Valine 904, Tyrosine 898, and Cysteine 908 in Na,K-ATPase α Subunits Are Important for Assembly with β Subunits

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A 26-amino acid sequence in an extracellular loop of the Na,K-ATPase α subunit between membrane-spanning segments 7 and 8 has been shown to bind to the β subunit of Na,K-ATPase and to promote αβ assembly (Lemas, M. V., Hamrick, M., Takeyasu, K., and Fambrough, D. M. (1994) J. Biol. Chem. 269, 8255–8259) When this 26-amino acid sequence of the rat Na,K-ATPase α3 subunit was replaced by the corresponding sequence of the rat gastric H,K-ATPase α subunit, the chimeric α subunit assembled preferentially with the rat gastric H,K-ATPase β subunit (Wang, S.-G., Eakle, K. A., Levenson, R., and Farley, R. A. (1997) Am. J. Physiol. 272, C923–C930). In the present study, these 26 amino acids (Asn886–Ala911) of rat Na,K-ATPase α3 were replaced by the corresponding amino acids Asn908–Ala933 of rat distal colon H,K-ATPase. Site-directed mutagenesis of the chimeric α subunits and Na,K-ATPase α3 showed that Val904, Tyr908, and Cys908 in the Na,K-ATPase α3 subunit are key residues in αβ subunit interactions. The V904Q mutation in Na,K-ATPase α3 reduced the Bmax for ouabain binding and the ATPase activity of αβ1 complexes by ~95%, and Y988R reduced the Bmax and ATPase activity by ~60%. The complementary mutations Q904V and R988Y increased the amount of ouabain bound by yeast membranes expressing the chimera with the colon H,K-ATPase sequence. The amount of ouabain bound by complexes assembled between Na,K-ATPase α3 containing the Y988R,C908G mutations and gastric H,K-ATPase β was less than 10% of wild type Na,K-ATPase α3 expressed with the same β subunit. The R898Y,G908C mutations in the chimeric α subunits also increased ouabain binding.

P-type ATPases are those ion-transporting ATPases that are phosphorylated transiently by ATP as part of the catalytic mechanism. This class of ion-motive ATPase, including Na,K-ATPase (NK), 1 H,K-ATPase (HK), and Ca-ATPase, is distributed in many tissues throughout the body. The rat Na,K-ATPase α subunit contains a large catalytic subunit with a molecular mass between 70 and 200 kDa, and the potassium-transporting ATPases such as HK and NK also require a second, smaller glycosylated β subunit (30–55 kDa) for their enzymatic functions. The α subunit has multiple transmembrane segments and contains all of the amino acids thus far identified with the enzymatic functions of ATP hydrolysis and cation transport. The β subunit is a glycoprotein with one transmembrane segment and most of its mass located on the noncytoplasmic side of the membrane. The role of the β subunit in active ion transport is not fully understood.

Lemas et al. (1) have identified 26 amino acids in NKα, predicted to be located in an extracellular loop between transmembrane segments 7 and 8, which mediate interactions between α and β subunits. These 26 amino acids correspond to Asn886–Ala911 of rat Na,K-ATPase α3 subunit. Wang et al. (2) examined αβ assembly using a chimeric α subunit (NGH26) formed by replacement of Asn886–Ala911 of rat Na,K-ATPase α3 with the corresponding amino acids Gln905–Val910 of rat gastric HKα. When NGH26 was expressed in yeast cells with HKβ, the number of ouabain binding sites was the same as for NKα expressed with either NKβ1 or HKβ. In contrast, only about 10% as many complexes were formed between NGH26 and NKβ1. Wang and colleagues concluded that some amino acids within the sequence Gln905–Val910 of rat gastric HKα probably destabilize αβ complexes formed with NKβ, leading to a reduction in the number of steady-state pumps. This conclusion is consistent with the observation that a chimeric α subunit with amino acids 1–519 from NKα and amino acids 519–1033 from gastric HKα did not form stable complexes with NKβ1.

