Conversion of the Low Affinity Ouabain-binding Site of Non-gastric H,K-ATPase into a High Affinity Binding Site by Substitution of Only Five Amino Acids*

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P-type ATPases of the IIC subfamily exhibit large differences in sensitivity toward ouabain. This allows a strategy in which ouabain-insensitive members of this subfamily are used as template for mutational elucidation of the ouabain-binding site. With this strategy, we recently identified seven amino acids in Na,K-ATPase that conferred high affinity ouabain binding to gastric H,K-ATPase (Qiu, L. Y., Krieger, E., Schaftenaar, G., Swarts, H. G. P., Willems, P. H. G. M., De Pont, J. J. H. M., and Koenderink, J. B. (2005) J. Biol. Chem. 280, 32349–32355). Because important, but identical, amino acids were not recognized in that study, here we used the non-gastric H,K-ATPase, which is rather ouabain-insensitive, as template. The catalytic subunit of this enzyme, in which several amino acids were not recognized in that study, was expressed with the Na,K-ATPase β1 subunit in Xenopus laevis oocytes. A chimera containing 14 amino acids, located in M4, M5, and M6, which are unique to Na,K-ATPase, displayed high affinity ouabain binding. Four of these residues, all located in M5, appeared dispensable for high affinity binding. Individual mutation of the remaining 10 residues to their non-gastric H,K-ATPase counterparts yielded five amino acids (Glu312, Gly319, Pro778, Leu795, and Cys802) whose mutation resulted in a loss of ouabain binding. In a final gain-of-function experiment, we introduced these five amino acids in different combinations in non-gastric H,K-ATPase and demonstrated that all five were essential for high affinity ouabain binding. The non-gastric H,K-ATPase with these five mutations had a similar apparent affinity for ouabain as the wild type Na,K-ATPase and showed a 2000 times increased affinity for ouabain in the NH4Cl-stimulated ATPase activity in membranes of transfected S9 cells.

Non-gastric H,K-ATPase is a member of the IIC subfamily of P-type ATPases. This subfamily includes two other members: Na,K-ATPase and gastric H,K-ATPase, which both transport K+ in exchange for either Na+ (Na,K-ATPase) or H+ ions (gastric H,K-ATPase) (1). All three enzymes are heterodimers, composed of a catalytic α-subunit that crosses the membrane 10 times and a glycosylated β-subunit that spans the membrane once. The members of this subfamily (also called X,K-ATPases) exhibit a high level of sequence homology (65% amino acid identity) between their α-subunits.

To date, α-subunits of non-gastric H,K-ATPase have been described in five different species: rabbit (2), rat (3), guinea pig (4), rabbit (5), and Bufo (6). No specific β-subunit for the non-gastric H,K-ATPase has been found. Most studies support the idea that the β1-subunit of Na,K-ATPase fulfills this function (7–10).

Ouabain, a cardiac glycoside, specifically inhibits the Na,K-ATPase activity and binds to the α-subunit (11). Ouabain binds from the extracellular side, so that the extracellularly located parts of this subunit are putative candidates for ouabain binding. Two polar amino acids (Gln111 and Asn122) located at the extracellular border of the M1/M2 hairpin have been established to be important for high affinity binding to Na,K-ATPase, since mutation of these amino acids into charged residues, as present in rat and mouse, lowers the ouabain affinity considerably (12). These two polar amino acids are present in both gastric and non-gastric H,K-ATPase of the rat. We previously demonstrated that the introduction of the transmembrane hairpins M3-M4 and M5-M6 of Na,K-ATPase into a backbone of gastric H,K-ATPase results in the formation of a high affinity ouabain-binding site (13). In follow-up studies we showed that a gastric H,K-ATPase in which only seven amino acids were replaced by their counterparts from Na,K-ATPase bound ouabain with the same affinity as native Na,K-ATPase (14, 15). These residues (Glu312, Val314, Ile315, Gly319, Phe358, Thr379, and Asp805) are all present in the extracellularly located parts of M4, M5, and M6 of Na,K-ATPase.

