The β-Subunits of Na\(^+\),K\(^+\)-ATPase and Gastric H\(^+\),K\(^+\)-ATPase Have a High Preference for Their Own α-Subunit and Affect the K\(^+\) Affinity of These Enzymes*

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The α- and β-subunits of Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^+\)-ATPase were expressed in Sf9 cells in different combinations. Immunoprecipitation of the α-subunits resulted in coprecipitation of the accompanying β-subunit independent of the type of β-subunit. This indicates cross-assembly of the subunits of the different ATPases. The hybrid ATPase with the catalytic subunit of Na\(^+\),K\(^+\)-ATPase and the β-subunit of H\(^+\),K\(^+\)-ATPase (NaKoHKβ) showed an ATPase activity, which was only 12 ± 4% of the activity of the Na\(^+\),K\(^+\)-ATPase with its own β-subunit. Likewise, the complementary hybrid ATPase with the catalytic subunit of H\(^+\),K\(^+\)-ATPase and the β-subunit of Na\(^+\),K\(^+\)-ATPase (HKoNaKβ) showed an ATPase activity which was 9 ± 2% of that of the recombinant H\(^+\),K\(^+\)-ATPase. In addition, the apparent K\(^+\) affinity of hybrid NaKoHKβ decreased, while the apparent K\(^+\) affinity of the opposite hybrid HKoNaKβ was increased. The hybrid NaKoHKβ could be phosphorylated by ATP to a level of 21 ± 7% of that of Na\(^+\),K\(^+\)-ATPase. These values, together with the ATPase activity gave turnover numbers for NaKoβ and NaKoHKβ of 8800 ± 310 min\(^{-1}\) and 4800 ± 160 min\(^{-1}\), respectively. Measurements of phosphorylation of the HKoNaKβ and HKoβ enzymes are consistent with a higher turnover of the former. These findings suggest a role of the β-subunit in the catalytic turnover. In conclusion, although both Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^+\)-ATPase have a high preference for their own β-subunit, they can function with the β-subunit of the other enzyme, in which case the K\(^+\) affinity and turnover number are modified.

Both Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^+\)-ATPase belong to the family of P-type ATPases, which transport ions across the plasma membrane (1). The Na\(^+\),K\(^+\)-ATPase is found in almost all animal cells and is essential for the maintenance of cellular ion gradients, whereas the gastric H\(^+\),K\(^+\)-ATPase is localized in parietal cells of the gastric mucosa, where it is responsible for acid secretion by the stomach. These X\(^+\),K\(^+\)-ATPases\(^1\) couple ATP hydrolysis to countertransport of X\(^+\) (Na\(^+\) or H\(^+\)) and K\(^+\) as can be described by the Post-Albers scheme (2–4). Both ATPases consist of an α- and a β-subunit, which assemble with a 1:1 stoichiometry to form a stable heterodimer. The catalytic α-subunits of Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^+\)-ATPase share a high degree of identity (63%), in contrast to their heavily glycosylated β1-subunits, which are structurally similar but only 30% identical.

Assembly of the α- and β-subunits is important for conformational stability of the functional holoenzyme (5, 6). This formation of a complex between α- and β-subunits is also essential for enzyme activity (7–9) and occurs before the subunits are transported from the endoplasmic reticulum to the plasma membrane (10). Lemas et al. (11) showed that the carboxy-terminal 161 amino acids of the Na\(^+\),K\(^+\)-ATPase α-subunit are sufficient for assembly with the β-subunit. More recently, Colonna et al. (12) demonstrated that only four amino acids (SYGQ) in the extracellular loop between the predicted transmembrane helixes 7 and 8 are crucial for α-β subunit interactions. These four evolutionarily conserved amino acids are also present in the β-subunit-binding region Arg\(^1003\)-Thr\(^1026\) of the H\(^+\),K\(^+\)-ATPase α-subunit (13). In addition, Wang et al. (14) revealed that the Na\(^+\),K\(^+\)-ATPase α-subunit containing Gin\(^305\),Val\(^320\) of the gastric H\(^+\),K\(^+\)-ATPase α-subunit (including SYGQ) preferentially assembles with the H\(^+\),K\(^+\)-ATPase β-subunit. Many investigators have maintained that the extracellular domain as well as the cytoplasmic and transmembrane domains of the β-subunit are important for assembly with the α-subunit (13, 15–23).

Eventually the enzymes are transported either to the plasma membrane (Na\(^+\),K\(^+\)-ATPase) or to the tubulovesicles (H\(^+\),K\(^+\)-ATPase). Elegant studies by Caplan and co-workers (24, 25) have localized the sorting signal of Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^+\)-ATPase to a sequence of eight amino acids present in the fourth predicted transmembrane domain of the α-subunit protein. In the cytoplasmic tail of the H\(^+\),K\(^+\)-ATPase β-subunit a functional endocytosis signal was found. The presence of this motif accounts for the returning of the pump from the apical cell membrane to its intracellular storage compartment resulting in inactivation of acid secretion (26).

Studies of the hybrid ATPase consisting of the Na\(^+\),K\(^+\)-ATPase α-subunit and the H\(^+\),K\(^+\)-ATPase β-subunit have sought to analyze the function of the β-subunit. This hybrid ATPase binds ouabain and transports cations across the membrane (27, 28). The extracellular region of the H\(^+\),K\(^+\)-ATPase β-subunit is probably responsible for the slightly higher apparent Na\(^+\) affinity and the lower apparent K\(^+\) affinity of this hybrid compared with the Na\(^+\),K\(^+\)-ATPase (20, 29). In recent gel electrophoresis; C\(_5\)E\(_4\), octaethylene glycol monodecyl ether; SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2-a]-pyridine.