Unlike gastric HKα, other HKα subunits do not appear to discriminate between different β subunits. For example, functional pumps are assembled between a distal colon HKα subunit and either NKβ1 or HKβ subunits. Codina et al. (4) showed that 86Rb uptake into Xenopus oocytes increased when colon HKα was expressed with either NKβ1 or gastric HKβ, and Cougnon et al. (5) observed an increase in the 86Rb uptake rate of oocytes injected with cRNA for colon HKα and an amphibian HKβ. A human HKα subunit (ATP1AL1) with 86% amino acid sequence identity to rat colon HKα was cloned by Modyanov et al. (6), who observed that any of several different β subunits could be coimmunoprecipitated with ATP1AL1 when expressed in Xenopus oocytes. Expression of rabbit gastric HKβ with ATP1AL1 in Xenopus oocytes resulted in a 3-fold increase in 86Rb uptake compared with uninjected cells (7).

To identify amino acids that are involved in interactions between the α and β subunits, a new chimeric α subunit (NCH26) was made by replacing the 26-amino acid sequence Asn886–Ala911 of the rat Na,K-ATPase α3 subunit with the corresponding sequence Asn908–Ala933 of rat distal colon HK α subunit. A series of mutations was introduced into NCH26 and the chimeric NGH26 and NCH26 subunits, and the presence of functional αβ complexes was measured by ouabain binding or ATPase activity.
activity after expression of the α polypeptides in yeast with either NKβ1 or gastric HKβ. Stability of the complexes was estimated from the ability of each αβ complex to bind ouabain at elevated temperatures. As a result of these measurements, Val\textsuperscript{904}, Tyr\textsuperscript{908} and Cys\textsuperscript{908} in NKO\textalpha have been identified as important amino acids for assembly of α subunits and β subunits.

MATERIALS AND METHODS

Construction of the Chimeric α Subunit NCH26—The plasmid pRD-CHK containing the cDNA of the rat colon HK α subunit was a gift of Dr. Gary Shull (University of Cincinnati). A 102-base pair fragment of pRD-CHK, encoding positions 2711–2812, was amplified by polymerase chain reaction, and ClaI and HpaI restriction sites were introduced at the same time. For construction of plasmid pNCH26m, the polymerase chain reaction fragment was digested with ClaI and HpaI and was ligated into the corresponding region of clone pNGH26m (2) whose ClaI-HpaI fragment (78 base pairs) had been removed. Three mutations (N886\textsuperscript{2}, A911V, and F912N), resulting from the introduction of ClaI and HpaI sites, were corrected by polymerase chain reaction. The resultant plasmid pNCH26 encodes the rat NKα3 subunit with the region (Asn\textsuperscript{886}–Ala\textsuperscript{911}) replaced by the 26 amino acids (Asn\textsuperscript{886}–Ala\textsuperscript{911}) of the rat colon HKα subunit. The A\textalphaIII-BgII fragment (1,486 base pairs) of the yeast expression plasmid YEPGNGH26 (2) was replaced by the corresponding A\textalphaIII-BgII fragment of pNCH26. The final plasmid (yEPCH2612) was analyzed by restriction digestion and by DNA sequencing.

Site-directed Mutagenesis—Mutations in the cDNA were made using the polymerase chain reaction, as described previously (2). Pfu DNA polymerase (Stratagene) was used to perform the site-directed mutagenesis, and the resultant mutants were screened by restriction enzyme version 2.0 (U. S. Biochemical Corp.).

Expression of NCH26 or α Mutants with β Subunits in Yeast Cells—The yeast strain 30-4 (MATα, trp1, ura3, Lin2, GAl+) obtained from R. Hitzeman (Genentech, South San Francisco) was transformed with different combinations of the chimeric α subunit or a mutant expression plasmid and one of the β subunit expression plasmids pG1T-Rβ1 and pG1T-HKβ1 (9) by the method of Elble (8). After identification of transformants containing α and β subunits on selective medium, frozen glycerol stocks from four different clones were made and were stored at –80 °C. Cultures for experiments described in this report were started from these glycerol stocks. A membrane fraction of transformed yeast cells was prepared as described previously (9). Membranes were extracted with 0.1% (w/v) SDS as described previously (10).

