Volume 314, number 3, 301–307 FEBS 11886 December 1992

Functional consequences of alterations to Pro^{328} and Leu^{332} located in the 4th transmembrane segment of the α-subunit of the rat kidney Na^+,K^+-ATPase

Bente Vilsen

Danish Biomembrane Research Centre, Institute of Physiology, University of Aarhus, DK-8000 Aarhus C, Denmark

Received 5 October 1992

Site-specific mutagenesis was used to analyse the functional roles of the residues Pro^{328} and Leu^{332} located in the conserved PEGLL motif of the predicted transmembrane helix M4 in the α_1-subunit of the ouabain resistant rat kidney Na^+,K^+-ATPase. cDNAs encoding either of the Na^+,K^+-ATPase mutants Pro^{328}→Ala and Leu^{332}→Ala, and wild type, were cloned into the expression vector pMT2 and transfected into COS-1 cells. Ouabain-resistant clones growing in the presence of 10 μM ouabain were isolated, and the Na^+, K^+, ATP and pH dependencies of the Na^+,K^+-ATPase activity measured in the presence of 10 μM ouabain were analysed. Under these conditions the exogenous expressed Na^+,K^+-ATPase contributed more than 95% of the Na^+,K^+-ATPase activity. The Pro^{328}→Ala mutant displayed a reduced apparent affinity for Na^+ ($K_{0.5}$ (Na^+) 13.04 mM), relative to the wild type ($K_{0.5}$ (Na^+) 7.13 mM). By contrast, the apparent affinity for Na^+ displayed by the Leu^{332}→Ala mutant was increased ($K_{0.5}$ (Na^+) 3.92 mM). Either of the mutants exhibited lower apparent affinity for K^+ relative to the wild type ($K_{0.5}$ (K^+) 2.46 mM for Pro^{328}→Ala and 1.97 mM for Leu^{332}→Ala, compared with 0.78 mM for wild type). Both mutants exhibited higher apparent affinity for ATP than the wild type ($K_{0.5}$ (ATP) 0.086 mM for Pro^{328}→Ala and 0.042 mM for Leu^{332}→Ala, compared with 0.287 mM for wild type). The influence of pH was in accordance with an acceleration of the E2(K)→E1 transition in the mutants relative to the wild type. These data are consistent with a role of Pro^{328} and Leu^{332} in the stabilization of the E2 form and of Pro^{328} in Na^+ binding. The possible role of the mutated residues in K^+ binding is discussed.

Na^+,K^+-ATPase; Amino acid substitution; E1–E2 equilibrium; Proline; Cation binding site

1. INTRODUCTION

The Na^+,K^+-ATPase belongs to the family of transport proteins that couple ion translocation across cell membranes to the hydrolysis of ATP through the formation of a phosphorylated intermediate ('P-type ATPases'). Other members of this family include the Ca^{2+}-ATPase of sarcoplasmic reticulum, the H^+,K^+-ATPase of gastric mucosa, the plasma membrane Ca^{2+}-ATPases, and the H^+-ATPase of fungi. The hydropathy profiles of these proteins are similar and eight to ten putative transmembrane hydrophobic segments appear to be a well-conserved feature for all the ionmotive P-type ATPases [1,2]. Although the P-type ATPases have been studied extensively, many questions concerning the mechanism of ion transport, ATP hydrolysis, and energy transduction remain unanswered. To provide further insight into the mechanism of cation translocation by these proteins it is essential to identify the amino acid residues involved in cation binding and in the protein conformational changes controlling rearrangements in the cation binding sites and the catalytic site during the transport process. An intramembranal location of the cation binding residues was recently proposed in a study that combined selective tryptic digestion of the Na^+,K^+-ATPase with measurement of cation occlusion [3]. Work based on site-directed mutagenesis on sarcoplasmic reticulum Ca^{2+}-ATPase has attributed the cation-binding sites to two prolines and six polar oxygen ligands within predicted transmembrane segments M4–M6 and M8 [4–6], and energy coupling between the cation sites and the phosphorylation site seems to depend on residues located near the cytoplasmic border of M4 [4,7].

In this study site-specific mutagenesis has been used to analyse the roles of the residues Pro^{328} and Leu^{332} in the motif 328-PEGLL predicted to lie within the 4th transmembrane segment in the rat kidney α_1-isoform of the Na^+,K^+-ATPase, by substitution of either of these residues with Ala. Pro^{328} is conserved throughout the family of P-type ATPases [2], whereas Leu^{332} is conserved only in the most closely related H^+,K^+-ATPase.

Correspondence address: Bente Vilsen, Institute of Physiology, University of Aarhus, Ole Worms Allé 160, DK-8000 Aarhus C, Denmark.