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\(^1\)The abbreviations used are: X\(^+\), Na\(^+\) or H\(^+\); HKo, H\(^+\),K\(^+\)-ATPase α-subunit; NaKo, Na\(^+\),K\(^+\)-ATPase α-subunit; NaKoβ, Na\(^+\),K\(^+\)-ATPase; NaKoHK, the hybrid consisting of the Na\(^+\),K\(^+\)-ATPase α-subunit and the H\(^+\),K\(^+\)-ATPase β-subunit; HKoβ, H\(^+\),K\(^+\)-ATPase; HKoNaKβ, the hybrid consisting of the H\(^+\),K\(^+\)-ATPase α-subunit and the Na\(^+\),K\(^+\)-ATPase β-subunit; Spodoptera frugiperda; PAGE, polyacrylamide gel electrophoresis; C\(_5\)E\(_4\), octaethylene glycol monodecyl ether; SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2-a]-pyridine.

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years, it has been demonstrated that the predicted transmembrane segments 4, 5, and 6 are involved in cation occlusion (32, 33). The \(\beta\)-subunits of both enzymes probably participate in stabilizing this occluded cation intermediate (32, 33). The question arises as to whether the complementary hybrid, consisting of the catalytic subunit of the H\(^{-}\)-K\(^{-}\)-ATPase and the \(\beta\)-subunit of the Na\(^+\)-K\(^+\)-ATPase, also possesses catalytic activity. We therefore measured both the Na\(^+\)-K\(^+\)-ATPase activity and the H\(^{-}\)-K\(^{-}\)-ATPase activity for both hybrid ATPases. Furthermore, this study examines the possible role of \(\beta\)-subunits in the apparent K\(^-\) affinity of both K\(^-\) dependent ATPases.

EXPERIMENTAL PROCEDURES

Expression Constructs—The rat gastric H\(^-\)K\(^+\)-ATPase \(\alpha\)-subunit cDNA (9) was digested with BglII and ligated into the BamHI site of the pFastbac vector (HK3a). The \(\beta\)-subunit cDNA of the rat gastric H\(^-\)K\(^+\)-ATPase (9) was digested with BamHI and ligated into the BbsI site of the pFastbac vector containing the H\(^-\)K\(^+\)-ATPase \(\alpha\)-subunit (HKnK). The rat Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit cDNA (34) HindIII fragment was ligated into the HindIII site of the pFastbac vector (NaK\(a\)). In a similarly procedured multiple cloning site vector the pFastbac (with BamHI) containing the H\(^-\)K\(^+\)-ATPase \(\beta\)-subunit (NaK\(a\)H\(\beta\)K). The sheep Na\(^+\)-K\(^+\)-ATPase \(\beta\)-subunit cDNA (35) was digested with SmaI and SpeI and ligated into the SmaI and NheI site of the pFastbac vector containing the Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit (NaK\(a\)K\(\beta\)K) or into the XhoI and NheI site of the pFastbac vector containing the H\(^-\)K\(^+\)-ATPase \(\alpha\)-subunit (HK\(n\)K\(\beta\)K). We therefore measured both the Na\(^+\)-K\(^+\)-ATPase activity and the H\(^-\)K\(^-\)-ATPase activity for both hybrid ATPases. Furthermore, this study examines the possible role of \(\beta\)-subunits in the apparent K\(^-\) affinity of both K\(^-\) dependent ATPases.

Production of Recombinant Viruses—Competent DH10bac Escherichia coli cells (Life Technologies, Breda, The Netherlands) harboring the baculovirus genome (bacmid) and a transposition helper vector, E. coli cells (Life Technologies, Breda, The Netherlands) harboring the pFastbacdual vector (HK\(\beta\)K). All \(\alpha\)-subunits were cloned downstream of the polyhedrin promoter, and all \(\beta\)-subunits downstream of the polyhedrin promoter. All recombinant helper baculoviruses were harvested and used to infect SF9 cells at a multiplicity of infection of 0.1. Four days after infection, the amplified viruses were harvested.

Preparation of Membranes—SF9 cells were grown at 27 °C in 100-ml spinner flasks cultures (9). For production of the ATPase subunits 1.0 × 10\(^6\) cells/ml were infected at a multiplicity of infection of 1–3 in Xpress medium (BioWittaker, Walkersville, MD) containing 1% ethanol (37) and incubated for 3 days. The SF9 cells were harvested by centrifugation at 2000 × g for 5 min, and resuspended at 0 °C in 0.25 M sucrose, 2 mM EDTA, and 25 mM Hepes/Tris (pH 7.0). The membranes were sonicated for 30 s without heating in 0.5 ml of 100 mM NaCl, 20 mM Hepes/Tris at 4 °C. Following centrifugation at 100,000 × g for 5 min, the supernatant was incubated with the antibodies bound to the protein A immobilized on agarose for 1 h at 4 °C. The immunoprecipitates were collected by centrifugation for a few seconds at 10,000 × g, washed 3 times in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl and solubilized in SDS-PAGE sample buffer. After SDS-PAGE the ATPase subunits in the precipitates were identified by immunoblotting, using the biotin-labeled antibodies L25 (43), M17-P5-F11 (44), and 2G11 (42). The antibody HK9 was used for detection of the H\(^-\)K\(^+\)-ATPase \(\alpha\)-subunit (47). The primary antibodies labeled with biotin were detected using streptavidin labeled with peroxidase (Jackson ImmunoResearch, West Grove, PA), while HK9 was detected with anti-rabbit secondary antibody, which was also labeled with peroxidase (DAKO A/S, Denmark).