[\textsuperscript{1}H]Ouabain Binding—Ouabain binding to yeast membranes was done as described previously (2). Experiments shown in Figs. 5 and 7 were done using approximately 20 nm [\textsuperscript{1}H]ouabain. To determine the number of steady-state pump complexes (B\textsubscript{max}) and the ouabain dissociation constant (K\textsubscript{d}), binding data were fit by a self-competition model (11) within the ouabain concentration range 0–1,000 nm. For determination of the effects of heat on αβ stability, 3 mg of yeast microsomal membrane protein was dissolved in 400 μl of 25 mM imidazole-HCl, 1 mM EDTA (sodium-free), pH 7.4, and was heated at different temperatures (40–50 °C) for 90 s. Membranes were placed in ice for 15–30 min, and the amount of ouabain bound at 37 °C was measured. The amount of ouabain bound by membranes without heating was used as 100%. SDS-Polyacrylamide Gel Electrophoresis and Immunoblots—100 μg of yeast microsomal membrane protein was separated on 10% SDS-polyacrylamide gels and then was transferred to Immobilon-P membranes (Millipore). The blots were first incubated with monoclonal antibody α5 (D. Fambrough, Johns Hopkins University), then incubated with the alkaline phosphatase-conjugated goat anti-mouse IgG (Calbiochem). The α subunits were visualized with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitro blue tetrazolium (Sigma). The density of each α band was determined by scanning with a Bio-Rad scanner (Imaging Densitometer, model GS-670). The average of the expression levels of NCH26 + NKβ1 and NCH26 + HKβ3 with three different clones was determined and compared with the NKO\textalpha + NKβ1 and NKO\textalpha + HKβ3 controls.

ATPase Activity—NK activity was determined in triplicate by measuring ouabain-inhibitable phosphate release, as described previously (12).

RESULTS

Expression Levels of NCH26 with NKβ1 or HKβ—The sequence Asn\textsuperscript{886}–Ala\textsuperscript{911} of rat NKα3 was replaced by the corresponding sequence Asn\textsuperscript{904}–Ala\textsuperscript{911} of rat colon HKα, and this chimeric α subunit NCH26 was expressed in yeast cells with either NKβ1 and gastric HKβ. Fig. 1A shows an immunoblot of membranes prepared from three different clones expressing either NKO\textalpha3 or NCH26 with either NKβ1 or HKβ. Three clones expressing only NKO\textalpha3 are also shown. Because the antibody α5 recognizes the same epitope on both NKO3 and NCH26, the relative abundance of each α subunit can be compared directly. The abundance of NCH26 expressed with NKβ1 is 82 ± 19% of NKO\textalpha3 expressed with NKβ1, and the amount of NCH26 expressed with HKβ3 is 87 ± 11% of NKO\textalpha3 expressed with HKβ3 (Fig. 1B). When expressed in the absence of a β subunit, NKO\textalpha3 is present at only 10 ± 10% of NKO\textalpha3 levels found with NKβ1. In the absence of the β subunit, the α subunit is degraded rapidly, and the higher steady-state abundance of α subunits expressed with either NKβ1 or HKβ reflects stabilization of the α subunit by association with a β subunit (13). These results show that the steady-state levels found with NKβ1 or HKβ are not significantly different from NKO\textalpha3 (p > 0.05).

Ouabain Binding by NCH26—Functional NCH26 + β complexes were quantitated by ouabain binding, and the B\textsubscript{max} and K\textsubscript{d} values for NKO\textalpha3 or NCH26 expressed with different β subunits were determined for three different clones each. The results presented in Fig. 2 demonstrate that functional complexes are formed equally well in yeast between NKO\textalpha3 and either NKβ1 or HKβ3. Although the NCH26 polypeptide is present at the same level as NKO\textalpha3, fewer functional complexes are formed between NCH26 and either NKβ1 or HKβ3 than with NKO\textalpha3. Yeast membranes containing NCH26 + HKβ3 form about 40% of the number of functional pumps as NKO\textalpha3 + NKβ1, and the number of functional NCH26 + HKβ3 complexes is about 20% of NKO\textalpha3 + HKβ3. The K\textsubscript{d} for ouabain binding by NCH26 + NKβ1 is 50 ± 13 nm, and for NCH26 + HKβ3 it is 206 ± 95 nm. These values are 8- and 27-fold higher than the K\textsubscript{d} of

