Glutamate 329 Located in the Fourth Transmembrane Segment of the α-Subunit of the Rat Kidney Na^+,K^+-ATPase Is Not an Essential Residue for Active Transport of Sodium and Potassium Ions†

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ABSTRACT: An allelic variant of the ouabain-insensitive rat kidney Na^+,K^+-ATPase α_1-isoform was identified by chance in a cDNA library. The variant differed from the wild-type rat kidney Na^+,K^+-ATPase by a single G to C base substitution in the cDNA, which on the amino acid level gave rise to a glutamine in place of the glutamate residue Glu329, previously suggested as a likely donator of oxygen ligands for Na^+ and K^+ binding. The variant cDNA was transfected into COS-1 cells and the transfectants expanded with success into stable cell lines that were able to grow in the presence of a concentration of ouabain highly cytotoxic to the parental cells containing only the endogenous COS-1 cell Na^+,K^+-ATPase. Under these conditions, the viability of the cells depended on the cation transport mediated by the ouabain-insensitive Glu329 → Gln variant, whose cDNA was shown by polymerase chain reaction amplification to be stably integrated into the COS-1 cell genome. Functional analysis on isolated plasma membranes demonstrated that the Glu329 → Gln variant was able to catalyze Na^+- and K^+-activated ATPase activity with a maximum turnover number similar if not identical to that of the wild type, but the variant exhibited a reduced affinity for both cations corresponding to a 2-fold increase in $K_{0.5}$ for Na^+ and a 6-fold increase in $K_{0.5}$ for K^+. Moreover, the apparent affinity for ATP was increased 15-fold in the variant relative to wild-type Na^+,K^+-ATPase. The Na^+,K^+-ATPase activity of the variant displayed an anomalous pH dependence with a down-shift of the pH optimum and a nearly constant rate in the range between pH 7.0 and 8.7.

The Na^+,K^+-ATPase belongs to the family of cation-transporting ATPases for which aspartyl phosphorylation as well as cation occlusion and alternation between two conformational states, E1[1] and E2, are essential aspects of the catalytic mechanism (Glynn & Karlish, 1990). Understanding how the free energy of hydrolysis of ATP is transduced into active exchange of 3 Na^+ for 2 K^+ across the plasma membrane requires knowledge of the structure of the ATP- and cation-binding sites as well as their interactions. Characterization of the structure of the cation sites would help to answer fundamental questions such as (i) whether the same residues bind to Na^+ and K^+ as proposed in the ping-pong sequential transport model, (ii) whether the cation and ATP sites are adjacent or quite separate in the protein structure, (iii) whether the cation sites are charged or neutral, and (iv) which factors confer cation selectivity on the protein.

Since carboxylate groups are likely cation ligands, studies aimed at localization of potential cation binding sites within the Na^+,K^+-ATPase molecule have used reagents which under certain controlled conditions appear to react specifically with the carboxylate groups of glutamic and aspartic acid residues (Shani-Sekler et al., 1988; Arguello & Kaplan, 1991). Carbodiimides have been most extensively used, and several reports have described Na^+- or K^+-protectable inactivation of Na^+,K^+-ATPase by these agents (Gorga, 1985; Pedemonte & Kaplan, 1986; Shani-Sekler et al., 1988). The finding that enzyme substrates such as ATP and Mg^{2+} also are capable of protecting against the inactivation by carbodiimides, along with the tendency of carbodiimides to form cross-links within proteins (Gorga, 1985; Pedemonte & Kaplan, 1986), may, however, argue against the notion of specificity of these agents for cation binding sites.

An intramembranal location of the cation-occluding structure was recently proposed on the basis of studies on the Na^+,K^+-ATPase that combined selective tryptic digestion and chemical modification using dicyclohexylcarbodiimide (DCCD) (Shani-Sekler et al., 1988; Karlish et al., 1990; Goldshleger et al., 1992). The entire mixture of trypsin-digested products, containing a 19-kDa COOH-terminal peptide and five smaller peptides derived from the membrane-bound part of the α chain, retained Rb^+ and Na^+ occlusion capacities similar to those of undigested enzyme. Rb^+ and Na^+ occlusion were inactivated by DCCD with identical rates and first-order dependence on concentration. These data were taken as indication that one or a small number of carboxylate groups in the transmembrane segments had been modified by DCCD and that Na^+ and K^+ might bind to these residues. Very recently these studies were further extended and it was found that each α-subunit contained two separate DCCD binding sites. Glu953 was identified as one of two labeled residues. In a recent mutagenesis study it was, however, shown that cation stimulation of Na^+,K^+-ATPase activity was unaffected by single amino acid substitutions of Glu953 (Van Huysse et al., 1993).

The exact location of the second DCCD site was not determined, but peptides corresponding to the transmembrane

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; E1 or E1-(Na), conformation with high Na^+ and ATP affinities; E2 or E2(K), K^+-occluded conformation with low affinity for ATP; E1P, ADP-sensitive phosphoenzyme intermediate; E2P, K^+-sensitive and ADP-insensitive phosphoenzyme intermediate; kb, kilobase pair; $K_{0.5}$, concentration giving half-maximal activation; MES, 2-(*N*-morpholino)ethanesulfonic acid; M1–M10, putative transmembrane helices numbered from the NH_2-terminal end of the peptide; Na^+,K^+-ATPase, sodium plus potassium-activated adenosinetriphosphatase (EC 3.6.1.3); PCR, polymerase chain reaction; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; T_m, melting temperature.

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hairpin loop M1 plus M2 or M3 plus M4 appeared to be labeled. Glu329 within the highly conserved motif 328-PEGL in the fourth transmembrane segment was suggested as the residue most likely labeled (Goldshleger et al., 1992). This was based on the assumption that DCCD labeling occurs at a carboxyl group and on analogy with the sarcoplasmic reticulum Ca^{2+}-ATPase. Site-directed mutagenesis studies of the latter enzyme have pinpointed six residues within putative transmembrane segments as essential for making up the Ca^{2+}-binding sites, of which four were negatively charged (Clarke et al., 1989, 1990; Andersen & Vilsen, 1992; Vilsen & Andersen, 1992), and Glu309, located at the position homologous to Glu329 in Na^+,K^+-ATPase, was one of these residues. Substitution of Glu309 with either Gln, Ala, or Lys led to Ca^{2+}-ATPase mutant enzymes that were unable to carry out Ca^{2+} transport as well as Ca^{2+}-activated ATP hydrolysis and phosphorylation from ATP, at Ca^{2+} concentrations at least 1000-fold higher than that required for half-maximum saturation of the wild-type enzyme. These data in conjunction with the results of studies of "back-door" phosphorylation from P_i in the Ca^{2+}-ATPase mutants have led to the suggestion that Glu309 provides oxygen ligands for the binding of one of the two Ca^{2+} ions at the high-affinity Ca^{2+} transport sites, and that Glu309 might also play a role in the countertransport of H^+ (Andersen & Vilsen, 1992; Vilsen & Andersen, 1992). Hence, on the basis of extrapolation of the results with Ca^{2+}-ATPase to Na^+,K^+-ATPase, Glu329 of the latter enzyme might provide ligands for the binding of Na^+ and/or K^+.

