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Significance of the glutamic acid residues Glu_{334}, Glu_{959}, and Glu_{960} of the α subunits of *Torpedo* Na^+,K^+ pumps for transport activity and ouabain binding

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Abstract

Glutamic acid residues in transmembrane segments of the α subunit of the Na^+,K^+-ATPase have been discussed as possible candidates for the binding sites of the transported cations. Here we report on effects of mutations of Glu_{334}, Glu_{959}, and Glu_{960} to alanine in ouabain-sensitive (OS) as well as ouabain-resistant (OR) ATPases of *Torpedo* electroplax expressed in *Xenopus* oocytes. All mutants are incorporated to about the same extend as the wild-type ATPases into the plasma membrane. None of the mutations produces complete inhibition of transport activity as judged from measurements of $^{86}Rb^+$ uptake, membrane current, and ATPase activity. After conversion of OS to OR by mutation of the bordering residues of the first extracellular loop Gln_{118} to Arg and Asp_{129} to Asn, the K_m value for inhibition by ouabain increases to 59 μM. Substitution of Glu_{334} to Ala in the OR pump variant restores ouabain sensitivity with a K_m value of 0.12 μM, which is similar to that of the endogenous *Xenopus* pump. After substitution of Glu_{960} by Ala in the OR pump, ouabain sensitivity is partially restored. The K_m values for pump stimulation by external K^+ appear to be reduced in the OR compared to the OS pump. Mutation of Glu_{959} and Glu_{960} to Ala has no pronounced effects on the potential-dependent K_m values at external pH 7.8; only in the Glu_{959}-mutated OR pump, the apparent K_m at 0 mV is raised. We conclude that none of the mutated glutamic acid residues is essential for cation coordination, but that Glu_{334}, and in part also Glu_{960}, seems to be involved in preserving the ouabain-resistant conformation of the enzyme. © 1998 Elsevier Science B.V.

Keywords: Sodium pump; Glutamic acid residue; Ouabain binding; Cation binding site; Transport activity; Voltage dependence

1. Introduction

The Na^+ pump or Na^+,K^+-ATPase belongs to the P-type ATPases that are temporarily phosphorylated at an aspartic acid residue and that couple the hydrolysis of ATP to translocation of the cations across the cell membrane. The enzyme is composed of a catalytic about 112 kDa α subunit with phosphorylation and cation binding sites, and of a glycosilated about 55 kDa β subunit. The heterodimers are possibly arranged as $(\alpha\beta)_2$ diprotomers (for review see [1]). Since the discovery of the Na^+,K^+-ATPase in the late 50's [2], the central question still remains open: how does this enzyme perform translocation of Na^+ and K^+ and what structural domains of the α subunit

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are involved in this process? It was proposed that, like in other cation binding proteins, the transported ions are ligated by oxygen-containing groups [3]. For the alkali metal ions, like K^+ and Na^+, the coordination number is nearly exclusively 4. In addition, it has been suggested that two positive charges of occluded K^+ or Na^+ cations are compensated by negative charges within the pump protein [4,5], indicating that glutamic and aspartic acid residues can be considered as candidates. Chemical modification of carboxyl groups with N,N′-dicyclohexylcarbodiimid (DCCD) [3,6,7] revealed that Glu_{953} and Glu_{327} may donate carboxyl groups critical for cation coordination. These findings stimulated investigation of the role of glutamic acid residues by means of site-directed mutagenesis. However, mutations of the negatively charged Glu_{953} and the neighbouring Glu_{954} or double mutations in the ouabain-resistant rat $\alpha 1$ subunit to Gln revealed no differences in cation stimulation of ATPase activity demonstrating that negative charges at these positions are not critical for cation coordination [8]. The investigated mutants still contained carbonyl oxygen that could contribute to the coordination structure. Replacement of both of these residues by the carboxyl-containing Asp with a shorter side chain [8], or to alanine or leucine [9] also left the enzyme functionally active. We have shown recently that effects of mutation may become visible only at positive membrane potentials, which can be achieved in voltage-clamp experiments [1]. In particular, the voltage-dependence of pump stimulation by external K^+ may be increased resulting in considerably elevated apparent K_m values [10] particularly at more depolarised membrane potentials.