\begin{figure}[h]
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\includegraphics{fig1.png}
\caption{Expression levels of NKO\textalpha3 and NCH26 expressed in yeast with NKβ1 or HKβ. Panel A, yeast membranes containing 100 μg of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blot was probed sequentially with monoclonal antibody α5 and alkaline phosphatase-conjugated goat anti-mouse IgG. Three different clones were used to determine the expression level of α subunit of each αβ complex. Panel B, relative expression levels were measured by densitometry. The averages of three clones of NKO\textalpha3 expressed with NKβ1 or HKβ are the 100% controls, and the expression levels of NCH26 expressed with NKO\textalpha3 or HKβ were normalized to the controls. Bars show means ± S.D.}
\end{figure}
values of NKα3 + NKβ1 (6 ± 2 nM) and NKα3 + gHKβ (7 ± 3 nM), respectively.

The number of ouabain-binding complexes formed by NCH26 and HKβ is only 20–40% of the number formed by either NKα3 + HKβ or NGH26 + HKβ (2). This result might be explained if residues within the sequence Asn898–Ala903 of rat colon HKα are less suitable for assembly with gastric HKβ than corresponding residues of rat NKα3 or rat gastric HKα. A comparison of the amino acid sequences Asn898–Ala911 of rat NKα3, Glu905–Val930 of rat gastric HKα, and Asn898–Ala933 of rat colon HKα shows that the three sequences have identical residues or conservative substitutions in all positions except at amino acids 898, 908, and 909. In both NKα3 and rat gastric HKα, a tyrosine or a phenylalanine is located at position 898, and a cysteine is located at position 908. In rat colon HKα these residues are arginine and glycine, respectively. In position 909, the amino acids are different for all three sequences. To see whether the amino acids in positions 898 and 908 are important for the assembly with HKβ, mutations R898Y and/or G908C were introduced into NCH26, and the amount of ouabain bound by the mutants expressed in yeast with different β subunits was measured.

Ouabain Binding by NCH26 Mutants—The maximum amount of ouabain bound (Bmax) by the R898Y, G908C, and R898Y,G908C mutants of NCH26 expressed in yeast cells with either NKβ1 or gastric HKβ was used to indicate the number of functional αβ complexes (Fig. 3). The mutation R898Y increases the Bmax of NCH26 + HKβ complexes to the same value as NKα3 + NKβ1. When expressed with HKβ, the Bmax of the R898Y mutant increases 2.7 times higher than that of NCH26 + HKβ but is still less than that of NKα3 + HKβ. The mutation G908C does not affect the number of NCH26 + HKβ complexes. When expressed with HKβ, however, the mutation G908C reduces the Bmax of NCH26 from 22% to 3% of NKα3 + HKβ. When the two mutations R898Y and G908C are made in NCH26, the Bmax for ouabain binding is the same as NKα3 + HKβ (p > 0.05), and is 1.8 times higher than that of NCH26/ R898Y + HKβ.

Ouabain Binding by NGH26 Mutants—Wang et al. studied the chimeric NGH26 α subunit and concluded that amino acids of gastric HKα between Glu905 and Val930 interact more stably with the extracellular domain of HKβ than NKβ (2). Charged amino acids have been implicated in the assembly of some membrane proteins (14, 15), and within the 26 residues that were exchanged during chimera formation, the charged amino acids Lys902 and Glu905 are conserved among all of the NKα subunits and also in the colon HKα subunits. In the gastric HKα subunits, these amino acids are leucine and glutamine, respectively. Because NGH26 forms fewer functional pumps with NKβ1 than with HKβ, charged residues in positions 902 and 905 may be important for interactions between α subunits and NKβ1. To test this possibility, the mutations L902K and Q905E were introduced separately or together into NGH26, and the α subunits were expressed in yeast with either NKβ1 or HKβ. Only the Q905E mutation reduced the ouabain binding capacity of yeast membranes containing the mutant α subunits assembled with HKβ (p < 0.05). Introduction of the double mutation (NGH26-KE), however, restored the binding capacity and reduced the Kd for ouabain binding by NGH26 from 72.7 to 23 nM (Table I).