This paper describes the cloning and functional expression of an allelic variant of the α_1-isoform of Na^+,K^+-ATPase. This variant, identified accidentally in a rat kidney cDNA library, differed from the wild-type enzyme by having a glutamine in place of the glutamic acid residue at position 329. A stable COS-1 cell line expressing the variant was constructed, and surprisingly the functional analysis revealed that the negative charge of Glu329 is not essential to Na^+- and K^+-activated ATPase activity and ion transport. The variant did, however, display several interesting differences from the wild type with respect to the dependence of ATPase activity on the concentrations of Na^+, K^+, H^+, and ATP.

MATERIALS AND METHODS

Cloning of the Variant Na^+,K^+-ATPase. The construction and screening of the rat kidney outer medulla plasmid cDNA library has previously been described in detail (Vilsen, 1992). Total RNA from the kidneys of three rats (Wistar strain, Møllegaard breeding laboratory, Denmark) was used. Approximately 3500 colonies were screened, out of which 42 gave a positive hybridization signal with an oligonucleotide probe defined by the sequence 5'AGTCTTGTCAGAGCAGAT3' derived from the phosphorylation site region of P-type ATPases. Previously, one of these clones was sequenced throughout, and the deduced amino acid sequence was found to be 100% identical with that described as the wild-type rat α_1-isoform of Na^+,K^+-ATPase (Shull et al., 1986). Examination by restriction enzyme analysis of the clones giving positive hybridization signal revealed that their sequences were not all identical. A *Hpa*II site present in the coding sequence in the wild-type Na^+,K^+-ATPase was absent in six of the clones, and by DNA sequencing it was revealed that this difference from the wild type was due to a single G to C base substitution and that no other base substitutions were present.

Expression of the Variant cDNA in COS-1 Cells. The full-length cDNAs encoding wild-type or variant Na^+,K^+-ATPases, carrying *Eco*RI/*Not*I adapters, were cloned into the *EcoRI* site of the expression vector pMT2 (Kaufman et al., 1989). To obtain cell lines with the Na^+,K^+-ATPase cDNA stably integrated into their chromosomes, the cesium chloride gradient-purified plasmids were transfected into COS-1 cells (Gluzman, 1981) by the calcium phosphate procedure. Following a 65-h incubation, ouabain was added to the medium at a final concentration of 5 μM. Individual ouabain-resistant colonies appeared after approximately 3 weeks. At least two colonies from each of six different master dishes were isolated by use of cloning cylinders and expanded into stable cell lines, which were stored in liquid nitrogen.

Isolation of Genomic DNA and Amplification by PCR. PCR was used to verify that the isolated COS-1 cell line contained the cDNA of the exogenous Na^+,K^+-ATPase stably integrated into the genome. Isolation of genomic DNA from the cells was carried out as described by Gross-Bellard et al. (1973), using an extraction buffer containing 0.1 mg/mL proteinase K and 0.5% sodium dodecyl sulfate. The primers used in PCR were designed to obtain an efficient amplification of the exogenous stably integrated cDNA encoding the Glu329 → Gln variant or wild-type Na^+,K^+-ATPase and at the same time avoid amplification of the endogenous COS-1 cell genomic DNA. The sense primer (5'CTGTGTTGAAGGAACTGCAC3') having a T_m of 60 °C was designed to hybridize across the boundary between exons 6 and 7 in the cDNA. The antisense primer (5'TCAGACATACCGTGACGGT3') having a T_m of 58 °C was designed to hybridize across the boundary between exons 7 and 8 in the cDNA. In this way a PCR product of an appropriate size of 290 bp corresponding to exon 7 was expected to be generated from the exogenous Na^+,K^+-ATPase cDNA, whereas the likelihood of obtaining a product from the endogenous genomic DNA (corresponding to exon 7 plus introns 7 and 8) was low. The buffer used in PCR contained 20 mM Tris-HCl (pH 8.3) and 50 mM KCl. In addition, all amplification reactions included 2.0 mM $MgCl_2$, 200 μM each dNTP, 50 pmol of each primer, 2 units of Taq DNA polymerase (Perkin-Elmer Cetus), and 400–600 ng of genomic DNA. The 60-μL reactions were then amplified for 35 cycles in a DNA thermal cycler (ABACUS/Hybaid). To analyze the PCR products, 5–8 μL was combined with 1–1.5 μL of dye (0.25% bromophenol blue/0.25% xylene cyanol/30% glycerol in water) and subjected to nondenaturing electrophoresis on a 5% polyacrylamide gel in 1× TBE (89 mM Tris–borate/89 mM boric acid/2 mM EDTA, pH 8.0). The PCR products were visualized by ethidium bromide fluorescence. The single 290-bp DNA fragment was extracted from the gel, phosphorylated at the 5' blunt ends by use of bacteriophage T_4 polynucleotide kinase (Pharmacia), and inserted into the *Sma*I site of the Bluescript vector (Stratagene, La Jolla, CA) by use of bacteriophage T_4 DNA ligase according to standard procedures (Sambrook et al., 1989). Sequencing was performed using the dideoxynucleotide chain-termination method (Sanger et al., 1977) with Sequenase, as described previously (Vilsen et al., 1989).

Isolation of Plasma Membranes and Assay of Na^+,K^+-ATPase. A crude plasma membrane fraction was prepared as previously described (Vilsen, 1992). Protein concentration was determined by the dye-binding method of Bradford (1976) using bovine serum albumin as standard. To open the vesicles, crude plasma membranes at a protein concentration of 0.25–0.45 mg/mL were incubated with 0.65 mg of sodium deoxycholate/mL, in the presence of 2 mM EDTA and 20 mM imidazole for 30 min at 20 °C. Na^+,K^+-ATPase activity was measured on 25 μL of the leaky membrane solution at 37 °C, essentially as described (Ottolenghi, 1975; Jørgensen

& Petersen, 1982). The rate of ATP hydrolysis was found to be constant over the incubation time (10–20 min) at all concentrations of ouabain, NaCl, KCl, ATP, and pH values tested. To determine the Na^+,K^+-ATPase activity contributed by the transfected wild type or variant rat kidney enzymes, assays were carried out as well in the presence of 10 μM ouabain, which inhibits endogenous COS-1 cell Na^+,K^+-ATPase, as in the presence of 20 mM ouabain, which inhibits all Na^+,K^+-ATPase activity. The ouabain-resistant Na^+,K^+-ATPase activity associated with the expressed exogenous enzyme was calculated by subtraction of the background ATPase activity measured at 20 mM ouabain from the ATPase activity measured at 10 μM ouabain. The background ATPase activity resistant to 20 mM ouabain, usually comprising approximately 20–30% of the total ATPase activity in the crude membrane preparation, was independent of the Na^+ and K^+ concentrations but depended on the pH and the ATP concentration. Therefore, in the pH and ATP concentration dependence studies the Na^+,K^+-ATPase activity was obtained by subtraction of the background activity measured at each individual pH value and ATP concentration. To obtain stable pH values in the studies of the pH dependence of Na^+,K^+-ATPase activity throughout the pH range 6.0–8.7, the following buffers were used: 30 mM MES (titrated with Tris) between pH 6.0 and 7.0, 30 mM TES (titrated with Tris) between pH 7.0 and 8.0, and 30 mM Tris (titrated with HCl) at pH values above 8.0.

In all titrations of Na^+,K^+-ATPase activity, at least three separate membrane preparations corresponding to different clonal isolates of cells transfected with the same cDNA were assayed in duplicate. There was no significant differences between the $K_{0.5}$ values obtained with the different clonal isolates.