Abbreviations: E1, 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; EGTA, ethylene-bis(oxyethylene-nitrilo)tetraacetic acid; $K_{0.5}$, concentration giving half maximal activation; M1–M10, the putative transmembrane helices numbered from the NH_2-terminal end of the peptide; Na^+,K^+-ATPase, sodium plus potassium-activated adenosine triphosphatase (EC 3.6.1.3); n_H, Hill coefficient.

The residue Glu^{329} in the PEGLL motif is homologous to one of the 4 carboxylic residues proposed as Ca^{2+} ligands in the sarcoplasmic reticulum Ca^{2+}-ATPase [5,6].

The functional properties of the Pro^{328}→Ala and Leu^{332}→Ala mutants were consistent with a role of Pro^{328} in Na^+ binding and of Pro^{328} as well as Leu^{332} in the E2–E1 conformational change.

2. MATERIALS AND METHODS

2.1. *Construction and screening of a rat kidney outer medulla plasmid cDNA library*

Total RNA from kidney outer medulla of male and female rats (Wistar strain, Møllegaard breeding laboratory, Denmark) was isolated by the guanidinium isothiocyanate procedure as described by Sambrook et al. [8]. RNA purification, cDNA synthesis and isolation were carried out as described previously [9]. The cDNA was ligated into the *Eco*RI site in the multiple cloning region of the Bluescript vector (Stratagene, La Jolla, CA). The Bluescript plasmid library was screened with an end-^{32}P-labeled oligonucleotide probe defined by the sequence 5'AGTCTTGTCAGAGCAGAT3' derived from the highly conserved phosphorylation site region of the P-type ATPases [9]. Approximately 3,500 colonies were screened, out of which 42 colonies gave a positive hybridization signal, and 12 of these were carried through 3 purification steps. Nucleotide sequencing [10] of the 5'-ends and 3'-ends revealed that these clones were identical in size and contained a full-length cDNA sequence encoding the Na^+,K^+-ATPase α_1-isoform. The coding sequence was flanked by a 143 nucleotide 5'-untranslated sequence and a 85 nucleotide 3'-untranslated sequence, both of which showed 100% identity with the brain α_1 nucleotide sequence [11]. One of the clones was sequenced throughout, and the deduced amino acid sequence of the coding region was 100% identical with that described previously [11].

2.2. *Site-directed mutagenesis*

Oligonucleotide-directed site-specific mutagenesis was carried out as described previously [4]. Base substitutions corresponding to the mutations Pro^{328}→Ala and Leu^{332}→Ala were introduced into a 586 base pair *Xho*I/*Bam*HI restriction fragment, which had been excised from the full-length cDNA encoding the rat kidney α_1-isoform of Na^+,K^+-ATPase and subcloned into the Bluescript vector. Mutant cDNA clones were sequenced [10] to verify that mutations were appropriate and to ensure that no undesired mutations or deletions had occurred. The mutated *Xho*I/*Bam*HI fragments were excised from the Bluescript vector and subsequently religated back into the original position in the full-length clone for reconstruction of full-length mutant cDNAs.

2.3. *Expression of the full-length cDNA in COS-1 cells*

The full-length cDNAs encoding wild type- or mutant Na^+,K^+-ATPases, carrying *Eco*RI/*Not*I adaptors, were cloned into the *Eco*RI site of the expression vector pMT2 [12]. 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 [13] by the calcium phosphate procedure. Following 65 h incubation, ouabain was added to the medium at a final concentration of 10 μM. Individual ouabain resistant colonies appeared after approximately 3 weeks. At least 2 colonies from each of 6 different master dishes were isolated by use of cloning cylinders and expanded into stable cell lines, which were stored in liquid nitrogen. The functional measurements described below were carried out on at least 3 different clonal isolates for each mutant and wild type.