Site-directed Mutagenesis of NGH26-KE—Although the mutations L902K and Q905E make the amino acid sequence of NGH26 more nearly like that of NKα3, the number of pumps is not increased above the NGH26 + NKβ1 level. This may be a result of the presence in NGH26 and NGH26-KE of amino acids whose side chains are sterically or electrostatically incompatible with assembly with NKβ1 or because of the absence in these α subunits of amino acids whose side chains are important for specific interactions with NKβ1. There are 10 amino acid differences between the NKα3 sequence and NGH26-KE (Fig. 4). Each of these amino acids was changed in NGH26-KE, either individually or in pairs, to those amino acids in NKα3, and ouabain binding was used to identify amino acids that are important for assembly of NKα3 with NKβ1.

Ouabain Binding by NGH26-KE and Related Mutants—Yeast membranes containing the α subunit mutants derived
amino acid substitutions in position 903 was not tested. The left panel of Fig. 6 shows the $B_{\text{max}}$ values of the NK3 mutants expressed with NKβ1, and the right panel shows the $B_{\text{max}}$ values for NK3 mutants expressed with HKβ. The $B_{\text{max}}$ of NK3/V904Q + NKβ1 is only 5% of the $B_{\text{max}}$ for NK3 + NKβ1, and the $B_{\text{max}}$ of NK3/V904Q + HKβ is 18% of NK3 + HKβ, confirming that Val904 is important for the functional assembly of NK3 with both β subunits. The $B_{\text{max}}$ of NK3/V904R + NKβ1 is 42% of NK3 + NKβ1, and the $B_{\text{max}}$ of NK3/V904R + HKβ is not significantly different from that of NK3 + HKβ. For the mutation C908G in NK3, no significant increase or decrease in the number of β subcomplexes was seen when assembled with either subunit. Even though the individual mutation Y989R or C908G has no significant effect on the number of NK3 + β complexes, the double mutation Y989R/C908G was associated with a reduction in the $B_{\text{max}}$ for ouabain binding to about 10% of NK3 + HKβ levels. Table II shows that for the V904Q mutation, no change in ouabain affinity was observed when the mutant NK3 subunit was expressed with either NKβ1 or HKβ.

**ATPase Activity of NK3 Mutants Expressed with NKβ1—** Yeast membranes containing the different α + NKβ1 complexes were extracted with SDS (16) to see whether the ATPase activity of these complexes could not be determined. The stability of the NK3 mutants expressed in yeast with NKβ1 or HKβ was investigated by heating the membranes at different temperatures (40–50°C) for 60 s and then measuring the amount of ouabain bound at 37°C. Fig. 7 (upper panel) shows that all of the mutants are as stable as NK3 + NKβ1 when expressed with NKβ1. In contrast, when expressed with HKβ, both NK3 and the mutants denature at much lower temperatures (lower panel). Compared with NK3 + HKβ, the α + HKβ complexes containing the mutations Y989R and C908G are significantly less stable. The mutant containing the double mutation Y989R/C908G is extremely unstable when expressed with HKβ. Ouabain binding by complexes of this mutant expressed with HKβ was not

from NGH26-KE together with either NKβ1 or gastric HKβ were equilibrated with 20 nM [3H]ouabain, and specific binding was measured as before. As shown in Fig. 5, when the double mutation Y903V,Q904V is made in NGH26-KE and this mutant α subunit is expressed with NKβ1, yeast membranes bind 10 times more ouabain than when NGH26-KE is expressed with NKβ1. In addition, the double mutation Y903V,Q904V in NGH26-KE increases the amount of ouabain bound by when expressed in yeast with HKβ. The amount of ouabain bound by the rest of the mutants when expressed with either NKβ1 or HKβ is not significantly different from that of NGH26-KE + NK3 or NGH26-KE + HKβ (p > 0.05), respectively.