Phosphoenzyme Formation. In the standard experiment, 25 μL of the leaky membrane suspension (corresponding to a total of 10 μg of protein) was phosphorylated at 0 °C in 100 μL of a reaction mixture containing 4 μM [γ-^{32}P]ATP, 20 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, and either 150 mM NaCl or 50 mM KCl. By varying the ATP concentration it was found that phosphoenzyme formation in the wild-type and variant Na^+,K^+-ATPases was more than 90% saturated at 4 μM ATP. In some experiments the ouabain-sensitive endogenous Na^+,K^+-ATPase present in the COS-1 cells was inhibited by formation of a stable ouabain-bound complex. This was achieved by preincubation of 25 μL of the leaky membrane suspension with 5 μM ouabain and 3 mM $MgCl_2$ in the absence of Na^+ and K^+ for 30 min at 20 °C, prior to initiation of the phosphorylation reaction under conditions identical to those in the standard experiment described above except for the presence of 5 μM ouabain. The phosphorylation reaction was terminated after 10 s by addition of 1 mL of ice-cold 7% (w/v) trichloroacetic acid containing 1 mM phosphate. The denatured protein was washed twice by centrifugation and subjected to SDS–polyacrylamide gel electrophoresis in 7% acrylamide gels under acid conditions (Vilsen et al., 1989). After the gel was dried, autoradiographs were produced by exposure at −70 °C. Quantitation of the phosphoenzyme was obtained by liquid scintillation counting of slices of the dried gels.

RESULTS

Construction of a Stable Cell Line Expressing the Na^+,K^+-ATPase Variant Glu329 → Gln. I have previously described the isolation of a cDNA encoding the ouabain-resistant rat kidney α_1-isoform of Na^+,K^+-ATPase from a plasmid cDNA library (Vilsen, 1992). Further examination of the library revealed, in addition to this wild-type cDNA, a variant Na^+,K^+-ATPase cDNA containing a single G to C base substitution, which on the amino acid level gave rise to a glutamate to glutamine substitution at position 329 within the motif 328-PEGL in the predicted fourth transmembrane helix. As described in the introduction, Glu329 has recently been attributed a role in cation binding, and it was therefore pertinent to examine whether the Glu329 → Gln variant was able to carry out active transport of Na^+ and K^+ and Na^+,K^+-activated ATP hydrolysis. The cDNAs encoding either the Glu329 → Gln variant or the wild-type rodent Na^+,K^+-ATPase were inserted in the expression vector pMT2 and transfected into COS-1 cells, which were grown in the presence of 5 μM ouabain. This strategy was based on the consideration that 5 μM ouabain preferentially inhibits the ouabain-sensitive Na^+,K^+-ATPase endogenously present in COS-1 cells (Vilsen, 1992). Thus, only COS-1 cells containing the exogenous cDNA encoding the ouabain-resistant rodent Na^+,K^+-ATPase stably integrated in their genome would be able to survive under these selective conditions. On the other hand, survival and sustained growth of the transformed COS-1 cells would require the ouabain-resistant Na^+,K^+-ATPase to be functional. Thus, if the amino acid substitution in the ouabain-resistant rodent enzyme led to a complete block of the Na^+,K^+-ATPase function or severely reduced the pump activity of the enzyme, the mutant, although expressed in the transfected COS-1 cells, would not be able to confer ouabain resistance to the cells, and the cells would die. Hence, failure of the modified Na^+,K^+-ATPase to confer ouabain resistance would indicate that the amino acid substitution was introduced at a critical site in the enzyme, while cell growth would indicate a relatively mild effect or a lack of effect on enzyme function.

Surprisingly, it was found that the Glu329 → Gln variant of the ouabain-resistant rodent Na^+,K^+-ATPase was able to confer ouabain resistance to COS-1 cells, as revealed by the appearance of ouabain-resistant colonies after approximately 2–3 weeks of growth in the presence of 5 μM ouabain. By contrast, no ouabain-resistant colonies were formed when the COS-1 cells were mock-transfected with the expression vector without insert, demonstrating that resistance was not conferred in the absence of the exogenous Na^+,K^+-ATPase cDNA. Establishment of ouabain resistance in COS-1 cells transfected with cDNA encoding the Glu329 → Gln variant clearly indicated that the expressed Glu329 → Gln modified enzyme was functional. Although the growth rate of these transfectants was similar to the growth rate of the transfectants expressing wild-type enzyme, the number of ouabain-resistant colonies appearing on each of the different master dishes was only around 10% that observed on master dishes containing COS-1 cells transfected with wild-type Na^+,K^+-ATPase cDNA. A possible reason is that the Glu329 to Gln amino acid substitution resulted in partial inactivation of the Na^+,K^+ pump function, so that the viability of COS-1 cells expressing the variant enzyme was critically dependent on the expression level. The latter depends on an optimal cDNA copy number and a favorable location in the chromosome, conditions fulfilled only in a fraction of the cells with stably integrated cDNA. As a consequence of the relatively small number of stable colonies formed when COS-1 cells were transfected with cDNA encoding the Na^+,K^+-ATPase Glu329 → Gln variant, it was important to verify that the base substitution encoding the Glu329 → Gln replacement was still present in the cDNA stably integrated into the chromosome of the isolated ouabain-resistant COS-1 cells, after these had been expanded into

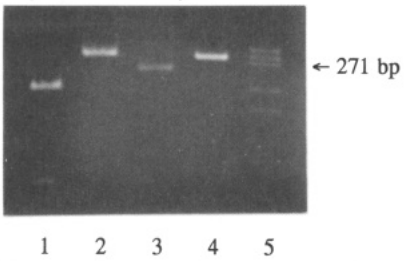

FIGURE 1: PCR amplification and restriction enzyme analysis of the 290-bp cDNA fragment corresponding to exon 7 of the exogenous stably integrated Na^+,K^+-ATPase cDNA. The amplification of genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu329 → Gln variant or wild-type Na^+,K^+-ATPase was carried out as described in Materials and Methods. The PCR products were analyzed on a 5% polyacrylamide gel. Lane 1, *Xho*I digestion of the PCR product amplified from genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu329 → Gln variant Na^+,K^+-ATPase; lane 2, *Hpa*II digestion of the PCR product amplified from genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu329 → Gln variant Na^+,K^+-ATPase; lane 3, *Hpa*II digestion of the PCR product amplified from genomic DNA isolated from COS-1 cells transfected with cDNA encoding the wild-type Na^+,K^+-ATPase; lane 4, undigested PCR product amplified from genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu329 → Gln variant Na^+,K^+-ATPase; lane 5, *Hae*III-digested ϕX174 RF DNA molecular size standard. The arrow points at the 271-bp fragment of the standard.