Substitutions of Glu_{327} led to more complicated results. It was demonstrated that mutation of the Glu_{329} (homologous to Glu_{327}) to Gln or Leu, thus removing either negative charge or carboxyl group at this position, led to expression of an active enzyme [11,12]. On the other hand, mutation of Glu_{329} in ouabain-insensitive rat $\alpha 1$ pumps expressed in COS cells [13], or in rat $\alpha 2^*$ pumps modified to be ouabain-insensitive and expressed in HeLa cells [11] to residues with shorter side chains, like Asp or Ala, did not allow transfected cells to survive in presence of 1 μM ouabain. This lead to the conclusion that the length of the side chain at the position 329 is important for cation interaction. On the other hand, expression of the ouabain-sensitive sheep $\alpha 1$ isoform in 3T3 cells containing ouabain-resistant endogenous pumps demonstrated that the Glu_{329} to Ala or Asp mutants had intact [^{3}H]ouabain binding sites and were able to bind K^+ and Na^+ [14]. Another, though not considered explanation for the inability of the COS cells transfected with the Glu_{329} to Ala or Asp mutants to grow in presence of low ouabain concentrations could be a changed ouabain sensitivity. The functional consequences of substitution of Glu_{329} to Ala, therefore, remains unclear.

In this work we investigated the functional significance of the putative intramembraneous glutamic acid residues Glu_{334}, Glu_{959}, and Glu_{960} in the α subunit of the Na^+,K^+-ATPase of *Torpedo* electroplax (homologous to Glu_{327}, Glu_{953} and Glu_{954} of the sheep $\alpha 1$ subunit) by substitution of these residues with Ala, which excludes any contribution of side chain oxygens at this position to the cation coordination. Effects of mutations are compared for ouabain-sensitive and ouabain-resistant variants of the mutants expressed in *Xenopus* oocytes. Particularly, effects on ouabain binding, expression of pump proteins, transport and hydrolytic activities as well as voltage dependencies of apparent K_m values for pump stimulation by external K^+ have been investigated. We will demonstrate that all mutated pumps are functionally active. Surprisingly, substitution of Glu_{334} or Glu_{960} to Ala increases the affinity to ouabain of the ''ouabain-resistant'' form of the *Torpedo* pump. Part of the results has been published previously [10,15].

2. Methods

2.1. Oocytes

Females of the clawed toad *Xenopus laevis* were anaesthetised with *m*-aminobenzoic acid ethylesther methane sulfonate (MS222, Sandoz, Basel (Switzerland) 1 g/l). Parts of the ovary were removed and treated with collagenase (1 mg/ml) overnight. Full-grown prophase-arrested oocytes were selected for experiments. For expression of Na^+ pumps, oocytes were injected with cRNA for wild-type or mutated α subunits (10–15 ng per oocyte) of the Na^+,K^+-ATPase of electroplax of *Torpedo californica* together with cRNA for the β subunit (5–10 ng per

oocyte). These cells and non-injected control oocytes were stored at 19°C in oocyte Ringer's solution (concentration in mM: NaCl 90; KCl 2; $CaCl_2$ 2; MOPS 5; pH 7.2) containing antibiotics (in mg/l: 25 streptomycin and 20 penicillin, or 70 gentamycin). Experiments were performed after 3 to 5 days of incubation.

2.2. Construction of the mutated α subunits

The plasmids pSPTαOS and pSPTβ containing cDNA encoding the α and β subunit of the Na^+,K^+-ATPase of *Torpedo californica* were constructed as described previously [16]. A 432-bp fragment [*EcoRI*(60)-*KpnI*(429)] was excised from pSPTαOS and used for mutation of Gln_{118} and Asn_{129} at the borders of the first extracellular domain to Arg and Asp, respectively, for construction of ouabain-resistant pumps [17]. Accordingly, a 610-bp fragment [*KpnI*(492)-*BamHI*(1102)] and a 556-bp fragment [*BsaAI*(2654)-*BsaAI*(3210)] were excised and used for mutation of Glu_{334}, and Glu_{959} or Glu_{960} to Ala, respectively. The 432-bp and 610-bp fragments were subcloned into pUC119, the 556-bp fragment was subcloned into pUC118. The following oligonucleotide primers used for the mutations were prepared in an Applied Biosystems DNA synthesiser (Model 380A).