The above-mentioned method cannot detect amino acid residues that are important for ouabain binding if these residues are similar in Na,K-ATPase and gastric H,K-ATPase. Whereas the gastric H,K-ATPase activity is not at all inhibited by ouabain, the non-gastric H,K-ATPase activity can be inhibited by relatively high ouabain concentrations (4, 10, 16). Apparently, the binding pocket for ouabain in non-gastric H,K-ATPase contains elements that are not suited for optimal ouabain binding. We therefore decided to use the non-gastric H,K-ATPase as a template to introduce dissimilar amino acids from Na,K-ATPase. This could also give an answer to the question why the affinity of the non-gastric H,K-ATPase for ouabain is so low. Because of the results obtained with the gastric H,K-ATPase (see above), we decided to restrict ourselves to the amino acids present in the extracellularly located parts of M4, M5, and M6.

In this study we found that a chimera with non-gastric H,K-ATPase as template and containing large parts of the M4, M5, and M6 membrane segments from Na,K-ATPase could bind ouabain with high affinity. In this chimera 14 amino acids were unique to Na,K-ATPase, but only 10 of these were shown to be needed for high affinity ouabain binding. By a systematic series of mutations we could reduce this number to five amino acid residues (Glu312, Gly319, Pro778, Leu795, and Cys802) without losing high affinity ouabain binding. This finding enlarges our knowledge on the ouabain-binding site in Na,K-ATPase.
Ouabain Binding to Non-gastric H,K-ATPase

and explains why the non-gastric H,K-ATPase has a low affinity for this drug.

EXPERIMENTAL PROCEDURES

Construction of Chimeras and Mutants—The chimeras and mutants were constructed from the rat Na,K-ATPase α₁-subunit containing the R111Q and D122N mutations (12, 13) and the rat non-gastric H,K-ATPase α₂-subunit (10). The rat Na,K-ATPase α₁- and β₁-subunits were cloned into the pTLN vector as described earlier (17). This vector is suitable for the Xenopus laevis oocyte expression system (18). The cDNA of the rat non-gastric H,K-ATPase α₂-subunit, a gift of Dr. H. Binder (19), was cloned with AvalI and EcoRV in the pTLN vector. 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.

The generation of the pFD vector containing the α₂-subunit of rat non-gastric H,K-ATPase and the β₁-subunit of rat Na,K-ATPase (pFD-HKα₂-NaKβ₁) that is suited for the baculovirus expression system has been reported before (10). The pFD vector containing the non-gastric H,K-ATPase α₂-subunit with the five mutations (D312E, S319G, A778P, I795L, F802C (numbering of pig Na,K-ATPase)); NaK, Na,K-ATPase; HK, H,K-ATPase) was cloned into the tPTN vector by cloning the AatII-EcoRI fragment in the pFD-HKα₂-NaKβ₁ construct.

Expression in X. laevis Oocytes—X. laevis were sacrificed with the modified Lowry method according to Peterson (24) using bovine serum albumin as a standard.

Western Blotting—The total membrane fraction of X. laevis oocytes was solubilized in sample buffer and separated on SDS-PAGE gels containing 10% acrylamide according to Laemmli (25). For immunoblotting, the separated proteins were transferred to Immobilon-P membranes (Millipore, Co., Bedford, MA). The α-subunits of non-gastric H,K-ATPase and the various chimeras were detected with the polyclonal antibody C384-M79 that recognizes the Glu128–Met156 region of the rat non-gastric H,K-ATPase α₂-subunit (10). The primary antibody was detected using an anti-rabbit secondary antibody, which was labeled with peroxidase (Dako Denmark A/S, Glostrup, Denmark).