**Ouabain Binding by NK3 Mutants—** The mutations Y903V,Q904V in the chimera NGH26-KE and R898Y,G908C in the chimera NCH26 lead to large increases in the amount of ouabain bound by yeast membranes containing these chimeric α subunits and either NKβ1 or HKβ. These increases could be due to changes in the affinity of the mutants for ouabain and/or to increased stability of the αβ complexes. If amino acids in positions 889, 903, 904, and/or 908 participate in interactions between α and β subunits, then the reverse mutations in NK3 should reduce the amount of ouabain bound by yeast membranes expressing each αβ complex. Thus, the mutations V904Q, Y898R, C908G, and Y898R,C908G were made in NK3 and the ouabain binding affinity and capacity of each αβ complex were measured after isolation of yeast membranes. Nonpolar amino acid side chains are conserved among NKα subunits in position 904 but not in position 903, and so the effect of

**Thermal Stability—** The stability of the NKα3 mutants expressed in yeast with NKβ1 or HKβ was investigated by heating the membranes at different temperatures (40–50°C) for 60 s and then measuring the amount of ouabain bound at 37°C. Fig. 7 (upper panel) shows that all of the mutants are as stable as NKα3 + NKβ1 when expressed with NKβ1. In contrast, when expressed with HKβ, both NKα3 and the mutants denature at much lower temperatures (lower panel). Compared with NKα3 + HKβ, the α + HKβ complexes containing the mutations Y989R and C908G are significantly less stable. The mutant containing the double mutation Y989R,C908G is extremely unstable when expressed with HKβ. Ouabain binding by complexes of this mutant was not...
all gastric HKα subunits in the position corresponding to Val\textsuperscript{904} in NKα.

**DISCUSSION**

It has been shown previously that both α and β subunits are required by NK and gastric HK to catalyze active ion transport (17–19). NKα subunits assemble equally well with NKβ1 and gastric HKβ (9), but gastric HKα does not form functional pumps with NKβ (3). In contrast to gastric HKα, coexpression of colon HKα in Xenopus oocytes with either gastric HKβ or NKβ (4) or with toad urinary bladder HKβ (5) leads to functional ion pumps. This result indicates that interactions between β subunits and colon HKα or gastric HKs are mediated by amino acids that are different in the two α subunits. Wang et al. (2) have shown that substitution of amino acids Gln\textsuperscript{905}–Val\textsuperscript{904} from rat gastric HKα for the corresponding sequence Asn\textsuperscript{886}–Ala\textsuperscript{911} of rat NKα reduces the assembly of this chimeric α subunit (NGH26) with NKβ compared with assembly with HKβ. In the experiments reported here, a chimeric α subunit (NCH26) was formed by replacing the sequence Asn\textsuperscript{886}–Ala\textsuperscript{911} of rat NKα with the corresponding sequence Asn\textsuperscript{890}–Ala\textsuperscript{933} of the rat colon HKα subunit to examine the structural basis for the selectivity of α subunits for assembly with different β subunits. The formation of functional pumps was compared when NKα3, NCH26, or NGH26 was expressed in yeast with either NKβ1 or gastric HKβ, and amino acids in the α subunits that are important for interactions between α and β subunits were identified after site-directed mutagenesis.

The steady-state abundance of the NCH26 polypeptides expressed in yeast with NKβ1 or HKβ is the same as that of NKα3 (Fig. 1A). About 40% of the NCH26 + NKβ1 complexes are functional, as determined by the ability to bind ouabain, and about 20% of the NCH26 + HKβ complexes are functional (Fig. 2). This result is consistent with the observation of Codina et al. (4) that the colon HKα is capable of functional assembly with either NKβ1 or gastric HKβ. The result also suggests that some NCH26 + β complexes are inactive in the yeast membranes.

Codina et al. (4) reported that high concentrations of ouabain inhibited colon HKα expressed in Xenopus oocytes with either NKβ1 or HKβ (IC\textsubscript{50} = 400–600 μM in the presence of 1 mM external KCl). In the absence of KCl, the K\textsubscript{d} for ouabain binding to NKα3 expressed in yeast with either NKβ1 or HKβ is 6–8 nM (Tables 1 and 2). The K\textsubscript{d} for ouabain binding to NCH26 + NKβ1 is 50 nM, and for NCH26 + gastric HKβ the K\textsubscript{d} is greater than 200 nM. It is likely, therefore, that the low affinity of colon HKα for ouabain is due at least in part to amino acids Asn\textsuperscript{890}–Ala\textsuperscript{933}. These amino acids are found in a loop predicted from hydropathy analysis to be located on the non-cytoplasmic side of the cell membrane, between transmembrane segments 7 and 8. The loop between these transmembrane segments of NKα has been implicated in ouabain binding by Schulteis et al. (20), who observed that mutations in Arg\textsuperscript{880} led to a reduction in the affinity of the sodium pump for ouabain.