2.4. *Isolation of plasma membranes and assay of Na^+,K^+-ATPase*

A crude plasma membrane fraction was prepared as follows. Six confluent 65 cm^2 tissue Petri dishes were washed twice with 5 ml phosphate-buffered saline ('PBS': 150 mM NaCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, 2.63 mM KCl). The cells were harvested at 20°C in a solution of 5 mM EDTA in PBS and then washed once with 5 ml of PBS at 0°C. The cells were lysed in 4 ml of a solution of 1 mM $NaHCO_3$ (pH 7.8), 2 mM $CaCl_2$, and 5 mM $MgCl_2$ ('lysis buffer') at 4°C for 10 min and then homogenized with 40 strokes in a glass Dounce homogenizer. After centrifugation for 5 min at 3,000 × g, the supernatant was isolated and stored at 0°C, while 4 ml of the lysis buffer was added to the pellet, which still contained some intact cells, cell debris and nuclei. The pellet was treated once again as described above except that the centrifugation was carried out at 1,500 × g. The two supernatants were combined and stirred for 10 min at 0°C with an equal volume of a solution of 2 M NaI, 20 mM EDTA, 5 mM $MgCl_2$, and 100 mM Tris (pH 8.3), followed by dilution 1:1 with 10 mM Tris (pH 7.4), 1 mM EDTA [14]. The plasma membranes were then pelleted by centrifugation at 48,000 × g for 45 min, and subsequently washed three times with 10 mM Tris, 1 mM EDTA (pH 7.4). Protein concentration was determined by the dye binding method of Bradford [15] using bovine serum albumin as a 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 [16,17]. 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 mutant rat enzymes, assays were carried out in the presence of as well 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 ATPase activity measured at 20 mM ouabain from that measured at 10 μM ouabain. The 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 ATP concentration. Therefore, in the ATP-concentration dependence studies the Na^+,K^+-ATPase activity was obtained by subtraction of a background activity measured at each individual ATP concentration.

3. RESULTS

To obtain COS-1 cell lines containing wild-type or mutant rat α_1-isoform Na^+,K^+-ATPase cDNA integrated into their chromosomes, the 1,000-fold difference between the ouabain sensitivities of the rat α_1-isoform of Na^+,K^+-ATPase and the endogenous enzyme in COS-1 cells (Fig. 1) was exploited to isolate stable transfectants by growing the transfected cells under selective conditions in 5–10 μM ouabain, which preferentially inhibited the endogenous Na^+,K^+-ATPase [18]. It was found that the COS-1 cells transfected with either wild-type or mutant cDNA were able to form ouabain-resistant colonies after 2–3 weeks of growth in the presence of 10 μM ouabain, whereas no ouabain-resistant colonies were formed, when the COS-1 cells were transfected with the expression vector without insert. The ability of transfected COS-1 cells to grow in the presence of 10 μM ouabain clearly demonstrated the expression of the ouabain-resistant Na^+,K^+-ATPase of wild-type and mutant rat α_1-isoform in the transfec-

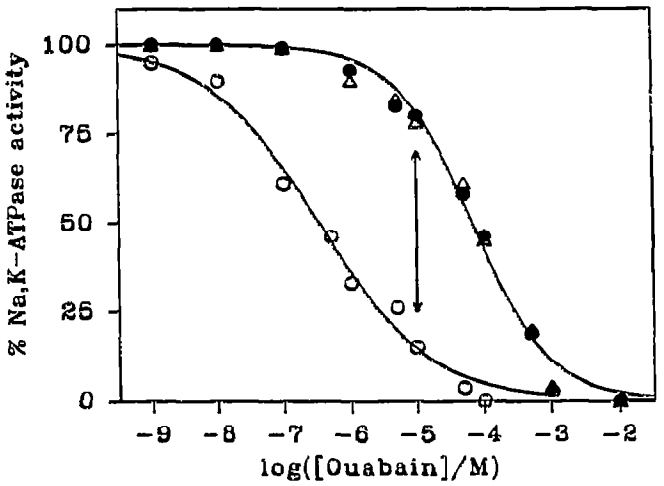

Fig. 1. Ouabain sensitivity of Na^+,K^+-ATPase activity of plasma membranes isolated from untransfected COS-1 cells and from COS-1 cells transfected with either wild-type or mutant cDNA. The Na^+,K^+-ATPase measurements were carried out at 37°C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, and varying [ouabain]. At least three separate membrane preparations corresponding to different clonal isolates of cells transfected with the same cDNA were assayed in duplicate, and 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 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 Figs. 2–4 and Table I. The specific Na^+,K^+-ATPase activities measured in the absence of ouabain (100% values) were as follows. Untransfected COS-1 cells grown in the absence of ouabain (○), 22.7 μmol/h/mg; COS-1 cells transfected with wild-type rat α_1-isoform cDNA and grown in the presence of ouabain (●), 8.9 μmol/h/mg; COS-1 cells transfected with the Pro^{328}→Ala mutant or the Leu^{332}→Ala mutant cDNA and grown in the presence of ouabain (△), 10–11 μmol/h/mg.

tants. The ouabain sensitivities of the wild-type and mutant rat α_1-isoforms were indistinguishable (Fig. 1).