Preparation of S9 Membranes—S9 cells were grown at 27 °C in 100-ml spinner flask cultures as described by Klaassen et al. (21). For production of H,K-ATPase, 1.5–10⁶ cells/ml⁻¹ were infected at a multiplicity of infection of 1–3 in the presence of 1% (v/v) ethanol (22) and 0.1% (w/v) pluronic F-68 (ICN, Aurora, OH) in Xpress medium (Bio-wittaker, Walkersville, MD) as described previously (23). After 3 days, S9 cells were harvested by centrifugation at 2000 × g for 5 min. The cells were washed once at 0 °C with 0.25 mM sucrose, 2 mM EDTA, and 25 mM Hepes/Tris (pH 7.0), resuspended in sucrose/EDTA/Tris buffer, and sonicated at 60 watts (Branson Power Co., Denbury, CT) for 30 s at 0 °C. After centrifugation for 30 min at 10,000 × g the supernatant was collected and recentrifuged for 60 min at 100,000 × g at 4 °C. The pelleted membranes were resuspended in the above-mentioned buffer and stored at −20 °C.

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Protein Determination—The protein concentrations were quantified with the modified Lowry method according to Peterson (24) using bovine serum albumin as a standard.

Materials—[^H]Ouabain (17 Ci mmol⁻¹) and [γ-³²P]ATP (3000 Ci mmol⁻¹) were purchased from Amersham Biosciences (Buckinghamshire, UK).

[^2]The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; S9 cells, Spodoptera frugiperda cells; Hkx₂-EGPLC, mutant of non-gastric HK-ATPase α₂ with the following mutations: D312E, S319G, A778P, I795L, F802C (numbering of pig Na,K-ATPase); NaK, Na,K-ATPase; Hkx₂, non-gastric H,K-ATPase.
RESULTS

Very recently we showed that substitution of seven unique amino acids present in extracellularly located parts of transmembrane segments M4, M5, and M6 of the \( \alpha_x \)-subunit of the rat gastric H,K-ATPase by the corresponding amino acids of the \( \alpha_x \)-subunit of rat Na,K-ATPase (Glu\( ^{312} \), Val\( ^{314} \), Ile\( ^{315} \), Gly\( ^{319} \), Phe\( ^{783} \), Thr\( ^{797} \), and Asp\( ^{804} \)) results in a high affinity ouabain-binding site in the gastric H,K-ATPase (15). In the present study we use the non-gastric H,K-ATPase that has a low affinity for ouabain to investigate the binding site in more detail. Since the seven amino acids mentioned above are all located in the M4, M5, and M6 regions that can be reached from the extracellular space, only amino acid residues located in this region were chosen for further investigation. The extracellularly located parts of M4, M5, and M6 contain 14 amino acids that are different between Na,K-ATPase and non-gastric H,K-ATPase (Fig. 1). To identify the amino acids in this region that are important for ouabain binding, the rat non-gastric H,K-ATPase residues were replaced by their rat Na,K-ATPase counterparts. Different combinations of these mutations were made (Table 1), and expressed together with the rat Na,K-ATPase \( \beta_x \)-subunit in X. laevis oocytes, whereupon ouabain binding was measured. The polyclonal antibody C384-M79 that recognizes the Glu\( ^{312} \)-Met\( ^{354} \) region of the rat non-gastric H,K-ATPase \( \alpha_x \)-subunit (10) was used to determine the expression levels in the total membrane proteins on a Western blot. Fig. 2A shows that the expression level of each of the mutants was rather similar to that of the recombinant non-gastric H,K-ATPase. [\(^3H\)]Ouabain binding levels were determined in the presence of either 1 mM ATP or 1 mM Pi to obtain the phosphorylated intermediate (15). In the present study, we use the non-gastric H,K-ATPase that has a low affinity for ouabain to investigate the binding site in more detail.