stable cell lines. To this end, genomic DNA was isolated from the stable cell lines and the polymerase chain reaction (PCR) was applied to amplify the genomic 290-bp cDNA fragment spanning the nucleotide region corresponding to residues Cys244–Leu339 (exon 7 in the genome), which contains the replaced residue. Information about the location of exon–intron boundaries was used to design primers that permitted selective amplification of the stably integrated cDNA free of contamination with endogenous COS-1 cell Na^+,K^+-ATPase genomic DNA (see Materials and Methods). As seen in Figure 1, this strategy worked, since the PCR product was detected as a single ethidium bromide-stained band on the electrophoretic gel, and this DNA fragment displayed exactly the size (290 bp) predicted from the target sequence (Figure 1, lane 4). Not even traces were observed of the larger fragment (introns 7 and 8 plus exon 7) expected if endogenous genomic COS-1 cell DNA had been amplified. The identity of the amplified 290-bp fragment was confirmed by digestion with the restriction enzyme *Xho*I, which cuts the target sequence at a single position, outside the region of the Glu329 to Gln substitution, resulting in two fragments of size 79 and 211 bp (Figure 1, lane 1). Since an endogenous *Hpa*II site was removed by the single G to C base substitution in the Glu329 → Gln variant, digestion with *Hpa*II could be used to reveal the presence of the base substitution. Figure 1 shows that the 290-bp fragment obtained by PCR amplification of genomic DNA isolated from COS-1 cells transfected with cDNA encoding the Glu329 → Gln variant was resistant to digestion with the *Hpa*II enzyme, as indicated by an unchanged mobility of the digested fragment relative to undigested fragment (compare lanes 2 and 4). By contrast, *Hpa*II digestion of the PCR-amplified 290-bp fragment obtained with genomic DNA from cells transfected with cDNA encoding the wild type led to formation of a fragment of size 254 bp, corresponding to the 290-bp fragment from which 36 bp had been removed, in accordance with the wild-type sequence (Figure 1, lane 3). These results tentatively demonstrated that the base substitution encoding the Glu329 to Gln replacement was retained in the cDNA stably integrated into the chromosome of the COS-1 cells. The definitive proof

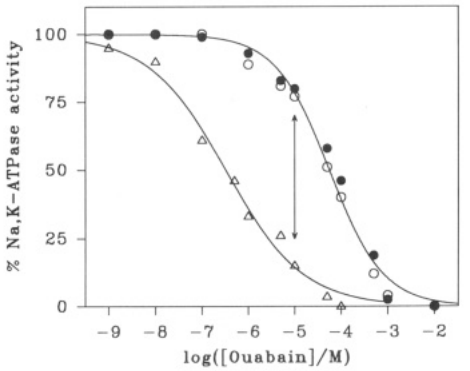

FIGURE 2: Ouabain sensitivity of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells mock-transfected with the PMT2 expression vector without insert and from COS-1 cells transfected with the expression vector containing cDNA encoding either wild type or the Glu329 → Gln variant. The Na^+,K^+-ATPase measurements were carried out in duplicate at 37 °C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, and varying [ouabain]. The average values are presented as percentage of the total Na^+,K^+-ATPase activity measured in the absence of ouabain, as calculated after subtraction of background ATPase activity measured at 20 mM ouabain. The differences between the duplicate measurements were smaller than the size of the symbols. The arrow indicates the maximum difference between the ATPase activities of the ouabain-insensitive exogenous rat Na^+,K^+-ATPase and the ouabain-sensitive COS-1 cell Na^+,K^+-ATPase, corresponding to the concentration of ouabain used in the measurements described in Figures 3, 4, 6, and 7. The specific Na^+,K^+-ATPase activities measured in the absence of ouabain (100% values) were as follows. Mock-transfected COS-1 cells grown in the absence of ouabain (△), 22.7 μmol h^{-1} mg^{-1}; COS-1 cells transfected with wild-type rat cDNA and grown in the presence of ouabain (●), 9.4 μmol h^{-1} mg^{-1}; COS-1 cells transfected with the Glu329 → Gln variant cDNA and grown in the presence of ouabain (○), 10.5 μmol h^{-1} mg^{-1}.

was obtained by sequencing the 290-bp PCR fragment throughout after insertion into the Bluescript vector. The sequence showed the expected single G to C base substitution corresponding to replacement of Glu329 by Gln.

Ouabain Sensitivities of Endogenous and Exogenous Na^+,K^+-ATPase. The Na^+,K^+-ATPase activity was examined in plasma membranes isolated from the stable COS-1 cell lines expressing wild-type rat kidney Na^+,K^+-ATPase or the Glu329 → Gln variant. First it was established that the wild type and the Glu329 → Gln variant possessed similar sensitivities to ouabain (Figure 2). Both of these rat kidney enzymes displayed an apparent affinity for ouabain which was about 500-fold lower than that of the endogenous COS-1 cell Na^+,K^+-ATPase (Figure 2). This result excludes that the functional differences between the wild type and the Glu329 → Gln variant described below were caused by a difference in ouabain sensitivity.

To characterize the Na^+,K^+-ATPase activity contributed by the ouabain-resistant wild-type or the Glu329 → Gln variant rat enzyme in the presence of the endogenous ouabain-sensitive Na^+,K^+-ATPase, ATPase activity measurements were performed in the presence of 10 μM ouabain. As described in a previous report (Vilsen, 1992), at this ouabain concentration the difference between the ATPase activities of the ouabain-sensitive endogenous enzyme and the ouabain-insensitive rat Na^+,K^+-ATPase was near maximal, and the relative contribution of the endogenous enzyme to the activity measured with the plasma membranes of the transfectants was estimated to be no more than 5% under these conditions (Vilsen, 1992). This permitted reliable determinations of the apparent affinities for the cations and ATP of the exogenous Na^+,K^+-ATPase as described below.

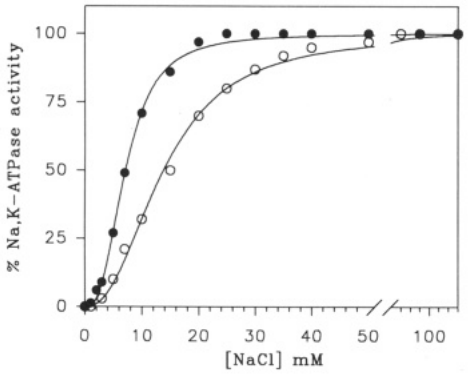

FIGURE 3: Na^+ dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild type or the Glu329 → Gln variant. The Na^+,K^+-ATPase measurements were carried out at 37 °C in the presence of 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, and varying [NaCl] and [choline chloride] (total concentration of [KCl] + [NaCl] + [choline chloride] = 150 mM). The assays were carried out in duplicate, and the average values are presented as percentage of the maximum, as calculated after subtraction of background ATPase activity measured at 20 mM ouabain. The differences between the duplicate measurements were smaller than the size of the symbols. The data were fitted to the relation Na^+,K^+-ATPase activity = $100[Na^+]^n/([Na^+]^n + K_{0.5}^n)$ giving the following respective values for the specific Na^+,K^+-ATPase activities corresponding to 100% and the $K_{0.5}(Na^+)$: wild-type (●), 7.9 μmol h^{-1} mg^{-1}, 7.13 mM; Glu329 → Gln variant (○), 8.3 μmol h^{-1} mg^{-1}, 13.79 mM.

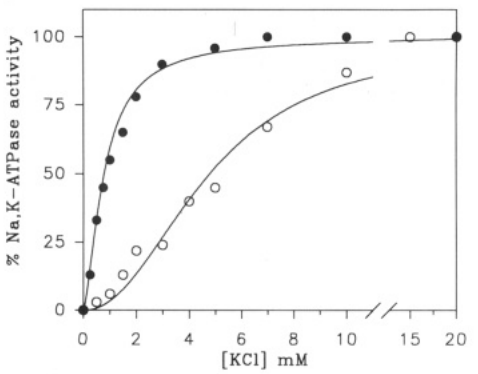

FIGURE 4: K^+ dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild type or the Glu329 → Gln variant. The Na^+,K^+-ATPase measurements were carried out at 37 °C in the presence of 40 mM NaCl, 3 mM ATP, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, and varying [KCl] and [choline chloride] (total concentration of [KCl] + [NaCl] + [choline chloride] = 150 mM). The assays were carried out in duplicate, and the average values are presented as percentage of the maximum, as calculated after subtraction of background ATPase activity measured at 20 mM ouabain. The differences between the duplicate measurements were smaller than the size of the symbols. The data were fitted to the relation Na^+,K^+-ATPase activity = $100[K^+]^n/([K^+]^n + K_{0.5}^n)$, giving the following respective values for the specific Na^+,K^+-ATPase activities corresponding to 100% and the $K_{0.5}(K^+)$: wild type (●), 7.9 μmol h^{-1} mg^{-1}, 0.78 mM; Glu329 → Gln variant (○), 8.3 μmol h^{-1} mg^{-1}, 4.79 mM.