ATPase Activity Assay—The ATPase activity was determined with a radiochemical method (48). For this purpose 0.6–7 μg of SF9 membranes were added to 100 μl of medium, which contained 1 mM MgCl\(_2\), 0.2 mM EGTA, 0.1 mM EDTA, 1 mM NaN\(_3\), and 25 mM Tris-HCl (pH 7.0). For determination of Na\(^+\)-K\(^+\)-ATPase activity, 10 μM (\(\gamma\)-\(32\)P)ATP, 100 mM NaCl, and 0.01 mM ouabain (in order to inhibit endogenous Na\(^+\)-K\(^+\)-ATPase from SF9 cells) were present. The specific activity is presented as the difference in activity with and without 0.1 mM SCH 28080. After incubation at 37 °C the reaction was stopped by adding 500 μl of 10% (w/v) charcoal in 6% (w/v) trichloroacetic acid and after incubation at 0 °C the mixture was centrifuged for 30 s (10,000 × g). To 200 μl of the clear supernatant, containing the liberated inorganic phosphate (\(\gamma\)-\(32\)P), 4 ml of OptiFluor (Canberra Packard, Tullburg, The Netherlands) was added and the mixture was analyzed by liquid scintillation spectrometry. Blanks were prepared by incubating in the absence of enzyme.

ATP Phosphorylation Capacity—ATP phosphorylation was determined as described (37). SF9 membranes (6–70 μg) were incubated at 0 °C in 25 mM Tris-acetic acid (pH 6.0), 1 mM MgCl\(_2\), in a volume of 50 μl. For phosphorylation of Na\(^+\)-K\(^+\)-ATPase, 100 mM NaCl was added to the incubation buffer. This phosphorylation is presented as the difference in phosphorylation of H\(^-\)K\(^-\)-ATPase from SF9 cells (42). The specific activity is presented as the difference with and without 0.1 mM SCH 28080. After incubation at 37 °C the reaction was stopped by adding 500 μl of 10% (w/v) charcoal in 6% (w/v) trichloroacetic acid and after incubation at 0 °C the mixture was centrifuged for 30 s (10,000 × g). To 200 μl of the clear supernatant, containing the liberated inorganic phosphate (\(\gamma\)-\(32\)P), 4 ml of OptiFluor (Canberra Packard, Tullburg, The Netherlands) was added and the mixture was analyzed by liquid scintillation spectrometry.

Calculations—The K\(_{50}\) value is defined as the concentration of effector (K) giving the half-maximal activation and the K\(_{50}\) as the value giving 50% inhibition of the maximal activation. Data are presented as mean values with standard error of the mean. Differences were tested for significance by means of the Student’s t test.

Materials—The cDNA clones of the H\(^-\)K\(^-\)-ATPase \(\alpha\) and \(\beta\)-subunits and the rat and sheep cDNA clones of the Na\(^+\)-K\(^+\)-ATPase \(\alpha\) and \(\beta\)-subunits were supplied by Drs. G. E. Shull and J. B. Lingrel, respectively. Collectin, competent DH10bac E. coli cells, and all enzymes were purchased from Promega Corp. (Madison, WI). \(\gamma\)-\(32\)P)ATP (3000 Ci mmol\(^{-1}\)) was purchased from Amersham (Buckinghamshire, United Kingdom). SCH 28080, kindly provided by Dr. A. Barnett, Schering-Plow, Kenilworth, NJ, was dissolved in ethanol and diluted to its final concentration of 0.1 mM in 0.2% ethanol. The antibiotics 2G11 and M17-P5-F11 were gifts from Drs. J. Forte (Berkeley) and W. J. Ball Jr. (Cincinnati), respectively. Dr. J. V. Moller (Aarhus,
Recombinant baculoviruses expressing \( \text{Na}^+,\text{K}^+\)-ATPase, \( \text{H}^+,\text{K}^+\)-ATPase, their \( \alpha \)-subunits, and their hybrids were produced and Sf9 cells were infected. The membrane fractions of these Sf9 cells expressing the recombinant proteins were isolated and Western blot analysis revealed comparable expression patterns (Fig. 1). Both \( \text{Na}^+,\text{K}^+\)-ATPase and \( \text{H}^+,\text{K}^+\)-ATPase \( \alpha \)-subunits detected with antibodies L25 (43) and HKB (41), respectively, had an apparent molecular mass of about 100 kDa. The antibody M17-P5-F11 (44) visualized both a carbohydrate-free and a core-glycosylated form of the \( \text{Na}^+,\text{K}^+\)-ATPase \( \beta \)-subunit. The monoclonal antibody 2G11 (42) also recognized a carbohydrate-free and a core-glycosylated form of the \( \text{H}^+,\text{K}^+\)-ATPase \( \beta \)-subunit. This carbohydrate-free form was of a similar molecular mass as the carbohydrate-free \( \text{Na}^+,\text{K}^+\)-ATPase \( \beta \)-subunit, but the core-glycosylated \( \text{Na}^+,\text{K}^+\)-ATPase \( \beta \)-subunit had a lower apparent molecular mass, due to the presence of three glycosylation sites in this subunit in contrast to the six glycosylation sites present in the \( \text{H}^+,\text{K}^+\)-ATPase \( \beta \)-subunit.

