. Biophys. Acta* **1061,** 141–148.
31. Arystarkhova, E., Gasparian, M., Modyanov, N. N. & Sweadner, K. J. (1992) *J. Biol. Chem.* **267,** 13694–13701.
32. O'Brien, W. J., Wallick, E. T. & Lingrel, J. B. (1993) *J. Biol. Chem.* **268,** 7707–7712.
33. Feng, J. N. & Lingrel, J. B. (1994) *Biochemistry* **33,** 4218–4224.
34. Lutsenko, S., Anderko, R. & Kaplan, J. H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7936–7940.
35. Gatto, C., Lutsenko, S., Shin, J. M., Sachs, G. & Kaplan, J. H. (1999) *J. Biol. Chem.* **274,** 13737–13740.