Cation Dependence of Na^+,K^+-ATPase Activity. Figure 3 shows the Na^+,K^+-ATPase activity measured at varying NaCl concentrations on membranes isolated from transfectants corresponding to the Glu329 → Gln variant and the wild-type Na^+,K^+-ATPase. The Glu329 → Gln variant displayed an apparent $K_{0.5}$ for Na^+ of 13.8 mM, while the $K_{0.5}$ for the wild type was 7.1 mM.

In the experiments described in Figure 4 the Na^+,K^+-ATPase activity was titrated as a function of the KCl concentration. The Glu329 → Gln variant exhibited an apparent $K_{0.5}$ for K^+ of 4.8 mM, whereas the $K_{0.5}$ for the wild-type enzyme was 0.8 mM.

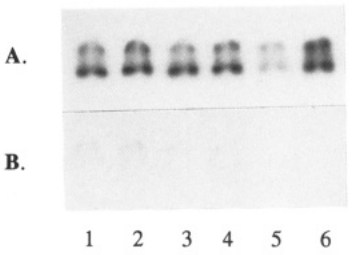

FIGURE 5: Phosphorylation from ATP of plasma membranes isolated from COS-1 cells mock-transfected with the PMT2 expression vector without insert and from COS-1 cells transfected with the expression vector containing cDNA encoding either wild type or the Glu329 → Gln variant Na^+,K^+-ATPase. Phosphorylation was carried out at 0 °C for 10 s in the presence of 20 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, 4 μM [γ-^{32}P]ATP, and either 150 mM NaCl without K^+ (panel A) or 50 mM KCl without Na^+ (panel B). The acid-quenched samples were subjected to SDS–polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels are shown. Each lane was loaded with 7 μg of plasma membrane protein. Lanes 1 and 2, plasma membranes of cells expressing wild-type rat kidney Na^+,K^+-ATPase; lanes 3 and 4, plasma membranes of cells expressing Glu329 → Gln variant Na^+,K^+-ATPase; lanes 5 and 6, plasma membranes of mock-transfected COS-1 cells grown in the absence of ouabain. Prior to phosphorylation the samples were preincubated for 30 min at 20 °C with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) 5 μM ouabain in the presence of 3 mM $MgCl_2$ and the absence of Na^+, K^+, and ATP. The maximum specific Na^+,K^+-ATPase activities of the samples used for phosphorylation were the following: wild type, 8.0 μmol h^{-1} mg^{-1}; Glu329 → Gln variant Na^+,K^+-ATPase, 8.2 μmol h^{-1} mg^{-1}; plasma membranes of mock-transfected COS-1 cells grown in the absence of ouabain, 22.7 μmol h^{-1} mg^{-1}.

Phosphorylation. It is of note that the Glu329 → Gln variant and the wild-type Na^+,K^+-ATPase displayed similar maximum specific activities (micromoles of P_i released per hour per milligram of crude membrane protein measured at saturating Na^+,K^+, and ATP concentrations). There are in principle two possible explanations for this. Either the wild type and the Glu329 → Gln variant were expressed to about identical levels in the COS-1 cells and their maximum molecular turnover numbers were similar or identical, or alternatively, the maximum molecular turnover number was reduced in the variant, but this was compensated for by a corresponding increase in the expression level of the mutant enzyme. To distinguish the correct possibility, the concentrations of active Na^+,K^+-ATPase sites present in the membrane preparations containing wild-type and Glu329 → Gln variant were determined. This was accomplished by phosphorylation experiments in which radioactive [γ-^{32}P]ATP was included under conditions where the dephosphorylation rate is low and phosphorylation is therefore nearly stoichiometric (0 °C, 150 mM Na^+ present, absence of K^+). Since the expressed Na^+,K^+-ATPase constituted such a minute fraction of the total protein present in the COS-cell plasma membranes, several measures were taken to avoid problems due to phosphorylation of proteins other than Na^+,K^+-ATPase. The ATP concentration dependence was titrated to determine the lowest ATP concentration at which the wild type and the Glu329 → Gln variant were saturated, and this ATP concentration (4 μM) was used in the experiments described in Figure 5. The Ca^{2+}-ATPase was inhibited by inclusion of EGTA in the assay to remove traces of Ca^{2+}. Furthermore, the phosphorylated Na^+,K^+-ATPase was separated from minor amounts of other phosphorylated proteins by SDS–polyacrylamide gel electrophoresis under acid conditions as previously described (Vilsen et al., 1989). As seen in Figure

5, all the phosphoprotein migrating corresponding to 100 kDa on the gel was Na^+,K^+-ATPase as judged from its disappearance upon addition of K^+. Importantly, there was no significant difference between the levels of phosphoenzyme obtained with the membranes containing wild-type rat kidney enzyme or the Glu329 → Gln variant, when preparations of similar specific ATPase activities were used. To estimate the fraction of the phosphoenzyme contributed by the ouabain-sensitive endogenous Na^+,K^+-ATPase present in the COS-1 cells, the reaction with radioactive ATP was carried out with and without preincubation with 5 μM ouabain. As expected, phosphorylation was completely prevented by preincubation with ouabain in membranes isolated from cells which had been mock-transfected with the expression vector without insert and maintained in the absence of ouabain, since these cells expressed only the endogenous ouabain-sensitive enzyme. By contrast, the preincubation with ouabain was without significant effect on the phosphoenzyme levels in the membranes isolated from cells expressing the rat kidney wild-type enzyme or Glu329 → Gln variant. This indicates, somewhat unexpectedly, that the endogenous enzyme was inactive or not present at all in these membranes. A likely explanation is that the ouabain-sensitive endogenous enzyme had already formed a stable ouabain-bound complex during propagation of the COS-1 cells in the presence of ouabain in the cell culture. Another possible explanation is that only minute amounts of active endogenous Na^+,K^+-ATPase were expressed due to competition with the exogenous α-chains for the assembly with endogenous β-subunits required for functional maturation and transport of the pumps to the plasma membrane (Geering, 1991). Due to the absence of a significant contribution from endogenous enzyme to the phosphorylation observed with the membranes containing the rat kidney wild-type enzyme or Glu329 → Gln variant, it can be deduced from the data shown in Figure 5 that the latter two preparations contained similar amounts of phosphorylated exogenous enzyme. This information in conjunction with the ATPase activity measurements permits the conclusion that the turnover number of the Glu329 → Gln variant is almost identical to that of the wild type. Rough estimates of 28 600 min^{-1} ± 3200 and 26 900 min^{-1} ± 3150 (average values of four experiments ± SD) for the respective turnover numbers of the Glu329 → Gln variant and the wild type were obtained on the basis of quantitation of the phosphorylation by liquid scintillation counting of gel slices.