To characterize the Na^+,K^+-ATPase activity contributed by the ouabain-resistant wild-type or mutant rat α_1-isoform in the presence of the endogenous ouabain-sensitive Na^+,K^+-ATPase enzyme, the ATPase activity measurements were performed in the presence of 10 μM ouabain. As indicated by the arrow in Fig. 1, at this ouabain concentration the difference between the ATPase activities of the ouabain-sensitive endogenous enzyme and the insensitive rat Na^+,K^+-ATPase was near maximal. The Na^+,K^+-ATPase activity of the endogenous enzyme was reduced to 15% at 10 μM ouabain, whereas the Na^+,K^+-ATPase activity of the transfectants expressing both exogenous and endogenous enzymes was reduced to 80%. This gives a maximum estimate of (100−80)/(100−15) = 23.5% for the relative contribution by the endogenous enzyme to the total Na^+,K^+-ATPase activity measured in the transfectants in the absence of ouabain. At 10 μM ouabain, i.e. the conditions used in the analysis of the functional properties of the mutants described below, the relative contribution of the endogenous enzyme was then no more than 23.5% · 0.15/0.8 = 4.4%.

The reason why the endogenous enzyme contributed such a relatively small fraction of the total ouabain inhibitable Na^+,K^+-ATPase in the transfectants could be the limitation imposed by the amount of endogenous β-subunit synthesized by the COS-1 cells, since the exogenous and endogenous α-subunits must compete for interaction with the endogenous β-subunit [19]. Moreover, a considerable fraction of the endogenous Na^+,K^+-ATPase presumably formed a stable complex with ouabain already during the growth in the cell culture. Experiments were conducted to test the rate of dissociation of the ouabain that had bound to Na^+,K^+-ATPase in the cell culture, to obtain a maximal estimate of the ouabain-inhibitable Na^+,K^+-ATPase activity that would appear during the 10–20 min Na^+,K^+-ATPase assay. It was found that the Na^+,K^+-ATPase activity increased by no more than 5% during 20 min preincubation at 37°C in the absence of ouabain and in the presence of 100 mM Na^+, in accordance with previous data demonstrating that the half-life of the complex of ouabain with the ouabain-sensitive Na^+,K^+-ATPase is

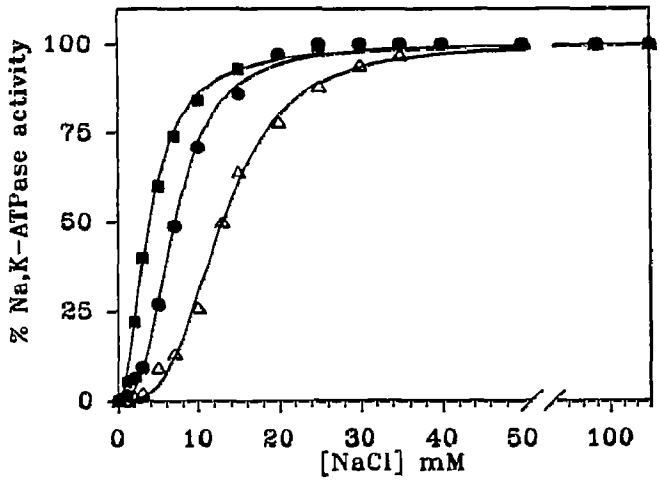

Fig. 2. Na^+ dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild type or the mutants Pro^{328}→Ala and Leu^{332}→Ala. The Na^+,K^+-ATPase measurements were carried out at 37°C in the presence of 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 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). At least three separate membrane preparations corresponding to different clonal isolates of cells transfected with the same cDNA were assayed 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 data were fitted to the relation Na^+,K^+-ATPase activity = $100 \cdot [Na^+]^n/([Na^+]^n + (K_{0.5})^n)$ giving the following respective values for the specific Na^+,K^+-ATPase activities corresponding to 100%, the $K_{0.5}$ (Na^+), and the Hill coefficients. Wild-type (●), 8.5 μmol/h/mg, 7.13 mM, n_H = 2.74; Pro^{328}→Ala mutant (△), 7.8 μmol/h/mg, 13.04 mM, n_H = 3.22; Leu^{332}→Ala mutant (■), 9.3 μmol/h/mg, 3.92 mM, n_H = 1.90.