Mutation	Oligonucleotide (changes underlined)
Gln_{118} to Arg	5′-TATGGTATCCGGGTTGCAACC-3′
Asn_{129} to Asp	5′-AAATGATGATTTGTA-3′
Glu_{334} to Ala	5′-ATGTGCCAGCAGCATTACT-3′
Glu_{959} to Ala	5′-GATTGTTTGCAGAGACTGC-3′
Glu_{960} to Ala	5′-GTTTGAAGCGACTGCACT-3′

The in vitro site-directed mutagenesis was performed by means of the Sculptor IVM system according to the supplier's instructions to the kit (Amersham, RPN, 1526).

The ouabain-resistant Glu-to-Ala mutants were constructed accordingly from pSPTαOR containing the substitution for Gln_{118} and Asn_{12}. Mutated fragments were sequenced to verify that the desired mutants were actually obtained. The gene fragments carrying the mutants were cut off from the recombinant plasmids, and used to replace the corresponding segments of the α subunit gene in pSPTαOS or pSPTαOR.

2.3. Antibody assay

After injection of cRNA, oocytes were incubated with 0.47 mM (5.55 MBq/ml) of [^{14}C]leucine for metabolic labelling of synthesised proteins. Triton X-100 extracts of yolk-free oocyte homogenates were subjected to immunoprecipitation using rabbit antiserum against the α subunit of the Na^+,K^+-ATPase of *Torpedo californica* according to [16]. The resulting immunoprecipitates were analysed on 10% SDS-polyacrylamide gels followed by fluorography.

2.4. Determination of ATPase activity

To assay Na^+,K^+-ATPase activity about 200–400 oocytes were homogenised, and the microsomal fraction was recovered from the homogenate by centrifugation at 160,000 g for 30 min [18]. The ATPase activity was taken as the ouabain-sensitive formation of inorganic phosphate from ATP according to the spectroscopic method of Martin and Doty with modification as described in [18], and was measured in a reaction mixture (0.1 ml) containing 50 mM imidazole/HCl buffer (pH 7.5), 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 1 mM ATP and microsomes (10–30 μg protein) either in absence or in presence of 10 μM or 10 mM ouabain. The reaction was started by adding ATP to a mixture that had been incubated at 37°C for 10 min. Na^+,K^+-ATPase activity was obtained by subtracting the rate of phosphate formation measured in the presence of 10 mM ouabain from that measured in the presence of 10 μM ouabain (for ouabain-resistant type) or in its absence (for ouabain-sensitive type).

2.5. Measurements of ouabain binding and $^{86}Rb^+$ uptake

The number of ouabain binding sites is identical to the number of pump molecules per oocytes, and was determined by incubating Na^+-loaded oocytes in K^+-free solution containing 2.5 μM cold ouabain and 2.5 μM [^{3}H]ouabain [19]. For determination of uptake of $^{86}Rb^+$ as a measure for maximum pump activity, Na^+-loaded oocytes were incubated in Na^+-free test solution containing 5 mM Rb^+ (925 kBq/ml) for 12 min [20]. Ouabain-sensitive $^{86}Rb^+$ uptake was