Interaction between the \( \alpha \)- and \( \beta \)-subunits in both \( \text{Na}^+,\text{K}^+\)-ATPase and \( \text{H}^+,\text{K}^+\)-ATPase is essential for a functionally active enzyme. To examine the interaction between \( \alpha \)- and \( \beta \)-subunits in hybrid ATPases an immunoprecipitation assay was performed. The \( \alpha \)-subunits of \( \text{Na}^+,\text{K}^+\)-ATPase and \( \text{H}^+,\text{K}^+\)-ATPase were immunoprecipitated with antibodies L25 (43) and 1F11 (46), respectively. In control experiments both the \( \text{Na}^+,\text{K}^+\)-ATPase isolated from sheep kidney and recombinant \( \text{Na}^+,\text{K}^+\)-ATPase were precipitated with antibody L25. Similarly, rat gastric \( \text{H}^+,\text{K}^+\)-ATPase and recombinant \( \text{H}^+,\text{K}^+\)-ATPase were precipitated by antibody 1F11. With the native enzymes only glycosylated \( \beta \)-subunits were precipitated, with apparent molecular masses significantly higher than those of their recombinant counterparts. This is due to the absence of complex glycosylation in Sf9 cells. When the same method was used for the hybrid ATPases, both \( \beta \)-subunits were also coprecipitated with the other \( \alpha \)-subunit (Fig. 2). This indicates that there is not only an interaction between the \( \alpha \)- and \( \beta \)-subunits of \( \text{Na}^+,\text{K}^+\)-ATPase and \( \text{H}^+,\text{K}^+\)-ATPase, but also between the \( \alpha \)- and \( \beta \)-subunits of the two hybrid ATPases. Although quantification in these experiments is rather difficult, there seemed to be less coprecipitated \( \beta \)-subunits in the hybrid ATPases than in the wild type ATPases (NaKβ, HKβ).

When HKOaNaKβ was present during immunoprecipitation with antibody L25 (directed against the \( \alpha \)-subunit of NaK+-ATPase), the \( \beta \)-subunit of NaK+-ATPase was unexpectedly precipitated. Also when the H+-ATPase \( \alpha \)-subunit was absent the NaK+-ATPase \( \beta \)-subunit was precipitated (data not shown). In the absence of antibody L25 the NaK+-ATPase \( \beta \)-subunit was not precipitated. These findings suggest that the expressed Na+-ATPase \( \beta \)-subunit assembles with the endogenous Na+-ATPase \( \alpha \)-subunit. Antibody L25 apparently recognizes this endogenous \( \alpha \)-subunit during immunoprecipitation, but not on a Western blot. The absence of unglycosylated \( \beta \)-subunit indicates that the assembly with the endogenous \( \alpha \)-subunit only occurred during the early stage of infection, when the glycosylation machinery is still functional.

Na+-ATPase activity was measured in the presence of 100 \( \mu \)M ATP and optimal concentrations of Na+ (100 mM) and K+ (10 mM). Because of the endogenous Na+-ATPase activity (50–100 nmol mg\(^{-1}\) protein h\(^{-1}\)) present in the Sf9 membrane preparations, we determined the ouabain sensitivity for the endogenously present Na+-ATPase in mock infected cells. The endogenous Na+-ATPase activity was completely inhibited at \( 1 \times 10^{-6} \) M ouabain, while the recombinant activity was hardly inhibited at this concentration. These findings are in line with those of Lui and Guidotti (49). Using \( 1 \times 10^{-5} \) M ouabain in the assay, we measured the recombinant ATPase activity as the difference between the activity with and without 10 mM K+ (Fig. 3A). The activity of NaKαHKβ was 12 ± 4% (\( n = 3 \)) of the activity of NaKα. NaKα and mock infected cells did not show Na+-ATPase activity in the presence of \( 10^{-5} \) M ouabain.

In order to measure K+-stimulated H+-ATPase activity we used a suboptimal (10 \( \mu \)M) ATP concentration since at higher ATP concentrations the endogenous activity increases.
Hybrids of Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase

ATPase activity was set at 100% (Fig. 5). The increasing part of this curve is due to K⁺ activation of the dephosphorylation step. The decreasing part is due to the competition of K⁺ with H⁺ or Na⁺ at the cytoplasmic activation sites. This directs the enzyme from the E₁ toward the E₂ conformation, which cannot be overcome by the low ATP concentration used. The K_{50} for K⁺ of NaKβ was 0.5 ± 0.2 mM and the IC_{50} was 50 ± 9 mM (Fig. 5A). The curve of the hybrid NaKαHKβ was shifted to the right compared with the curve of NaKβ. The K_{50} and the IC_{50} values of this hybrid were slightly increased to 0.7 ± 0.3 mM (K_{50}) and 103 ± 24 mM (IC_{50}), respectively, when compared with NaKβ. In contrast, the K⁺ activation curve of HKβNaKβ was shifted to the left compared with the curve of HKβ (Fig. 5B). Moreover, the K_{50} for HKβB (0.07 ± 0.01 mM) was decreased for the hybrid HKβNaKβ (0.02 ± 0.004 mM) and also the IC_{50} was decreased from 10 ± 0.2 to 3.5 ± 0.7 mM. These shifts in K_{50} and IC_{50} values were significant (p < 0.05, n = 3). Thus HKβNaKβ had a slightly decreased K⁺ affinity compared with HKβ, while the opposite hybrid HKβNaKβ had an increased K⁺ affinity compared with HKβ. These findings indicate that the β-subunits of both Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase influence the K⁺ sensitivity of these enzymes.