To assess which of these 10 amino acids are important for high affinity ouabain binding, we generated individual mutants of mutant 2, in which each of these ten amino acids was back mutated into the original non-gastric H,K-ATPase residue. Western blotting showed again similar expression levels of the new mutants compared with mutant 2 (Fig. 3A). All mutants could be phosphorylated by ATP (data not shown), which excludes the possibility that the loss of ouabain binding capacity of these mutants is due to the impossibility to form a phosphorylated intermediate. Fig. 3, B and C, show that the [\(^3H\)]ouabain binding levels of mutants Mut2-E312D, Mut2-G319S, Mut2-P778A, Mut2-L795I, and Mut2-W310V were not significantly different from those of mutant 2 (p > 0.05). The [\(^3H\)]ouabain binding level of mutant Mut2-W310V was even significantly higher than that of mutant 2 (p < 0.05). Also in this case the results in the presence of ATP (Fig. 3B) were rather similar to those in the presence of Pi (Fig. 3C). In the latter case significance was reached for all mutants except mut2-E312D, which is probably due to the relatively larger experimental variations. Taken together, these observations indicate that Glu\( ^{312} \), Gly\( ^{319} \), Pro\( ^{778} \), Leu\( ^{797} \), and Cys\( ^{802} \) are essential for high affinity ouabain binding.

However, these kinds of "loss-of-function" experiments could not exclude the possibility that the loss of ouabain binding capacity of the mutants is due to indirect effects on ouabain binding. Therefore we performed "gain-of-function" experiments by introducing different combinations of the above-mentioned five amino acids into non-gastric H,K-ATPase. Fig. 4A shows similar expression levels of all tested

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**TABLE 1**

*Description of the used mutants*

| Construct | Mutations |
|-----------|-----------|
| Mutation A | Y090T-V310W-D312E-I314V-S319G |
| Mutation B | A778P-C781T |
| Mutation C | V786F-G788I-G790N-L791I |
| Mutation D | F795L-J797V-F802C |

The mutants used in this study have the rat non-gastric H,K-ATPase \( \alpha_x \)-subunit as a backbone and contain the \( \beta_x \)-subunit of rat Na,K-ATPase.
mutants. None of the mutants showed higher $[^3H]$ouabain binding levels than those of non-gastric H,K-ATPase except the mutant with all five mutations, HK$_{H9251}$H$_{9251}$2-EGPLC (Fig. 4, B and C). Since the individual mutants all could be phosphorylated, it is unlikely that the lack of ouabain binding of most of these mutants is due to an inability to become phosphorylated. These findings confirm that the combination of Glu312, Gly319, Pro778, Leu795, and Cys802 is essential for high affinity ouabain binding.

To compare the apparent affinity for ouabain of chimera HK$_{H9251}$H$_{9251}$2-EGPLC and Na,K-ATPase, we performed an ouabain replacement assay (Fig. 5) in the presence of 1 mM Pi. The figure shows that the apparent ouabain binding affinity of chimera HK$_{H9251}$H$_{9251}$2-EGPLC (0.22 $\pm$ 0.03 $M$) is not significantly different from that of Na,K-ATPase (0.19 $\pm$ 0.01 $M$) ($p > 0.05$), indicating that chimera HK$_{H9251}$H$_{9251}$2-EGPLC has gained high affinity ouabain binding.

Finally, we expressed chimera HK$_{H9251}$H$_{9251}$2-EGPLC and the rat Na,K-ATPase $\beta_2$-subunit in Sf9 cells using the baculovirus expression system. The expressed non-gastric H,K-ATPase EGPLC had an NH$_4^+$-stimulated ATPase activity of 9.8 $\pm$ 0.9 pmol/mg of protein/h that was about 40% of that of the wild type enzyme (26.8 $\pm$ 2.8 pmol/mg of protein/h). The phosphorylation level of the chimera was 9.0 $\pm$ 0.8 pmol/mg of protein that is also about 40% of that of the wild type enzyme (22.5 $\pm$ 2.3 pmol/mg of protein). This means that the mutations had no effect on the turnover number of this enzyme.

Next, we determined the effect of ouabain on the NH$_4^+$-stimulated ATPase activity in membranes isolated from these cells. Fig. 6 shows that the IC$_{50}$ for ouabain under these conditions is a factor 2000 lower than that of the same preparation without these mutations. This strongly supports the conclusion of this paper that the presence of these five amino acid residues in non-gastric H,K-ATPase is sufficient for yielding a high affinity ouabain-binding site.