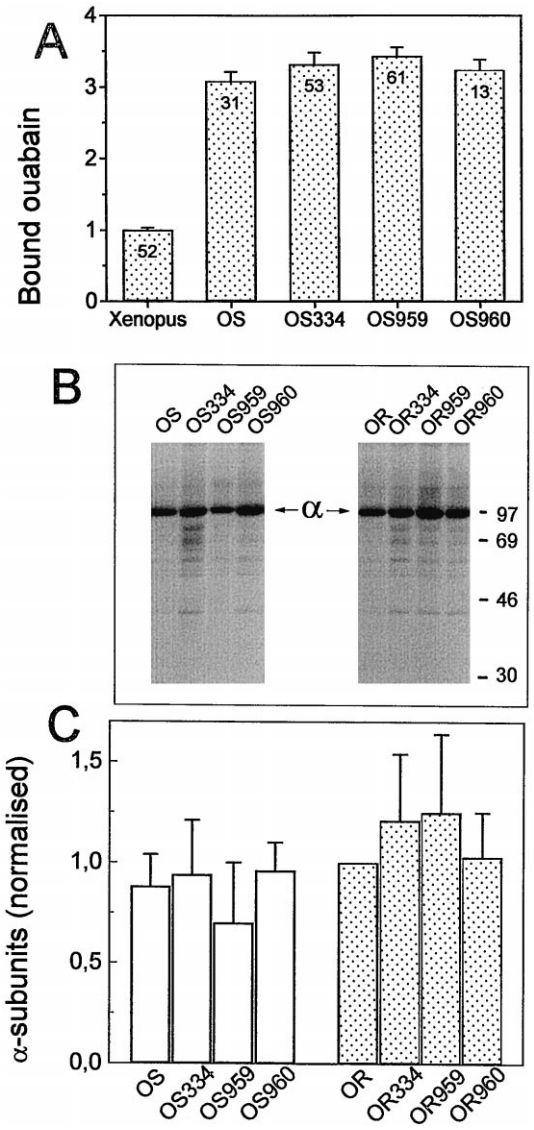

Fig. 1. Expression of OS and OR pump variants in *Xenopus* oocytes. (A) [^{3}H]ouabain bound to single oocytes. Data are normalised to the value of endogenous pumps (309 ± 10 dpm/oocyte, corresponding to about 10 fmol/oocyte), and represent averages ± SEM (numbers within the bars give numbers of analysed oocytes). (B) Synthesis of α subunits in the oocytes. Oocytes were subjected to immunoprecipitation with anti α subunit antiserum. The immunoprecipitates were resolved by SDS-PAGE and visualised by fluorography. From oocytes without expressed *Torpedo* pumps, no signal can be detected [16]. Numbers on the right indicate the position of proteins with known molecular masses (in kDa). (C) Quantified amounts of α subunits precipitated with antibodies against the α subunit of *Torpedo* pumps. Data are averaged from three sets of experiments as shown in (B), and normalised to the values obtained for OR α subunit.

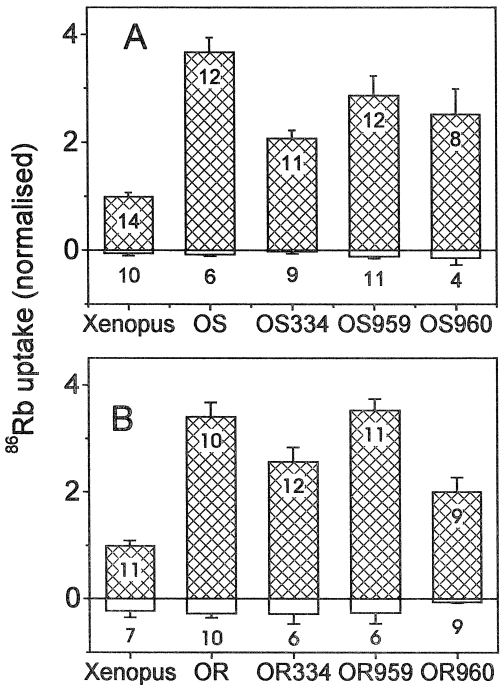

Fig. 2. Ouabain-specific ^{86}Rb uptake in the oocytes with expressed OS and OR pump variants. Ouabain-inhibitable uptake of ^{86}Rb for OS (A) or OR pumps (B) was determined using the assay for ouabain-sensitive pumps, and was calculated as a difference in total uptake in absence of ouabain and in presence of 10 mM ouabain. Data were obtained from 3 different batches of oocytes and were normalised to ouabain-sensitive uptake in non-injected control oocytes from the same oocyte batch (2055 ± 531 dpm). Numbers within or below bars indicate number of oocytes. Negative bars indicate contributions of unspecific $^{86}Rb^+$ uptake.

taken as a difference of the uptake in absence and in presence of 10 mM ouabain.