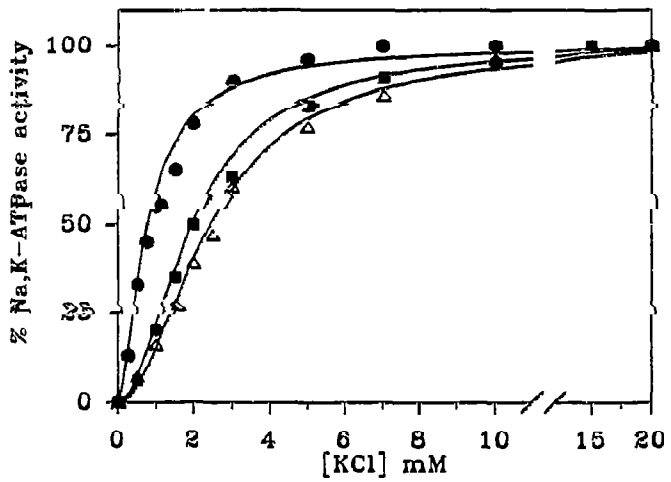

Fig. 3. K^+ dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild type or the mutants Pro^{328}→Ala and Leu^{332}→Ala. The Na^+,K^+-ATPase measurements were carried out at 37°C in the presence of 40 mM NaCl, 3 mM ATP, 3 mM $MgCl_2$, 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). At least three separate membrane preparations corresponding to different clonal isolates of cells transfected with the same cDNA were assayed 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 data were fitted to the relation Na^+,K^+-ATPase activity = $100 \cdot [K^+]^n/([K^+]^n + (K_{0.5})^n)$ giving the following respective values for the specific Na^+,K^+-ATPase activities corresponding to 100%, the $K_{0.5}$ (K^+), and the Hill coefficients. Wild-type (●), 8.2 μmol/h/mg, 0.78 mM, n_H = 1.51; Pro^{328}→Ala mutant (△), 7.0 μmol/h/mg, 2.46 mM, n_H = 1.91; Leu^{332}→Ala mutant (■), 8.7 μmol/h/mg, 1.97 mM, n_H = 1.89.

of the order of hours [20]. Thus, it must be concluded that the ATPase activity contributed by endogenous COS-1 Na^+,K^+-ATPase was insignificant under the present conditions for Na^+,K^+-ATPase activity measurements at 10 μM ouabain.

Fig. 2 illustrates the results of experiments in which the ouabain-resistant Na^+,K^+-ATPase activity was measured at varying NaCl concentrations on membranes isolated from COS-1 cells transfected with either wild-type or mutant cDNA. The Pro^{328}→Ala mutant displayed a 2-fold lower apparent affinity for Na^+ relative to the wild type. By contrast the apparent affinity for Na^+ was increased around 2-fold in the Leu^{332}→Ala mutant relative to the wild type. The KCl concentration was 20 mM to minimize competition at Na^+ sites and at the same time keep the K^+ sites on the extracellular side saturated. The curve representing Na^+ activation of Na^+,K^+-ATPase activity of the Leu^{332}→Ala mutant is less sigmoid (n_H = 1.90) than each of the curves representing the wild-type (n_H = 2.74) and the Pro^{328}→Ala mutant (n_H = 3.22) enzymes. These changes in sigmoidicity imposed by the mutations can be explained either by a shift of the E1–E2 equilibrium [21] or by a selective change in the intrinsic affinity of one or two of the three ion-binding sites [22].

In Fig. 3 the Na^+,K^+-ATPase activity was measured at varying KCl concentration. Both mutants exhibited lower apparent affinity for K^+ relative to the wild-type enzyme, 3- to 4-fold for the Pro^{328}→Ala mutant and 2- to 3-fold for the Leu^{332}→Ala mutant.

The changes in the cation affinities induced by the Leu^{332}→Ala mutation may be explained by a shift in the E1–E2 conformational equilibrium in favor of E1. It was feasible to test this hypothesis by measurement of the apparent affinity for ATP in the Na^+,K^+-ATPase assay, since the E1 conformation possesses more than 1000-fold higher affinity for ATP compared to the E2 conformation [23,24]. The results of experiments in which the Na^+,K^+-ATPase activity was measured at varying ATP concentrations are shown in Fig. 4. It can be seen that both mutants displayed higher apparent affinity for ATP relative to the wild-type Na^+,K^+-ATPase, which seems to suggest that the E1–E2 equilibrium was shifted in favor of E1 in either mutant. For the Leu^{332}→Ala mutant the apparent affinity for ATP was increased approximately 6- to 7-fold and for the Pro^{328}→Ala mutant 3- to 4-fold.