DISCUSSION

This paper shows that the low affinity binding site for ouabain in the rat non-gastric H,K-ATPase can be converted into a high affinity binding site by replacement of only five amino acids by their counterparts present in the catalytic subunit of rat Na,K-ATPase. This result was
obtained by a systematic mutational approach. Advantage was taken from our previous study (15) in which we were able to transfer the high affinity binding site for ouabain to the ouabain-insensitive gastric H,K-ATPase by mutation of only seven amino acids, originating from Na,K-ATPase. All these seven amino acids are present in extracellularly located parts of M4, M5, and M6 of Na,K-ATPase.

It is known that ouabain binds to the Na,K-ATPase from the extracellular side and that the highest affinity for ouabain is obtained when the enzyme is in the phosphorylated E2-P form (27). In the extracellular half of M4, M5, and M6 there are 14 amino acids that are different between non-gastric H,K-ATPase and Na,K-ATPase. Introduction of these 14 amino acid residues from Na,K-ATPase into non-gastric H,K-ATPase resulted in an enzyme with a high affinity for ouabain. These 14 amino acid residues were divided into four groups, A, B, C, and D, containing five, two, four, and three mutated amino acid residues, respectively. We next showed that mutant C, which represents a region with a relatively large species difference, did not alter ouabain binding and that only the 10 amino acid residues originating from the groups A, B, and D might be important for the high affinity ouabain binding. Next, individual mutational analysis revealed that only mutants mut2-E312D, mut2-G319S, mut2-P778A, mut2-L795I, and mut2-C802F partly or completely lost the ability to bind [3H]ouabain. Finally, we introduced Glu312, Gly319, Pro778, Leu795, and Cys802 into the ouabain-insensitive non-gastric H,K-ATPase, resulting in chimera HKα2-EGPLC that dem-
Ouabain Binding to Non-gastric H,K-ATPase

FIGURE 7. Model for the ouabain-binding pocket in Na,K-ATPase. The model is an extension of the model presented by Qiu et al. (15). The five amino acid residues that were detected in the present study are indicated by arrows. The backbone of the seven amino acids already found in the model of Qiu et al. (15) are yellow. Nitrogen is blue, oxygen red, and carbon cyan or green (in ouabain). Hydrogen bonds are shown as yellow disks. The surrounding parts of the structure have been hidden for clarity. Images were created with Yasara.

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role in ouabain binding (14). Taken together, these findings show that the loop region (PLP1795–GTV) is clearly important for the interaction between ouabain and Na,K-ATPase. Therefore, replacement of Leu795 with Ile might modify the loop structure that harbors Leu793 and Thr797 in such a way that ouabain cannot bind very well.

We also found that replacement of Cys802 by a Phe resulted in a loss of ouabain binding. Cys802 is located in the neighborhood of the cation-binding site and cannot be part of the ouabain-binding site. This mutation gives a large structural change from a hydrophilic residue with side chains containing sulfur atoms to hydrophobic amino acid with an aromatic ring. It is possible that this large structural change affects the enzyme conformation and so changes the ouabain affinity. Surprisingly, a similar change in our previous study with gastric H,K-ATPase had much less effect (14). The direct environment of Cys802 in gastric H,K-ATPase is different from that of non-gastric H,K-ATPase, which might explain the different result. For instance, in gastric H,K-ATPase a Glu is present on position 804, whereas in non-gastric H,K-ATPase, like in Na,K-ATPase, an Asp is present on this position. We showed before that the presence of Asp804, which is part of the cation-binding pocket, is necessary for high affinity ouabain binding (13, 14). Therefore, it is likely that Cys802 may alter the ouabain binding affinity indirectly by changing enzyme-cation interactions.

In summary, we presented evidence that the presence of Glu312, Gly319, Pro278, Leu795 and Cys802 in Na,K-ATPase is necessary for obtaining high affinity ouabain binding. Moreover, we demonstrated that only these five amino acids present in the extracellular half of M4, M5, and M6 of Na,K-ATPase are sufficient to confer high affinity ouabain binding to non-gastric H,K-ATPase.

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Ouabain Binding to Non-gastric H,K-ATPase