2.6. Voltage-clamp experiments

During the normal transport cycle of the Na^+,K^+-ATPase three Na^+ ions are transported out of the cell and two K^+ ions into the cell. As a result of the $3Na^+$–$2K^+$ stoichiometry, the pump generates a current that can be taken as a measure for transport activity. This current was determined as the current activated by external K^+ (if all other K^+-sensitive currents are blocked) or as the current component blocked by Na^+,K^+-ATPase specific inhibitors like the cardiac glycoside ouabain. Over a wide range of

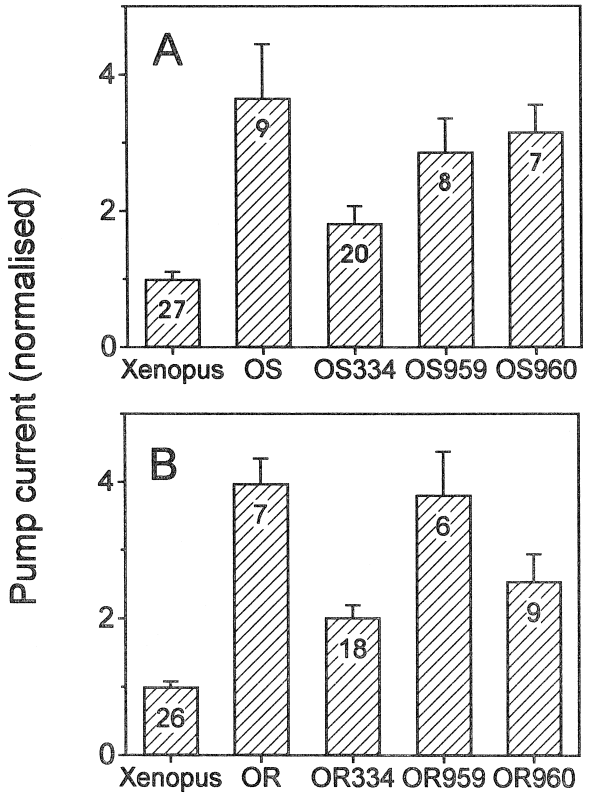

Fig. 3. Current generated by OS (A) and OR (B) pump variants. Measurements were performed at −60 mV under conditions of maximum, voltage-independent pump activation (5 mM K^+ and 0 Na^+ in the external solution). Pump current was determined as a difference of total membrane current in absence and presence of 5 mM external K^+. Data are averages from 3 to 8 batches of oocytes normalised to pump current of non-injected oocytes (38 ± 3 nA).

potentials, the currents determined by these two assays are identical [21]. The electrophysiological protocol for the characterisation of the Na^+ pump was the same as described previously [22]. In brief, steady-state membrane currents were recorded at the end of 500-ms, rectangular voltage-clamp pulses (from −150 to +30 mV in 10 mV steps) that were applied from a holding potential of −60 mV. To increase the activity of the Na^+ pump, cytoplasmic Na^+ was elevated by incubating the oocytes for about 40 min in ''Na-loading solution'' (concentrations in mM: Na-citrate 2.5; NaCl 110; MOPS 5; pH 7.4) and then for at least half an hour in post-loading solution (in mM: NaCl 90; $CaCl_2$ 2; MOPS 5; pH 7.4). Current generated by the Na^+,K^+-ATPase was determined in Na^+-free test solutions (in mM: tetramethylammonium chloride (TMA)Cl 100; $BaCl_2$ 10; tetraethylammonium chloride (TEA)Cl 20; $NiCl_2$ 5; MOPS 5; pH 7.8) as current activated by 5 or 10 mM extracellular K^+. $BaCl_2$ and (TEA)Cl were added to block K^+-sensitive currents. To reduce background currents mediated by Ca^{2+}-activated channels (Cl^- channels), no Ca^{2+} was added to test solutions. To reduce an additional inward current generated by the pump in solution without K^+ and Na^+ [23–25], measurements were performed at external pH 7.8. In the nominally Na^+- and K^+-free solutions, their actual concentrations were below 5 μM as determined by flame photometry.