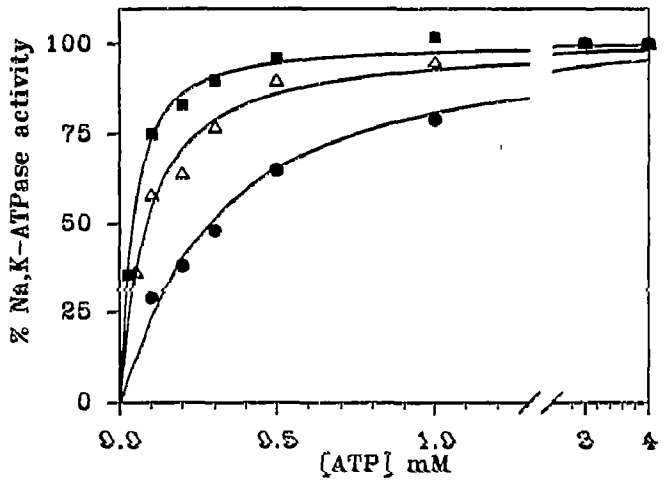

Fig. 4. ATP dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either wild type or the mutants Pro^{328}→Ala and Leu^{332}→Ala. The Na^+,K^+-ATPase measurements were carried out at 37°C in the presence of 130 mM NaCl, 20 mM KCl, 0.1–5 mM $MgCl_2$ (keeping [Mg^{2+}] constant), 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, and varying [ATP]. At least three separate membrane preparations corresponding to different clonal isolates of cells transfected with the same cDNA were assayed 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 data were fitted to the relation Na^+,K^+-ATPase activity = $100 \cdot [ATP]^n/([ATP]^n + (K_{0.5})^n)$ giving the following respective values for the specific Na^+,K^+-ATPase activities corresponding to 100%, the $K_{0.5}$ (ATP), and the Hill coefficients. Wild-type (●), 9.0 μmol/h/mg, 0.287 mM, n_H = 1.15; Pro^{328}→Ala mutant (△), 7.6 μmol/h/mg, 0.086 mM, n_H = 1.05; Leu^{332}→Ala mutant (■), 9.2 μmol/h/mg, 0.042 mM, n_H = 1.17.

In the series of experiments described in Table I, the influence of pH on the maximum turnover rate and on the apparent affinities for Na^+ and K^+ was examined in the two mutants and the wild-type enzyme, in order to investigate the effects of the mutations under conditions where the relative contributions of each of the partial reaction steps to rate limitation of the overall Na^+,K^+-ATPase turnover cycle varied [25]. The pH dependence of the maximum activity was in accordance with the bell-shaped curve described previously, with a pH maximum between 7.5 and 8.0 [26,27]. Moreover, it can be seen that at pH 6.4 the relative turnover rate was higher in the Pro^{328}→Ala and Leu^{332}→Ala mutants than in wild type (compare 78.0% and 72.3% with 57.2%). This is in accordance with the hypothesis that the mutations enhanced the rate of the E2(K)→E1 transition, which is rate limiting for the overall turnover at low pH [25]. Table I also shows that the relative differences in Na^+ and K^+ affinity among the wild-type enzyme and the two mutants remained approximately the same over the wide pH range from 6.4 to 8.4. There was a slight tendency for the $K_{0.5}$ for Na^+ to decrease and for $K_{0.5}$ for K^+ to increase with increasing pH in both mutants and the wild type in accordance with the pH dependence of the E2(K)→E1 transition [27,28].

4. DISCUSSION

COS-1 cells transfected with cDNA encoding either of the rat kidney Na^+,K^+-ATPase mutants Pro^{328}→Ala and Leu^{332}→Ala were able to grow in the presence of 10 μM ouabain, a drug concentration that is cytotoxic to the parental COS-1 cells. Since the survival of the transfectants under these selective conditions solely depended on the activity of the ouabain-resistant mutant pumps, it must be concluded that the two Na^+,K^+-ATPase mutants were able to carry out active transport of Na^+ and K^+. The residues Pro^{328} and Leu^{332} are therefore not indispensable to the function of the Na^+,K^+-ATPase. The maximum specific activities (μmol P_i released per hour per mg of crude membrane protein) measured at saturating substrate concentrations and neutral pH were found to be very similar for the Na^+,K^+-ATPase mutants and the wild-type enzyme (legends to figures and Table I). If it is assumed that the COS-1 cells expressed identical numbers of mutant and wild-type Na^+,K^+-ATPase molecules per mg crude membrane protein, their turnover numbers would be approximately equal.

By using a relatively high ouabain concentration (10 μM) to knock out the endogenous enzyme during selection of transfectants and during activity measurement, it was possible to analyse cation- and ATP-concentration dependencies of the Na^+,K^+-ATPase activity of expressed exogenous wild-type and mutant Na^+,K^+-ATPases under conditions, where the endogenous enzyme contributed less than 5% of the activity. In this way reliable estimates could be made of the apparent affinities for Na^+, K^+, and ATP in the mutant Na^+,K^+-ATPases.

The Leu^{332}→Ala mutant displayed an increased apparent affinity for either of the ligands, Na^+ and ATP, and a reduced apparent affinity for K^+ relative to the wild type. These findings are consistent with a shift in the E1–E2 conformational equilibrium in favor of the E1 form, which possesses high affinity to Na^+ as well as ATP.