ATP Dependence of Na^+,K^+-ATPase Activity. The results of experiments in which the Na^+,K^+-ATPase activity was measured at varying ATP concentrations are shown in Figure 6. It can be seen that the Glu329 → Gln variant displayed a 15-fold higher apparent affinity for ATP relative to wild-type Na^+, K^+-ATPase. It is well-established that the E1 conformation possesses more than 1000-fold higher affinity for ATP compared to the E2 conformation (Nørby & Jensen, 1971; Jensen & Ottolenghi, 1983). Therefore, the increase in the apparent affinity for ATP induced by the Glu329 → Gln substitution may be explained by a displacement of the E1–E2 equilibrium in favor of the E1 conformation.

pH Dependence of Na^+,K^+-ATPase Activity. The pH has a pronounced effect on the turnover of the pump, and depending on the pH, different steps in the pump cycle may limit the overall turnover rate (Forbush & Klodos, 1991). Furthermore, protons (or more likely H_3O^+) can act as surrogate Na^+ ions at low pH and low Na^+ conditions (Blostein & Polvani, 1991). It was therefore of interest to compare the pH dependencies

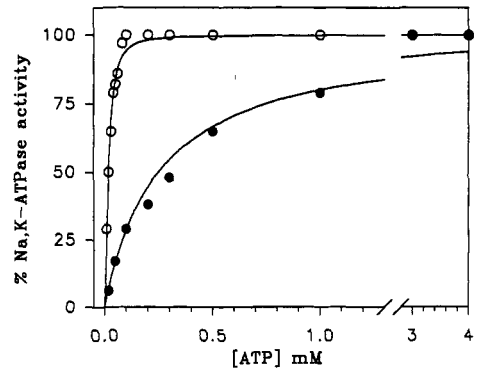

FIGURE 6: ATP dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild type or the Glu329 → Gln variant. The Na^+,K^+-ATPase measurements were carried out at 37 °C in the presence of 130 mM NaCl, 20 mM KCl, 0.1–5 mM $MgCl_2$ (keeping $[Mg^{2+}]$ constant), 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, and varying [ATP]. The assays were carried out in duplicate, and the average values are presented as percentage of the maximum, as calculated after subtraction of background ATPase activity measured at 20 mM ouabain at the same ATP concentrations. The differences between the duplicate measurements were smaller than the size of the symbols. The data were fitted to the relation Na^+,K^+-ATPase activity = $100[ATP]^n/([ATP]^n + K_{0.5}^n)$, giving the following respective values for the specific Na^+,K^+-ATPase activities corresponding to 100% and $K_{0.5}$(ATP): wild type (●), 7.6 μmol h^{-1} mg^{-1}, 0.279 mM; Glu329 → Gln variant (○), 8.1 μmol h^{-1} mg^{-1}, 0.019 mM.

of the maximum turnover rates of the Glu329 → Gln variant and the wild type (Figure 7A). For comparison, pH titration was in addition carried out on the previously characterized mutant Pro328 → Ala (Figure 7B), which like the Glu329 → Gln variant displayed reduced apparent affinities for Na^+ and K^+ and an increased affinity for ATP (Vilsen, 1992). From the results shown in Figure 7 it can be seen that the pH dependence of the expressed rat-kidney wild-type enzyme had the classic bell-shaped appearance, with a maximum between pH 7.4 and 8.0 (Skou, 1957; Forbush & Klodos, 1991). Such a bell-shaped pH titration curve was also found for the Pro328 → Ala mutant and reflects the involvement during the enzyme cycle of at least two groups with different pK values, one titrating between pH 5 and 7 and another with a pK around 9. Surprisingly, the pH titration curve representing the Glu329 → Gln variant did not display the normal bell-shaped form. The turnover rate of the Glu329 → Gln variant was almost unaffected in the alkaline pH range, decreasing only from 100% at pH 7.0 to around 94% at pH 8.7. By contrast, the turnover rate dropped to 66% and 53% in the wild-type enzyme and the Pro328 → Ala mutant, respectively. This indicates that the group titrated in the higher pH range did not exist in the Glu329 → Gln variant or that its titration did not affect the turnover of the variant. In the lower pH range the activity of the Glu329 → Gln variant was also enhanced relative to that of the wild type. The resulting pH dependence of the variant showed an optimum which was displaced toward lower pH values relative to that of the bell-shaped curve of the wild type. At low pH, the E2(K) → E1(Na) transition is known to be rate-limiting for the overall turnover rate in the wild-type enzyme. Therefore, the observed difference between the Glu329 → Gln variant and the wild type in the lower pH range may reflect an acceleration of the E2(K) → E1(Na) conversion (Skou, 1982). This would be in line with the finding described above that the Glu329 → Gln variant displayed a higher apparent affinity for ATP than the wild-type enzyme (Figure 6). A less pronounced down-

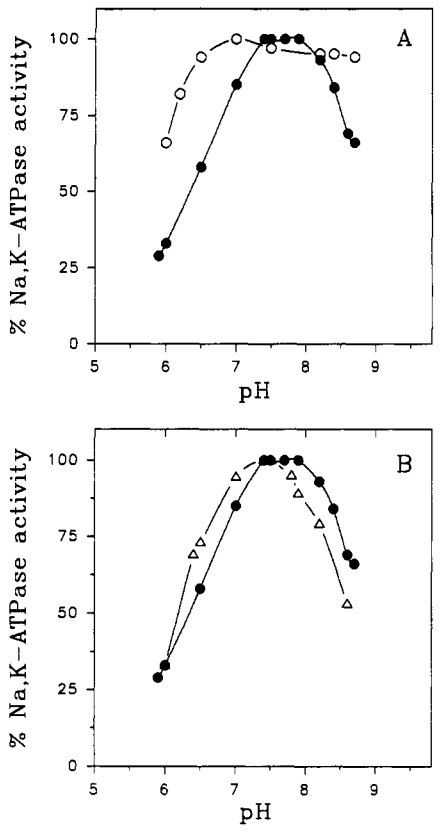

FIGURE 7: pH dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild-type Na^+,K^+-ATPase, the Glu329 → Gln variant, or the Pro328 → Ala mutant. The Na^+,K^+-ATPase measurements were carried out at 37 °C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, 10 μM ouabain, and 30 mM MES/Tris (pH 6.0–7.0), TES/Tris (pH 7.0–8.0), or Tris-HCl (pH 8.0–8.7). The assays were carried out in duplicate, and the average values are presented as percentage of the maximum Na^+,K^+-ATPase activity calculated after subtraction of background ATPase activity measured at 20 mM ouabain at the same pH. The differences between the duplicate measurements were smaller than the size of the symbols. (A) Wild-type Na^+,K^+-ATPase and the Glu329 → Gln variant. (B) Wild-type Na^+,K^+-ATPase and the Pro328 → Ala mutant. The specific Na^+,K^+-ATPase activities corresponding to 100% were the following: wild-type Na^+,K^+-ATPase (●), 7.6 μmol h^{-1} mg^{-1}; Glu329 → Gln variant (○), 8.1 μmol h^{-1} mg^{-1}; Pro328 → Ala mutant (Δ), 7.7 μmol h^{-1} mg^{-1}.

shift of the pH optimum was observed for the Pro328 → Ala mutant (Figure 7B), which also displayed a less pronounced increase in apparent affinity for ATP (Vilsen, 1992).