The formation of an acid-stable phosphorylated intermediate during the catalytic cycle is a characteristic property of the P-type ATPases. Phosphorylation of Na⁺,K⁺-ATPase was measured with Na⁺ present in the preincubation in order to shift the equilibrium of the enzyme forms toward NaE₁. NaKβHKβ was phosphorylated for 28 ± 11% compared with NaKβ (n = 3, Fig. 6A). Unlike phosphorylation of HKβNaKβ, phosphorylation of HKβNaKβ was not visible, whereas HKβNaKβ was normally phosphorylated (Fig. 6B). Changing the temperature to 21°C (31), longer incubation periods, inhibition with K⁺ instead of SCH 28080, higher ATP concentrations, or addition of triallylamine (51) still did not result in any measurable amount of phosphorylated intermediate. In order to determine the maximal phosphorylation level we measured the phosphorylation level at different ATP concentrations (0.006–2 μM) at 21°C. The data are plotted as the phosphorylation level versus the ATP concentration (Fig. 7A) and the same data are also visualized in a Woolf-Augustinson-Hofstee plot (Fig. 7B). In the last plot the apparent ATP affinity and the maximal phosphorylation level can be determined more easily. The ATP affinity is equal to the slope of the graph, while the intercept with the y-axis is equal to the maximal phosphorylation level. For this interpretation it must be assumed that the distribution of E₁ forms does not change over the range of concentrations of ATP used. For HKβ a maximal phosphorylation level of 6.3 ± 0.9 pmol mg⁻¹ protein with an apparent ATP affinity of 23 ± 3 nM (n = 3) was measured. In the reaction mixture where the Na⁺,K⁺-ATPase α-subunit was present oligomycin was included, which increased the phosphorylation level by about 30%. The apparent ATP affinities for NaKβ (12 ± 2 nM, n = 3) and NaKβHKβ (12 ± 1 nM, n = 3) are similar. However, the maximal phosphorylation level for NaKβ was 3.3 ± 1.0 pmol mg⁻¹ protein, whereas the maximal phosphorylation level for NaKβHKβ was 0.71 ± 0.10 pmol mg⁻¹ protein (which was 21 ± 7% of that of NaKβ, n = 3). These values, together with the maximal ATPase activity (determined at infinite ATP concentrations) give turnover numbers for NaKβ and NaKβHKβ of 8800 ± 310 min⁻¹ and 4800 ± 160 min⁻¹, respectively. These data are not corrected for the suboptimal K⁺ concentration for the hybrid ATPase in the ATPase reaction, which probably will increase the turnover number by about 15%.

FIG. 3. ATPase activity in Sf9 membranes infected with recombinant baculoviruses. Membranes obtained from Sf9 cells, expressing Na⁺,K⁺-ATPase, H⁺,K⁺-ATPase, and their hybrids, were isolated and incubated at 27°C and pH 7.0 with 10 μM ATP and 1 mM KCl for H⁺,K⁺-ATPase activity, while Na⁺,K⁺-ATPase activity was determined in the presence of 100 μM ATP, 100 mM NaCl, and 10 mM KCl. The Na⁺,K⁺-ATPase activity (A) and the H⁺,K⁺-ATPase activity (B) were measured as described under “Experimental Procedures.” The values presented are the mean ± S.E. of three experiments. * significantly different from the mock and α-subunit ATPase activity (p < 0.05).

Relative ATPase activity of the hybrid ATPases could be due to changed ATP affinities, we measured the ATPase activity at different ATP concentrations (0.03–1 mM). The results are given in a Woolf-Augustinson-Hofstee plot (Fig. 4). The intercept with the y-axis is equal to the maximal ATPase activity at infinite ATP concentrations. The maximal NaKβ ATPase activity was 1.8 ± 0.6 μmol mg⁻¹ protein h⁻¹, whereas the activity for NaKαHKβ was 0.21 ± 0.03 μmol mg⁻¹ protein h⁻¹ (which was 12 ± 4% of that of NaKβ, n = 3). The maximal HKβNaKβ ATPase activity was 1.4 ± 0.1 μmol mg⁻¹ protein h⁻¹, whereas the activity for HKβNaKβ was 0.12 ± 0.02 μmol mg⁻¹ protein h⁻¹ (which was 9 ± 2% of that of HKβ, n = 3). The slope of this graph represents the apparent ATP affinity. The apparent ATP affinities for NaKβ (197 ± 27 μM, n = 3) and NaKβHKβ (206 ± 5 μM, n = 3) are similar. Also the apparent ATP affinities for HKβ (17 ± 0.7 μM, n = 3) and HKβNaKβ (13 ± 1.3 μM, n = 3) are not significantly different. This indicates that the lower ATPase activity of the hybrids is not due to a change in ATP affinity.