For analysis of the voltage-dependent stimulation of the pump by external K^+, voltage dependencies of

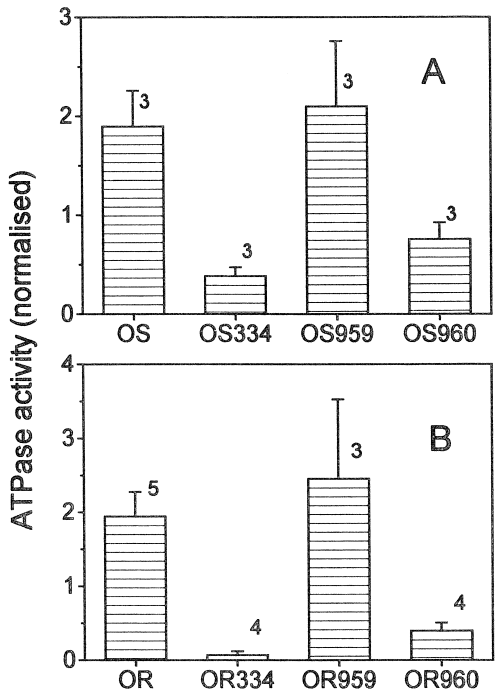

Fig. 4. ATPase activity of OS (A) and OR (B) pump variants. ATPase activities were normalised to the activity of the endogenous pumps (1.48 ± 0.29 μmol P_i/mg/h). Data represent averages from 3–5 batches of oocytes (indicated by the numbers above bars). (A) For the ouabain-sensitive pumps, ATPase activity was determined as the difference between ATPase activity in absence and in presence of 10 mM ouabain. The contribution of endogenous pumps has been subtracted from the total ATPase activity in oocytes with expressed pumps. (B) For ouabain-resistant pumps ATPase activity was determined as the difference between activity in 10 μM and 10 mM ouabain.

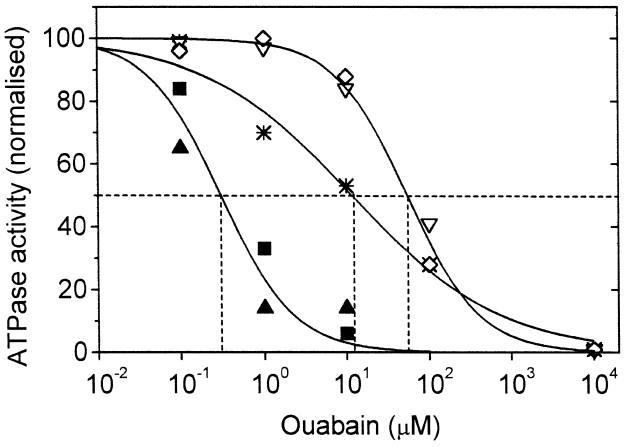

Fig. 5. Inhibition of ATPase activity in OS and OR pump variants by ouabain. Filled symbols: ■ refer to *Xenopus*, ▲ to OS960, open symbols: ∇ to OR, ◇ to OR959, and ∗ to OR960. The contribution of endogenous pumps has been subtracted from the total ATPase activity in oocytes with expressed pumps. The $K_{1/2}$ values for inhibition (marked by vertical broken lines) are: $0.39 \pm 0.03\,\mu M$ for *Xenopus* and OS960 pumps; $58.6 \pm 5.0\,\mu M$ for OR and OR959 pumps and $11.7 \pm 4.1\,\mu M$ for OR960 pump. Solid lines represent fits of $100 * K_{1/2}^n / (K_{1/2}^n + [\text{ouabain}]^n)$ to the data with a Hill coefficient of $n = 1$ for *Xenopus*, OS960, OR and OR959 pumps, and with $n = 0.5$ for OR960 pump.

pump current were determined at external K^+ concentrations of 0.05, 0.1, 0.25, 0.5, 2.5 and 5 mM or 10 mM in the absence of external Na^+ (see inset to