DISCUSSION

In the present study it has been demonstrated that the glutamic acid residue Glu329 is not an essential residue for the active transport of Na^+ and K^+ and Na^+,K^+-activated ATP hydrolysis by the Na^+,K^+-pump. Glu329 is located within the motif 328-PEGL in the predicted transmembrane segment M4 and has previously been pinpointed as a potential cation binding residue by the results of chemical modification and by extrapolation to the Na^+,K^+-ATPase of results with the sarcoplasmic reticulum Ca^{2+}-ATPase (see the introduction). The conclusion that Glu329 is nonessential for the function of the Na^+,K^+-pump was drawn on the basis of the finding that COS-1 cells transfected with exogenous cDNA encoding the Glu329 → Gln variant of the ouabain-resistant rat α_1-isoform of Na^+,K^+-ATPase were able to form ouabain-resistant colonies, of which several were expanded with success into stable viable cell lines in the presence of a concentration of ouabain which is highly cytotoxic to the parental COS-1 cells containing only the endogenous ouabain-sensitive isoform of Na^+,K^+-ATPase. By polymerase chain reaction amplification and DNA sequencing it was unambiguously documented that the Glu329 to Gln substitution was still intact in the nucleotide sequence of the genome of the transfectants after several months of maintenance in the presence of ouabain. In the presence of saturating substrate concentrations, the plasma membranes harvested from the cell lines expressing the variant displayed a maximum specific Na^+,K^+-ATPase activity very similar to that of the wild-type enzyme, and the measurement of site concentration by phosphorylation with ATP showed that the expression levels of the Glu329 → Gln variant and the wild type were indistinguishable. This permits the conclusion that their maximum molecular turnover numbers were similar if not identical.

All the Na^+,K^+-ATPase activity measurements were carried out on leaky plasma membranes opened up by treatment with sodium deoxycholate, and thus equal concentrations of the cations were present on the two sides of the membrane. It is well-established that the apparent affinities for cytoplasmic Na^+ and extracellular K^+ can be studied with this system, due to the large difference between the Na^+–K^+ affinity ratios on the two sides of the membrane, which allows Na^+ to bind preferentially at the cytoplasmic sites and K^+ to bind preferentially at the extracellular sites when both ions are present (Skou, 1990). Despite the fact that the Glu329 → Gln variant was functional, it did not respond to variation of the concentrations of Na^+, K^+, and ATP in the same way as the wild-type enzyme. The Glu329 → Gln variant displayed a 15-fold increase in apparent affinity for ATP relative to the wild type and a reduced apparent affinity for either of the cations corresponding to a 2-fold increase in $K_{0.5}$ for cytoplasmic Na^+ and a 6-fold increase in $K_{0.5}$ for extracellular K^+. The increased apparent affinity for ATP displayed by the Glu329 → Gln variant suggests that the Glu to Gln substitution displaced the E1–E2 equilibrium in favor of the E1 conformation possessing high affinity for ATP (Nørby & Jensen, 1971; Jensen & Ottolenghi, 1983). In further support of this conclusion was the finding that the Glu329 → Gln variant displayed a pronounced change in the pH optimum toward a lower pH value (Figure 7A), a change which was associated with a higher turnover rate in the Glu329 → Gln variant relative to the wild-type enzyme at low pH conditions, where the E2(K) → E1(Na) conversion is known to be rate-limiting in the wild-type enzyme. This is analogous to the change in pH optimum induced by an increase in the ATP concentration (Karlish & Yates, 1978; Skou, 1979) as well as by other means that displace the equilibrium between the E2(K) form and the E1(Na) form in favor of E1(Na) (Skou, 1982). The finding that the Glu329 → Gln variant exhibited a 2-fold reduced affinity for Na^+, however, cannot be understood by reference to the conformational equilibrium, since the displacement in favor of E1 would tend to increase the apparent affinity for cytoplasmic Na^+. It is necessary, then, to postulate that in addition to the displacement of the equilibrium in favor of the E1 conformation the intrinsic affinity of the E1 conformation for Na^+ was reduced in the Glu329 → Gln variant. Hence, it seems that the Glu329 → Gln substitution disturbed one or more of the high-affinity cytoplasmic Na^+ sites. The reduction of intrinsic affinity may have amounted to more than 2-fold, since the observed decrease in apparent affinity may be the net effect resulting from a combination of reduced intrinsic affinity with the displacement of the conformational equilibrium toward the

Na^+ form. It is significant, however, that replacement of Glu329 had such a small effect on the apparent Na^+ affinity. An effect of similar magnitude was observed in a previous study, in which the juxtaposed residue Pro328 was substituted with Ala (Vilsen, 1992). In the sarcoplasmic reticulum Ca^{2+}-ATPase the effect of replacing Pro308, homologous to Pro328 in Na^+,K^+-ATPase, was likewise a 2–3-fold reduction in apparent Ca^{2+} affinity (Vilsen et al., 1989), but the replacement of Glu309 in Ca^{2+}-ATPase (homologous to Glu329 in Na^+,K^+-ATPase) with Gln resulted in a more than 1000-fold reduction in apparent Ca^{2+} affinity for one of the two bound Ca^{2+} ions (Clarke et al., 1989; Andersen & Vilsen, 1992). Therefore, the role played by Glu329 in Na^+,K^+-ATPase is not as crucial as the role played by the homologous residue in Ca^{2+}-ATPase.

Although the effect of the Glu329 → Gln substitution on Na^+ affinity is limited, the data are not totally inconsistent with a direct role of Glu329 in Na^+ binding. If in analogy with ionophores and protein cation-binding sites of known structure it is assumed that a Na^+ binding site consists of as many as six to eight oxygen-containing ligands (Eisenman & Dani, 1987), the removal of one or two of these ligands would not necessarily be expected to disrupt the site. On the other hand, one might speculate whether the negative charge of Glu329 would make this residue more crucial to cation binding than neutral oxygen ligands. It has been proposed that the Na^+,K^+-ATPase possesses a cation binding pocket consisting of a total of only two negative charges (Glynn & Karlish, 1990). In this connection it is instructive to compare the distribution of partial charges on oxygen ligands of those groups which most likely donate electrons to cation binding, such as carboxylate, carboxamide, and main-chain carbonyl. In the isolated state, a carboxylate group has its total charge of –1 divided equally between its two oxygen atoms. Due to its electronegativity, the oxygen atom of a carboxamide group has a comparable partial charge of approximately –0.3, and the partial charge of a main-chain carbonyl oxygen atom is about –0.4 (Rogers, 1989). Hence, if only one of the two oxygen atoms of the carboxylate group of Glu329 is involved in cation binding, the loss of partial charge from the cation coordination sphere resulting from the Glu → Gln substitution could be smaller (–0.2) than the loss resulting from removal of a "neutral" main-chain carbonyl group. A more pronounced reduction in apparent charge (reduction from –1.0 to –0.3) in the coordination sphere could, on the other hand, be the result of the Glu329 → Gln substitution, if each of the two oxygen atoms in the carboxylate group of Glu329 contributes to the coordination of the ion. The exact magnitude of the apparent charges would depend on the local environment in the protein. Of particular importance are the number of hydrogen bonds formed between the oxygen atoms and the main-chain or side-chain NH groups and the distances between the oxygen atoms and the cation. On the basis of these considerations it seems reasonable to speculate that the loss of charge from the cation coordination sphere resulting from the Glu329 → Gln substitution might be moderate.