Both Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase occlude K⁺ and transport this ion across the plasma membrane. We compared the K⁺ dependence of the overall ATPase activity of Na⁺,K⁺-ATPase, H⁺,K⁺-ATPase, and their hybrids. All ATPases showed a biphasic activation curve, in which the maximal ATPase activity was set at 100% (Fig. 5). The increasing part of this curve is due to K⁺ activation of the dephosphorylation step. The decreasing part is due to the competition of K⁺ with H⁺ or Na⁺ at the cytoplasmic activation sites. This directs the enzyme from the E₁ toward the E₂ conformation, which cannot be overcome by the low ATP concentration used. The K_{50} for K⁺ of NaKβ was 0.5 ± 0.2 mM and the IC_{50} was 50 ± 9 mM (Fig. 5A). The curve of the hybrid NaKαHKβ was shifted to the right compared with the curve of NaKβ. The K_{50} and the IC_{50} values of this hybrid were slightly increased to 0.7 ± 0.3 mM (K_{50}) and 103 ± 24 mM (IC_{50}), respectively, when compared with NaKβ. In contrast, the K⁺ activation curve of HKβNaKβ was shifted to the left compared with the curve of HKβ (Fig. 5B). Moreover, the K_{50} for HKβB (0.07 ± 0.01 mM) was decreased for the hybrid HKβNaKβ (0.02 ± 0.004 mM) and also the IC_{50} was decreased from 10 ± 0.2 to 3.5 ± 0.7 mM. These shifts in K_{50} and IC_{50} values were significant (p < 0.05, n = 3). Thus HKβNaKβ had a slightly decreased K⁺ affinity compared with HKβ, while the opposite hybrid HKβNaKβ had an increased K⁺ affinity compared with HKβ. These findings indicate that the β-subunits of both Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase influence the K⁺ sensitivity of these enzymes.

The formation of an acid-stable phosphorylated intermediate during the catalytic cycle is a characteristic property of the P-type ATPases. Phosphorylation of Na⁺,K⁺-ATPase was measured with Na⁺ present in the preincubation in order to shift the equilibrium of the enzyme forms toward NaE₁. NaKβHKβ was phosphorylated for 28 ± 11% compared with NaKβ (n = 3, Fig. 6A). Unlike phosphorylation of HKβNaKβ, phosphorylation of HKβNaKβ was not visible, whereas HKβNaKβ was normally phosphorylated (Fig. 6B). Changing the temperature to 21°C (31), longer incubation periods, inhibition with K⁺ instead of SCH 28080, higher ATP concentrations, or addition of triallylamine (51) still did not result in any measurable amount of phosphorylated intermediate. In order to determine the maximal phosphorylation level we measured the phosphorylation level at different ATP concentrations (0.006–2 μM) at 21°C. The data are plotted as the phosphorylation level versus the ATP concentration (Fig. 7A) and the same data are also visualized in a Woolf-Augustinson-Hofstee plot (Fig. 7B). In the last plot the apparent ATP affinity and the maximal phosphorylation level can be determined more easily. The ATP affinity is equal to the slope of the graph, while the intercept with the y-axis is equal to the maximal phosphorylation level. For this interpretation it must be assumed that the distribution of E₁ forms does not change over the range of concentrations of ATP used. For HKβ a maximal phosphorylation level of 6.3 ± 0.9 pmol mg⁻¹ protein with an apparent ATP affinity of 23 ± 3 nM (n = 3) was measured. In the reaction mixture where the Na⁺,K⁺-ATPase α-subunit was present oligomycin was included, which increased the phosphorylation level by about 30%. The apparent ATP affinities for NaKβ (12 ± 2 nM, n = 3) and NaKβHKβ (12 ± 1 nM, n = 3) are similar. However, the maximal phosphorylation level for NaKβ was 3.3 ± 1.0 pmol mg⁻¹ protein, whereas the maximal phosphorylation level for NaKβHKβ was 0.71 ± 0.10 pmol mg⁻¹ protein (which was 21 ± 7% of that of NaKβ, n = 3). These values, together with the maximal ATPase activity (determined at infinite ATP concentrations) give turnover numbers for NaKβ and NaKβHKβ of 8800 ± 310 min⁻¹ and 4800 ± 160 min⁻¹, respectively. These data are not corrected for the suboptimal K⁺ concentration for the hybrid ATPase in the ATPase reaction, which probably will increase the turnover number by about 15%.
**DISCUSSION**

The $\beta$-subunits of $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase}$ and $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ are involved in correct folding (10) of the $\alpha$-subunits. In addition, the $\beta$-subunit of $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ plays a role in endocytosis of the enzyme (24). Most probably, both $\beta$-subunits are also involved in the modulation of the enzyme activity: $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase}$ affinities of the $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase}$ were changed when the $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase} \beta$-subunit was replaced by the $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase} \beta$-subunit (20, 29). In the present study we demonstrate that the hybrid consisting of the $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase} \alpha$-subunit and the $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase} \beta$-subunit has a low ATPase activity with a high apparent $\mathrm{K}_1$ affinity and probably a high turnover number as compared with $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$.

![FIG. 4. Woolf-Augustinsson-Hofstee plot of the ATPase activity versus the ATP concentration. Membranes obtained from Sf9 cells, expressing $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase}$ (A, $\square$), Na$\alpha$HK$\beta$ (B, $\square$), $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ (A, $\bigcirc$), or HKoNa$\beta$ (B, $\bigcirc$), were isolated and incubated at 37 °C and pH 7.0 with 1 mM KCl for $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ activity, while $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase}$ activity was determined in the presence of 100 mM NaCl and 10 mM KCl. The ATPase activity was measured as described under "Experimental Procedures" using varying concentrations of ATP (0.03–1 mM). The activities obtained with Na$\alpha$HK$\beta$ and Na$\alpha$HK$\beta$ were measured as the difference in activity in the presence of 0.01 and 1 mM ouabain.](image)