The Pro^{328}→Ala mutant, on the other hand, exhibited an increased apparent affinity for ATP and a reduced apparent affinity for either of the cations, corresponding to a 2-fold increase in $K_{0.5}$ for Na^+ and a 3 to 4-fold increase in $K_{0.5}$ for K^+. The increased affinity for ATP in the Pro^{328}→Ala mutant suggests that this amino acid substitution shifted the E1–E2 equilibrium in favor of the E1 conformation [23,24], and in further support of this conclusion the relative turnover rate was found to be higher in the mutant than in the wild-type enzyme under conditions (pH 6.4) where the E2(K)→E1 transition is known to be rate limiting for the overall turnover cycle in the wild-type enzyme (Table I). As this confor-

Table I

Effect of pH on the maximum turnover rate and on the apparent affinities for Na^+ and K^+

	Maximum turnover rate (%)[a]		
Mutants	pH 6.4	pH 7.4	pH 8.4
Wild type	57.2	100	93.0
Pro^{328}→Ala	78.0	100	93.0
Leu^{332}→Ala	72.3	100	91.8
	$K_{0.5}$ (Na^+) (mM)[b]		
Mutants	pH 6.4	pH 7.4	pH 8.4
Wild-type	7.2	7.1	5.5
Pro^{328}→Ala	17.2	13.0	13.0
Leu^{332}→Ala	4.7	3.9	3.9
	$K_{0.5}$ (K^+) (mM)[b]		
Mutants	pH 6.4	pH 7.4	pH 8.4
Wild-type	0.8	0.8	1.1
Pro^{328}→Ala	1.8	2.5	2.6
Leu^{332}→Ala	1.8	2.0	2.2

[a] Measured at 37°C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 30 mM histidine, 1 mM EGTA, 10 μM ouabain. pH was adjusted to the indicated values with Tris base or HCl. The percentages were calculated relative to the rates measured at pH 7.4 after subtraction of background ATPase activity measured at 20 mM ouabain. The numbers corresponding to 100% were as follows. Wild type, 8.3 μmol/h/mg; Pro^{328}→Ala mutant, 7.4 μmol/h/mg; Leu^{332}→Ala mutant, 9.1 μmol/h/mg. Each value represents the average of 3 determinations differing maximally 7%.

[b] The $K_{0.5}$ values were obtained from curve fitting as described for Figs. 2 and 3.

mational displacement per se would tend to increase the apparent affinity for Na^+, i.e. the opposite of the observed effect on Na^+ affinity, it is necessary to postulate that in addition the intrinsic affinity of the E_1 conformation for Na^+ had been reduced. Hence, it seems that one or more of the high affinity cytoplasmic Na^+ sites must have been distorted in the Pro^{328}→Ala mutant. The role of the Pro^{328} residue might be to donate the electrons associated with the backbone carbonyl group in the preceding peptide bond to ion binding, or to kink the helix in such a way that the juxtaposed glutamic acid residue Glu^{329} is brought into an optimal position for coordinating Na^+.

Either of the mutants Pro^{328}→Ala and Leu^{332}→Ala displayed a reduced apparent affinity for K^+. There are at least 3 possible explanations for this finding, none of which are mutually exclusive.

First, assuming the consecutive reaction sequence of the Post–Albers scheme to hold, and the rate of the E2(K)→E1 transition to be enhanced due to the amino acid 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 transition [29].

Second, the mutations may have shifted the E1P–E2P conformational equilibrium in favor of the E1P form in parallel with the displacement of the E1–E2 equilibrium towards E1, in line with the basic idea underlying the E1–E2 notation, that the phosphorylated and unphosphorylated conformations are similar. The rates of the E2(K)→E1 and E2P→E1P transformations may then both have been enhanced in the Pro^{328}→Ala and Leu^{332}→Ala mutants relative to the wild-type enzyme.

Third, Pro^{328} and/or Leu^{332} may be directly involved in formation of the high-affinity extracellular K^+ transport sites. Leu^{332} does not contain any liganding group, but its bulky hydrophobic side chain might in some way serve to stabilize a closed bracelet structure around the potassium ion. A reduction in the free energy of interaction between the enzyme and K^+ would be consistent with an acceleration of the E2(K)→E1 transition, if it is assumed that the deocclusion of K^+ taking place in this step implies K^+ dissociation from the same liganding groups that originally took part in the binding of K^+ at the extracellular surface.

It is of interest to compare the results obtained with the Na^+,K^+-ATPase mutants Pro^{328}→Ala and Leu^{332}→Ala in this study with results previously obtained in mutagenesis work on sarcoplasmic reticulum Ca^{2+}-ATPase, in which the residues at the homologous positions, Pro^{308} and Pro^{312}, were substituted [4].