Very recently, Karlish and co-workers have used heavily trypsinized Na^+,K^+-ATPase enzyme to show that cation occlusion is prevented by modification of one or both of two residues in the membrane domain with DCCD (see the introduction). One of these residues was located in a tryptic fragment presumed to contain the putative transmembrane segments M3 and M4, and Glu329 was suggested as the most likely candidate. While the present study shows that Na^+ and K^+ binding take place in the Glu329 → Gln variant, it does not exclude that the residue modified by DCCD was Glu329. The bulky DCCD molecule might have disturbed ion binding and occlusion, even if the modified residue were located at the periphery of the cation-binding pocket without being an essential component.

A possibility which also should be considered is that the binding site for one of the three sodium ions was completely disrupted in the Glu329 → Gln variant without serious disturbance of the binding and transport of the remaining two sodium ions. That this might be realistic is indicated by experiments demonstrating transport stoichiometries of $2H^+/2K^+$ and $(1Na^+ + 1H^+)/2K^+$ instead of the usual $3Na^+/2K^+$ stoichiometry at very low Na^+ and high H^+ concentrations (Blostein & Polvani, 1991). These and other data seem to indicate that the Na^+,K^+-ATPase can adapt to variable cation conditions by different modes of exchange (Cornelius, 1991). It is plausible that protonation reduces the number of Na^+ ions bound to the cation sites, and one might speculate whether protonation of the enzyme is imitated by the removal of a negative charge in the Glu329 → Gln variant. The data obtained with the Glu329 → Gln variant cannot exclude the possibility that the variant catalyzed an electroneutral $2Na^+/2K^+$ exchange or an electrogenic $2Na^+/1K^+$ exchange. Hence, in the future it would be of interest to investigate whether the Glu329 → Gln variant enzyme mediates an altered charge transfer relative to the wild-type enzyme.

A striking finding was that the maximum turnover rate in the Glu329 → Gln variant was almost unaffected by varying pH between 7 and 8.7, while in the wild type the turnover rate began to decrease above pH 8.0 and was only 66% at pH 8.7 (Figure 7A). By contrast, in the previously characterized Pro328 → Ala mutant (Vilsen, 1992), which in many other functional aspects resembled the Glu329 → Gln variant, the activity decreased with increasing pH in a way similar to that of the wild type (Figure 7B). In the isolated state of the amino acids, the ϵ-amino group of lysine and the α-amino groups titrate at pH values above 7.5, whereas carboxylate groups titrate below pH 5. However, when carboxylate groups and amino groups are buried within the protein structure they may titrate with anomalously high or low pK values depending on the local environment. Assuming that Glu329 is located within a hydrophobic region in Na^+,K^+-ATPase and that such a location leads to an increase in the pK of Glu329 relative to its isolated state, due to destabilization of the anionic form of the carboxylate group, the simplest explanation of the present data would be that Glu329 is the residue whose deprotonation is responsible for the decrease in activity observed with the wild-type enzyme at pH values above 8.0. Another possibility is that the titration of an amino group is responsible for the decrease in enzyme activity in the wild-type enzyme. Maximal Na^+,K^+-ATPase activity may require that Glu329 is neutralized by formation of a salt bridge with the protonated amino group. An increase in pH above 8.0 would lead to deprotonation of the amino group resulting in a weakening or breaking of the salt bridge, thereby leaving the negative charge of Glu329 unshielded.

Experiments with purified enzyme have led to the proposal that at high pH values the Na^+,K^+-ATPase activity is limited by the steps involved in the Na^+-translocating E1 → E1P → E2P transition (Forbush & Klodos, 1991). It is possible that to maintain the turnover rate at maximum, the residue at position 329 may in one or some of the partial steps in the E1 → E1P → E2P transition have to be moved into a hydrophobic region in the protein by a conformational change. In the wild-type enzyme, a key event in this respect might be the

neutralization of the negative charge of Glu329, either directly through protonation of its carboxylate group or indirectly through salt bridge formation. In the Glu329 → Gln variant there is less negative charge to be neutralized, explaining why the enzyme activity of the Glu329 → Gln variant remained high at alkaline pH.

The Glu329 → Gln variant exhibited a 6-fold reduction in apparent affinity for extracellular K^+. There are several possible explanations for this finding, none of which are mutually exclusive. The reduced K^+ affinity may be related to the displacement of the E1–E2 equilibrium in favor of E1 revealed by the increase in the apparent affinity for ATP. Hence the rate of E2(K) → E1(Na) transformation may have been enhanced in the Glu329 → Gln variant. This would be consistent with the higher overall turnover rate of the variant relative to the wild-type enzyme at low pH values, where the E2(K) → E1(Na) transformation is rate-limiting in the wild type. Assuming the consecutive reaction sequence of the Post–Albers scheme to hold and the rate of the E2(K) → E1(Na) transition to be enhanced due to the Glu329 → Gln substitution, an increase in $K_{0.5}$ for K^+ binding at the extracellular activatory K^+ sites would be expected for kinetic reasons. This is analogous to the effect on the apparent affinity for external K^+ caused by ATP-induced acceleration of the E2(K) → E1(Na) transition (Eisner & Richards, 1981).

Glu329 might also be directly involved in K^+ binding at the high-affinity extracellular K^+ sites. A direct involvement of one or both side-chain oxygens of Glu329, not only in Na^+ binding but also in K^+ binding, would be in line with the hypothesis that the Na^+ and K^+ sites are composed of the same ligands interacting consecutively with the ions during the transport cycle (Glynn & Karlish, 1990). A destabilization of the interaction between the enzyme and K^+ in the Glu329 → Gln variant would also be in accordance with the acceleration of the E2(K) → E1(Na) transition discussed above, since the rate-limiting step in this transition is thought to be the deocclusion of K^+.

Recently, it was shown that the apparent affinity for K^+ decreases when lipophilic cations such as TPP^+ (tetraphenylphosphonium) are allowed to adsorb to the membrane (Läuger, 1991; Klodos & Plesner, 1992). The inclusion of TPP^+ in the membrane may be considered analogous to the removal of negative charge by the Glu329 → Gln substitution, since in either case there was a net increase in positive charge in the membrane phase, which interfered selectively with K^+ binding at the externally facing sites. From the voltage dependence of cation binding it has been suggested that some of the putative transmembrane helices form a narrow access channel connecting the K^+ occlusion sites to the external membrane surface. The existence of such a channel is in line with the sequential release of occluded K^+ or Rb^+ to the exterior (Glynn et al., 1985; Forbush, 1987). The reduced apparent affinity for K^+ displayed by the Glu329 → Gln variant and by the enzyme in TPP^+-doped membranes may be explained by a common mechanism in which the passage of the potassium ions from the external membrane surface through the access channel to the occlusion sites is hindered by electrostatic effects due to the presence of excess positive charge in the membrane. The electrostatic effects may be superimposed on effects resulting from a direct involvement of Glu329 in cation binding, providing an additional explanation of why the Glu329 → Gln substitution had a more pronounced influence on the apparent K^+ affinity compared to its influence on apparent Na^+ affinity. There seems to be no evidence of a low conductance access channel for migration of Na^+ between the cytoplasmic membrane surface and the Na^+ occlusion sites, and therefore electrostatic effects are less significant modulators of Na^+ binding at the cytoplasmic sites than of K^+ binding at the extracellular sites.

Further studies are under way in this laboratory to reveal whether one or more of the proposed explanations of the reduced cation affinities of the Glu329 → Gln variant is correct. It will also be of interest to examine whether animals homozygous for the Glu329 → Gln replacement are viable, as might be speculated on the basis of the present results with the transformed cells.

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