![FIG. 5. Effects of $\mathrm{K}^+$ on the ATPase activity in Sf9 membranes infected with recombinant baculoviruses. Membranes obtained from Sf9 cells, expressing Na$^+,\mathrm{K}^+\text{-ATPase}$ (A, $\square$), Na$\alpha$HK$\beta$ (B, $\square$), $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ (A, $\bigcirc$), or HKoNa$\beta$ (B, $\bigcirc$) were isolated and incubated at 37 °C and pH 7.0 with 10 mM ATP for $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ activity, while Na$^+,\mathrm{K}^+\text{-ATPase}$ activity was determined in the presence of 100 mM NaCl and 10 mM KCl. The $\mathrm{K}^+$-activated ATPase activity was measured as described under "Experimental Procedures" using varying concentrations of $\mathrm{K}^+$. The specific $\mathrm{Na}^+,\mathrm{K}^+\text{-ATPase}$ activity is presented as the activity in the presence of $10^{-5}$ M ouabain, minus the ATPase activity of mock infected membranes (A). The specific $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ activity is presented as the activity in the presence of $10^{-5}$ M ouabain, minus the ATPase activity of mock infected membranes (A). The specific $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ activity is presented as the difference in activity with and without SCH 28080 (B). The maximal ATPase activity for each preparation was set at 100% and the values presented are the mean ± S.E. of three experiments. * significantly different from the mock and $\alpha$-subunit phosphorylation levels ($p < 0.05$).](image)

![FIG. 6. ATP-phosphorylation level in Sf9 membrane preparations infected with recombinant baculovirus. Membranes obtained from Sf9 cells, expressing Na$^+,\mathrm{K}^+\text{-ATPase}$, $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$, and their hybrids, were isolated and incubated for 10 s at 0 °C and pH 6.0 in the presence of 0.1 mM ATP for $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$, while for Na$^+,\mathrm{K}^+\text{-ATPase}$ also 100 mM NaCl was present. The Na$^+,\mathrm{K}^+\text{-ATPase}$ phosphorylation level (A) and the $\mathrm{H}^+,\mathrm{K}^+\text{-ATPase}$ phosphorylation level (B) were measured as described under "Experimental Procedures." The values presented are the mean ± S.E. of three experiments. * significantly different from the mock and $\alpha$-subunit phosphorylation levels ($p < 0.05$).](image)
Recombinant Na\(^+\),K\(^+\)-ATPase, H\(^+\),K\(^-\)-ATPase, and their hybrids were produced with the baculovirus expression system. The molecular mass of all \(\alpha\)-subunits expressed was similar to that of the isolated \(\alpha\)-subunits. However, the recombinant \(\beta\)-subunits were less glycosylated than the isolated \(\beta\)-subunits, as has been reported before (9, 52). Expression levels of the subunits of each hybrid ATPase were comparable to those of Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^-\)-ATPase, respectively.

Assembly of \(\alpha\) and \(\beta\)-subunits is a crucial step in the formation of active X\(^+\),K\(^-\)-ATPases (7–9). The minimal \(\beta\)-subunit-binding site (12) in the \(\alpha\)-subunits of Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^-\)-ATPase are identical and therefore an interaction between the \(\alpha\) and \(\beta\)-subunits of the different ATPases seems likely. Both \(\beta\)-subunits are only 30% identical, but have a high structural similarity. In the hybrid ATPase NaK\(\alpha\)K\(\beta\) the subunits cross-assembled in a co-immunoprecipitation experiment. Others also observed an interaction between the Na\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit and the H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit (27, 28, 53). A possible interaction between the H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit and the Na\(^+\),K\(^+\)-ATPase \(\beta\)-subunit has not been demonstrated so far. When in our experiments the \(\alpha\)-subunit of the hybrid ATPase HK\(\alpha\)NaK\(\beta\) was precipitated by anti-Na\(^+\),K\(^+\)-ATPase antibodies, the \(\beta\)-subunit of Na\(^+\),K\(^+\)-ATPase coprecipitated. However, for both hybrid ATPases there seemed to be less coprecipitated \(\beta\)-subunits compared with the wild type enzymes, indicating a less efficient assembly between the different subunits of the hybrid ATPases. This could be due to the 70% difference in amino acid composition or to the difference in glycosylation between the two \(\beta\)-subunits.

Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^-\)-ATPase need hydrolysis of ATP to transport cations across the membrane. Accordingly, functional hybrid ATPases should also have ATPase activity. We determined that the hybrid ATPase NaK\(\alpha\)HK\(\beta\) possessed a low ATPase activity which was about 12% of the activity of recombinant Na\(^+\),K\(^-\)-ATPase. Hybrid ATPase activity has not been measured so far, although in yeast expressing the Na\(^+\),K\(^-\)-ATPase \(\alpha_1\)-subunit and the H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit ouabain binding was observed (27). ATPase activity has been measured in membranes isolated from yeast expressing the Na\(^+\),K\(^-\)-ATPase \(\alpha_1\)-subunit and a chimeric \(\beta\)-subunit (29). In oocytes expressing NaK\(\alpha\)K\(\beta\) a small Na\(^+\),K\(^-\)-pump current and Rb\(^+\) uptake has been detected (28), although this could not be confirmed by Ueno et al. (54), which might be due to the very low activity of this hybrid ATPase. None of these assays were performed for the hybrid ATPase HK\(\alpha\)NaK\(\beta\). When we measured the SCH 28080-sensitive ATPase activity, this hybrid exhibited also a low ATPase activity, which was only 9% of the activity of recombinant H\(^+\),K\(^-\)-ATPase. Both hybrid ATPases have ATP hydrolyzing activity, although this activity is much lower than that of Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^-\)-ATPase. The ATP affinities of the hybrids are similar to those of the wild type ATPases. So, the lower ATPase activity of hybrid ATPases might result from a decreased amount of functional hybrid ATPase molecules.