The replacement of Pro^{312} in Ca^{2+}-ATPase with the less bulky residues alanine and glycine led to mutants that were defective in the E1P to E2P conformational transition and in addition displayed an increased apparent affinity for Ca^{2+}. This can be explained by a displacement of the conformational equilibrium very similar to that proposed to explain the present data with the homologous Na^+,K^+-ATPase Leu^{332}→Ala mutant. Since at the position corresponding to Leu^{332} in Na^+,K^+-ATPase, the family of P-type ATPases contain either a proline or a leucine residue, these two residues may share some properties that are essential to the E1/E1P–E2/E2P conformational changes. It seems possible that the presence at this position of either the bulky side chain of a leucine or a kink dictated by the presence of a proline residue would help to introduce defects in helix packing in the membrane required for the stabilization of E2/E2P [4,30]. The present data show that the small side chain of alanine would be insufficient in this respect.

Replacement of Pro^{308} in Ca^{2+}-ATPase with alanine or glycine led to mutants that were characterized by reduced apparent affinities for Ca^{2+}. This is similar to the finding of a reduced apparent affinity for Na^+ in the homologous Na^+,K^+-ATPase Pro^{328}→Ala mutant. It seems likely that at this position the proline residue is involved in formation of the high affinity cation binding sites.

Acknowledgements: I would like to thank Janne Petersen and Jytte Jørgensen for their expert and invaluable technical assistance; Dr. Jens Peter Andersen for discussion, and for his encouragement of this work; and Dr. R.J. Kaufman, Genetics Institute, Boston, for the gift of the expression vector pMT2. This research was supported by grants from the Danish Biomembrane Research Centre, the Danish Medical Research Council, the NOVO Foundation, and the Carlsberg Foundation (Denmark).

REFERENCES

[1] Jørgensen, P.L. and Andersen, J.P. (1988) J. Membr. Biol. 103, 95–120.
[2] Green, N.M. (1989) Biochem. Soc. Trans. 17, 972–974.
[3] Karlish, S.J.D., Goldshleger, R. and Stein, W.D. (1990) Proc. Natl. Acad. Sci. USA 87, 4566–4570.
[4] Vilsen, B., Andersen, J.P., Clarke, D.M. and MacLennan, D.H. (1989) J. Biol. Chem. 264, 21024–21030.
[5] Clarke, D.M., Loo, T.W., Inesi, G. and MacLennan, D.H. (1989) Nature 339, 476–478.
[6] Vilsen, B. and Andersen, J.P. (1992) FEBS Lett. 306, 247–250.
[7] Vilsen, B., Andersen, J.P. and MacLennan, D.H. (1991) J. Biol. Chem. 266, 18839–18845.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[9] Vilsen, B. and Andersen, J.P. (1992) FEBS Lett. 306, 213–218.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[11] Shull, G.E., Greeb, J. and Lingrel, J.B. (1986) Biochemistry 25, 8125–8132.
[12] Kaufman, R.J., Davies, M.V., Pathak, V.K. and Hershey, J.W.B. (1989) Mol. Cell. Biol. 9, 946–958.
[13] Gluzman, Y. (1981) Cell 23, 175–182.
[14] Matsui, H. and Schwartz, A. (1966) Biochim. Biophys. Acta 128, 380–390.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Ottolenghi, P. (1975) Biochem. J. 151, 61–66.
[17] Jørgensen, P.L. and Petersen, J. (1982) Biochim. Biophys. Acta 705, 38–47.

[18] Kent, R.B., Emanuel, J.R., Neriah, Y.B., Levenson, R. and Housman, D.E. (1987) Science 237, 901–903.
[19] Geering, K. (1991) FEBS Lett. 285, 189–193.
[20] Hansen, O. (1984) Pharmacol. Rev. 36, 143–163.
[21] Tanford, C., Reynolds, J.A. and Johnson, E.A. (1985) Proc. Natl. Acad. Sci. USA 82, 4688–4692.
[22] Karlish, S.J.D. and Stein, W.D. (1985) J. Physiol. 359, 119–149.
[23] Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116.
[24] Jensen, J. and Ottolenghi, P. (1983) Curr. Top. Membr. Trans. 19, 223–227.
[25] Forbush III, B. and Klodos, I., (1991) in: The Sodium Pump: Structure, Mechanism and Regulation, (J.H. Kaplan and P. De Weer, Eds.), The Rockefeller University Press, NY, 1991, pp. 211–225.
[26] Skou, J.C. (1957) Biochim. Biophys. Acta 23, 394–401.
[27] Forbush, B., III. (1987) J. Biol. Chem. 262, 11104–11115.
[28] Skou, J.C. (1982) Biochim. Biophys. Acta 688, 369–380.
[29] Eisner, D.A. and Richards, D.E. (1981) J. Physiol. 319, 403–418.
[30] Andersen, J.P. and Vilsen, B. (1992) Acta Physiol. Scand. 146, 151–159.