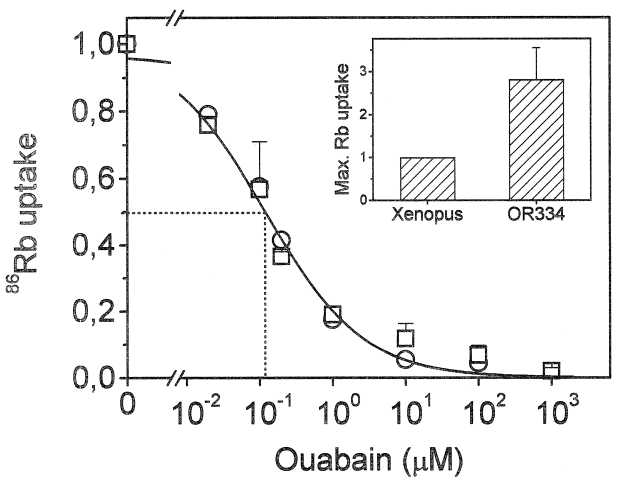

Fig. 6. Dependence of Rb^+ uptake on ouabain concentration for *Xenopus* (O) and OR334 (□) pumps. Data points represent averages (±SEM) of 10–50 oocytes from 5 independent experiments (2–10 oocytes for each concentration). The inset gives a relative maximal ouabain-sensitive uptake of Rb^+, indicating that the uptake by the OR334 mutant composes 2/3 of the total Rb^+ uptake.

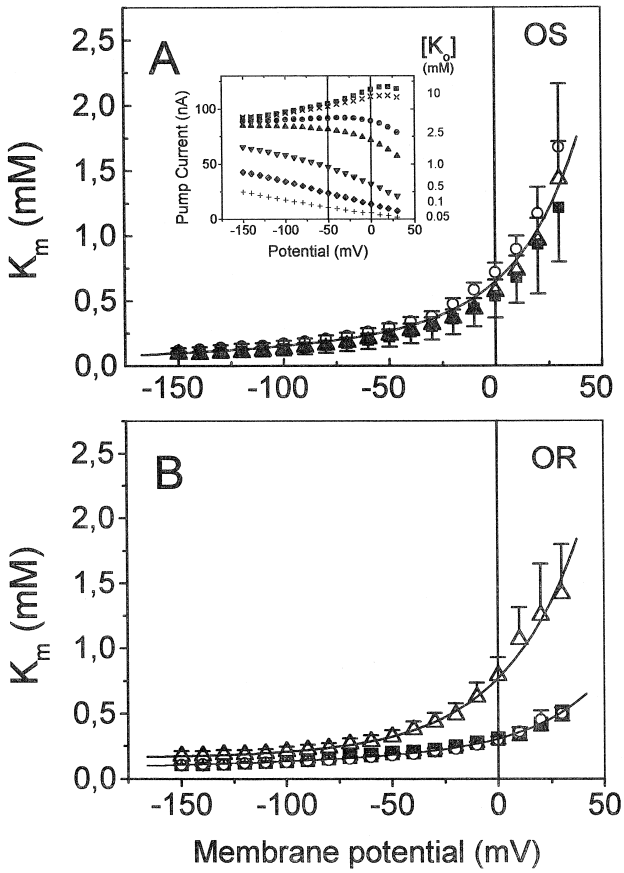

Fig. 7. Potential dependence of apparent K_m values for stimulation of pump-generated currents for OS (A) and OR (B) pump variants by external K^+. K_m values were determined from the $[K^+]$ dependence of pump currents measured at different potentials (see inset). ■ refer to OS and OR wild types, respectively, Δ to Glu_{959} mutants, and O to Glu_{960} mutants. For the analysis of expressed pump variants contribution of endogenous *Xenopus* pumps was either subtracted from the total current (for the OS pumps) or blocked with 1 μM ouabain (for OR pumps). Lines represent fits of Eq. (2) to the data; corresponding fit parameters are listed in Table 1. Data represent averages of 6 to 8 experiments. The inset in (A) illustrates an example of the voltage dependence of pump current generated by OS ATPases for a series of external K^+ concentrations ranging from 50 μM to 10 mM (numbers on the right).

Fig. 7, for details see [22]). The concentration dependencies of pump current at different membrane potentials (see e.g. data points on vertical lines in the inset of Fig. 7) were fitted by:

$$I = I_{max} \frac{[K^+]}{K_m + [K^+]} \quad (1)$$

and apparent K_m-values were extracted and plotted versus corresponding potentials (Fig. 7).

2.7. Nomenclature

We are using the wording ''ouabain-resistant'', which means that these pumps are much less sensitive to ouabain but their activity still can be inhibited by high concentrations of ouabain (see Fig. 5). The different types of pumps are abbreviated as follows:

Xenopus	endogenous Na^+-pump of *Xenopus* oocytes;
OS	ouabain-sensitive *Torpedo* pumps;
OR	ouabain-resistant *Torpedo* pumps.