X\(^+\),K\(^-\)-ATPases need K\(^+\) occlusion before they can dephosphorylate (31) and probably the \(\beta\)-subunit is involved in this \(\mathrm{K}^+\) occlusion (32, 33). When the Na\(^+\),K\(^+\)-ATPase \(\beta\)-subunit was replaced by the H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit the K\(^+\) affinity in the ATPase reaction decreased similarly as has been reported in ouabain binding experiments by Eakle et al. (20). However, the K\(^+\) affinity of the complementary hybrid ATPase HK\(\alpha\)NaK\(\beta\) was increased as compared with that of H\(^+\),K\(^-\)-ATPase. Thus the apparent K\(^+\) affinities of Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^-\)-ATPase are partly modulated through their \(\beta\)-subunits. In summary, the \(\beta\)-subunit of Na\(^+\),K\(^+\)-ATPase, as compared with the \(\beta\)-subunit of H\(^+\),K\(^-\)-ATPase, gives the enzyme a higher apparent K\(^+\) affinity.

When K\(^+\) ions are absent during incubation, the enzymes are presumably accumulated in the phosphorylated state. The hybrid ATPase NaK\(\alpha\)HK\(\beta\) was phosphorylated to 21% of the Na\(^+\),K\(^+\)-ATPase phosphorylation level, which is slightly higher than the percentage activity obtained in the ATPase reaction. This lower ATPase activity and phosphorylation level of the hybrid must mainly be caused by a less efficient subunit assembly. Surprisingly, with the hybrid HK\(\alpha\)NaK\(\beta\) we were not able to measure any specific phosphorylation. Although no K\(^+\) is added to the reaction mixture it still contained about 5 \(\mu\)M K\(^+\) as determined by flame photometry. The decrease in \(K_{0.5}\) directs the enzyme from the \(E_2P\) into the \(E_3\) conformation, while the decrease in IC\(_{50}\) directs the enzyme from the \(E_1\) into the \(E_2\) conformation. Both these processes inhibit accumulation of hybrid HK\(\alpha\)NaK\(\beta\) in the \(E_2P\) conformation and drive the enzyme into the \(E_3\) conformation. It is unlikely, however, that the low amount of K\(^+\) present accounts for the total absence of any phosphorylated intermediate.

Another explanation for the absence of a phosphorylated intermediate in the hybrid HK\(\alpha\)NaK\(\beta\) is an increased turnover number. The H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit decreases the turnover number of the hybrid NaK\(\alpha\)HK\(\beta\) as compared with that of NaK\(\alpha\)\(\beta\) significantly. The assumption that the Na\(^+\),K\(^-\)-ATPase \(\beta\)-subunit increases the turnover number of the hybrid HK\(\alpha\)NaK\(\beta\), as compared with HK\(\alpha\)\(\beta\), seems likely. This higher

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**Fig. 7. ATP dependent phosphorylation level.** Membranes obtained from Sf9 cells, expressing Na\(^+\),K\(^+\)-ATPase ( ), NaK\(\alpha\)HK\(\beta\) ( ), or H\(^+\),K\(^-\)-ATPase ( ), were isolated and incubated for 10 s at 21°C and pH 6.0 for H\(^+\),K\(^-\)-ATPase, while for Na\(^+\),K\(^+\)-ATPase 100 mM NaCl was also present. The phosphorylation level was measured as described under "Experimental Procedures" varying the concentrations of ATP (0.006–0.2 \(\mu\)M). The phosphorylation levels obtained with NaK\(\alpha\)\(\beta\) and NaK\(\alpha\)HK\(\beta\) were corrected for that of NaK\(\alpha\). A, phosphorylation level as a function of the ATP concentration. B, data from A represented as a Woolf-Augustinsson-Hofstee plot.
turnover number would then be responsible for a lower phosphorylation level for HKNaKβ. If the increase in turnover number is of the same magnitude as the 1.8-fold decrease in the turnover number of the hybrid NaKαHKβ as compared with NaKβ, then the maximal EP level of HKNaKβ is 0.3 pmol mg⁻¹ protein, which is below the detection limit.

The suggestion of a relationship between the K⁺ affinity and the turnover of the ATPases, which both are influenced by the β-subunit, is tempting. The deocclusion step for Na⁺,K⁺-ATPase is the rate-limiting step, while for the H⁺,K⁺-ATPase the dephosphorylation step (this is the occlusion of K⁺) is rate-limiting. This rate-limiting step must be accelerated if the turnover number is raised. The hybrid HKNaKβ then not only has an increased K⁺ affinity but also a higher rate of K⁺ stimulated dephosphorylation as compared with HKβ. Thus, HKNaKβ occludes K⁺ faster and with a higher affinity than HKβ, which directly increases the turnover number. The opposite is true for the other hybrid NaKαHKβ, although in this case the K⁺ occlusion becomes rate-limiting.

The findings reported here show that both Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase require their own β-subunits for optimal activity. Probably the subunit assembly in the hybrid ATPases is less efficient than in the wild type ATPases. When the β-subunits are exchanged, the enzyme activity decreases and the apparent K⁺ affinity of both hybrid ATPases is modified. The Na⁺,K⁺-ATPase β-subunit gives the enzyme a higher K⁺ affinity and probably a higher turnover number than the H⁺,K⁺-ATPase β-subunit.

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