The chimeric α subunit NGH26 contains amino acids Gln\textsuperscript{905}–Val\textsuperscript{904} from rat gastric HKα substituted for Asn\textsuperscript{886}–Ala\textsuperscript{911} of rat NKα3. When this chimera was expressed in yeast, about 10 times as many pumps were assembled with gastric HKβ as with NKβ (4). The difference in the number of pumps assembled in yeast from NKα, gastric HKα, or from the chimeric polypeptides NCH26 and NGH26 and either NKβ1 or HKβ probably reflects differences in assembly or in the stability of the different αβ complexes. Consequently, mutations were made in the chimeras NGH26 and NCH26 and also in NKα3 to identify amino acid side chains that might mediate αβ subunit interactions. Introduction of charged amino acids in positions 902 and 905 of NGH26 (L902K and Q905E) increased the

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**TABLE II**

Ouabain binding affinities of NKα3 and NKα3-related mutants expressed with NKβ1 or HKβ

| Mutation        | NKβ1 (nM) | HKβ (nM) |
|-----------------|-----------|----------|
| None            | 6.1 ± 1.7 | 7.5 ± 3.2 |
| Y898R           | 13.0 ± 5.4 | 49.6 ± 6.3 |
| C908G           | 11.2 ± 2.7 | 33.5 ± 1.5 |
| Y898R,C908G     | 77.8 ± 8.4 | 90.1 ± 50.5 |
| V904Q           | 3.5 ± 1.5 | 6.8 ± 2.0 |

**TABLE III**

Comparison of the effects of mutations in NKα3 on ouabain binding capacity and on ouabain-inhibitable ATPase activity in yeast membranes

| Mutation        | B\textsuperscript{max} | ATPase activity % | % |
|-----------------|------------------------|-------------------|---|
| None            | 100 ± 18               | 100 ± 3           |
| V904Q           | 5 ± 2                  | 4 ± 2             |
| Y898R           | 42 ± 10                | 44 ± 2            |
| C908G           | 119 ± 22               | 90 ± 9            |
| Y898R,C908G     | 31 ± 2                | 28 ± 3            |

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**Fig. 7. Heat inactivation of ouabain binding.** Upper panel, NKα3 and related mutants expressed with NKβ1. Lower panel, NKα3 and related mutants expressed with gastric HKβ. 3 mg of yeast membranes containing different αβ complexes were heated at different temperatures (40–50°C) for 90 s, and then the amount of ouabain binding was determined in quadruplicate at 37°C by incubation with 20 nM \[^{3}H\]ouabain, 4 mM MgCl\textsubscript{2}, 4 mM P\textsubscript{i}, pH 7.5. Values shown are expressed as percent of \[^{3}H\]ouabain bound by the unheated membranes. Standard deviations are indicated by error bars. Filled triangles, NKα3; open circles, Y898R; filled squares, C908G; filled circles, Y898R,C908G; open squares, V904Q

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detected after the membranes were heated at 45°C for 90 s (data not shown). In contrast to Y898R and C908G, the mutation V904Q in NKα3 had no effect on the thermal stability of the functional α + HKβ complexes. Glutamine is conserved in...
affinity of the pump for ouabain but did not increase the number of pumps assembled with HKβ (Table I). Because the single mutations alone did not influence the dissociation constant of ouabain binding of NGH26 + HKβ, it is likely that neither Leu902 nor Gln905 interacts directly with ouabain. The lower \( K_d \) of the double mutant is probably caused by an induced tertiary structure in the ouabain binding site of the double mutant similar to that of the NKα3 subunit. Because neither the single mutations (L902K and Q905E) nor the double mutation (L902K,Q905E) increase the \( B_{max} \) of NGH26 expressed with either NKβ1 or HKβ, these amino acids also are probably not located in the \( \alpha \beta \) interaction interface.

The chimera containing the L902K and Q905E mutations (NGH26-KE) was used as a template for additional mutations that changed amino acids in the chimera to those found in NKα3. Most of the mutations in NGH26-KE did not affect ouabain binding by the chimera; however, the mutations Y903V and Q904Y led to a significant increase in the amount of ouabain bound when the mutant/chimeric \( \alpha \) subunit was expressed with NKβ1 (Fig. 5). This result suggests that one or both of these small hydrophobic amino acids may be important for complex formation with NKβ1. The valine at position 903 is not conserved among NKα subunits. Polar amino acids including threonine, glutamine, and glutamic acid are also found at this position in some isoforms or species. A non-polar amino acid such as valine, leucine, or isoleucine is conserved at position 904 in all NKα subunits, and a glutamine is found at this position in all gastric HKα subunits. Because gastric HKα subunits do not assemble with NKβ subunits, the mutation V904Q was made in NKα3 to test whether the glutamine in position 904 of gastric HKα could be the reason that gastric HKα does not assemble with NKβ1. The V904Q mutation in NKα3 caused a reduction in both the number of functional \( \alpha \beta \) complexes (Fig. 6) and the ATPase activity (Table III) to only 5% of NKα3 + NKβ1, without affecting the affinity of the mutant for ouabain (Table II). This result shows that the presence of glutamine at position 904 in gastric HKα subunits is sufficient to prevent assembly of HKα subunits with NKβ1. It also suggests that valine or another small hydrophobic amino acid in position 904 in NKα subunits is important for assembly with NKβ1.

In addition to Val904, Tyr908 also appears to be important for assembly of \( \alpha \) subunits with NKβ1. The mutation R898Y in NGH26 caused a 2-fold increase in the number of functional \( \alpha + \beta \) complexes (Fig. 3), and the reverse mutation Y898R in NKα3 caused a 50% reduction in the number of functional pumps when assembled with NKβ1 (Fig. 6). Arginine is conserved in colon HKα subunits at the position corresponding to amino acid 898 of NKα3, and the positive charge may limit assembly of colon HKα subunits with NKβ3 subunits. Interestingly, the Y898R mutation did not lead to a decrease in the number of pumps formed with gastric HKβ (Fig. 6).

The thermal stability of pumps containing the V904Q or Y898R mutation in NKα3 is comparable to that of nonmutated NKα3 (Fig. 7). This result indicates that the reduced number of functional pumps containing these mutations is probably not the consequence of unstable \( \alpha \beta \) complexes. The limiting factor may be the initial assembly of the two polypeptides, such that the V904Q or Y898R mutation in NKα3 prevents the majority of the two subunits from forming functional complexes.

et al. (21) reported that mutations to hydrophobic amino acids near the carboxyl terminus of NKβ3 interfere with \( \alpha \beta \) complex formation in Xenopus oocytes (21). In particular, the double mutation V269N,F271N abolished the cellular accumulation of \( \alpha \) subunits, which is an indication of \( \alpha \beta \) complex formation. The finding here that Val904 and Tyr908 in NKα3 are important for assembly with NKβ1 is intriguing in this context. Perhaps the valine-aromatic amino acid pair on each subunit interacts with one another to provide a stable contact between the subunits.

The double mutation Y898R,C908G in NKα3 caused a reduction in the number of functional pumps assembled with HKβ3 by 90%, despite the absence of an effect of either mutation alone (Fig. 6). This effect of the double mutation on the assembly of NKα3 with HKβ is consistent with the observation that the reciprocal mutations R898Y,G908C in NCH26 led to a 4-fold increase in the amount of ouabain bound when this chimeric/mutant \( \alpha \) subunit was expressed in yeast with HKβ. The thermal stability profile (Fig. 7) demonstrates that NKα3 with the double mutation Y898R,C908G is extremely unstable when assembled with HKβ. Thus, the small number of pumps assembled either from NKα3 containing either these mutations or from NCH26, and HKβ3, may be the consequence of instability in \( \alpha + \beta \) complexes caused by arginine and glycine at these positions. The double mutations Y898R,C908G in NKα3 also caused a 13-fold reduction in the ouabain affinity of pumps assembled with NKβ1 (Table II) with little effect on complex stability (Fig. 7). Because neither Y898R nor C908G alone affected ouabain binding, it is likely that neither Tyr908 nor Cys908 is located within the ouabain binding site, and the double mutation reduces ouabain affinity by indirectly affecting the ouabain binding site.

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