Numbers 334, 959, 960 following OS or OR refer to the respective glutamic acid residue substituted by alanine. For numbering of the amino acids we will use the nomenclature of *Torpedo* ATPase if not stated otherwise.

3. Results

The objective of our investigation is to evaluate whether the glutamic acid residues of the putative IVth and IXth membrane domains contribute to the interaction of the transported cations with the pump molecule. For this we determined (1) whether pumps are expressed in the oocytes after mutations of Glu_{334}, Glu_{959} and Glu_{960} to Ala in the α subunit of the *Torpedo* ATPase, (2) whether functional pump molecules are formed, and, in case functioning and electrogenic pumps are expressed, whether (3) ouabain sensitivity and (4) voltage-dependent pump stimulation by external K^+ is modified. To determine functional activity of expressed pumps, the contribution of endogenous pumps has to be subtracted from the overall signal. This can be achieved by either subtracting the endogenous contribution from the total signal using data obtained from control oocytes of the same batches, or, if the OR mutants are expressed, by adding micromolar concentrations of ouabain, which blocks the endogenous pumps. This latter assay is much more effective, but one has to assume that the additional mutations do not interfere with the ouabain resistance. In the present investigation we, therefore, analysed in parallel experiments the effects of mutation of the glutamic acid residues to alanine in ouabain-sensitive as well as ouabain-resistant ATPases.

3.1. Expression of pump molecules

The binding of the pump-specific inhibitor ouabain to the α subunit of the Na^+,K^+-ATPase is considered to be a characteristic of correctly folded and assembled pump molecules. The amount of [^{3}H]ouabain bound to the oocyte surface at saturating concentration in the incubation medium is, therefore, a measure for the number of the heterodimers incorporated into the plasma membrane. Fig. 1(A) illustrates that all ouabain-sensitive pumps, wild-type as well as the mutants, are expressed in the oocytes. The number of exogenous *Torpedo* pumps is about twice as that of the endogenous *Xenopus* pumps, and the degree of expression is about the same for all OS pump species.

Since the [^{3}H]ouabain binding assay could not be applied routinously for evaluation of expression of OR pumps because of the high cost of experiment, we used the immunoprecipitation assay with anti-*Torpedo* α antibodies, which poorly react with the endogenous *Xenopus* pump [16]. Fig. 1(B) shows fluorography of ^{14}C-labelled, immunoprecipitated α subunits of OS and OR pumps. Quantitative values of the amount of the labelled α subunits were averaged from three sets of experiments. Data normalised to the amount of OR are presented in Fig. 1(C); the OS and also OR α subunits of wild type and Glu mutants are all expressed to about the same extend.

3.2. Transport activity of expressed pump molecules

(a) To investigate whether expressed pumps are able to transport K^+, we used $^{86}Rb^+$ as a tracer, which can replace K^+ with nearly the same efficiency [26]. Fig. 2 shows the result of measurements of ouabain-sensitive $^{86}Rb^+$ uptake. All the pump species are capable to transport Rb^+ into the cell. Even OR334 and OS334 are definitely active in transporting Rb^+. This observation is in contrast to previous reports [13,14] that mutation of Glu_{334} to Ala in ouabain-resistant mutants leads to an inactive enzyme. The increase in $^{86}Rb^+$ uptake of the OS and OR pumps without additional mutations of Glu residues beyond the endogenous signal is proportional to the increase in the number of expressed

pumps (Fig. 1). In contrast, $^{86}Rb^+$ uptake mediated by both OR334 and OS334 mutants and by OR960 mutant is less that one would expect from the expression level (compare Fig. 1 and Fig. 2) suggesting that maximal pump turnover may be reduced in these mutants.

(b) Current generated by the pump as a consequence of unequal stoichiometry of transported Na^+ and K^+ during the reaction cycle is a direct measure of transport activity [22]. Together with measurement of $^{86}Rb^+$ uptake, the current measurement serves for determination of transport stoichiometry and extrusion of Na^+. Fig. 3 shows pump-mediated currents determined at 5 mM external K^+ in Na^+-free solution and at a membrane potential of -60 mV; qualitatively the same results are obtained for the entire potential range from -150 to $+30$ mV. All pump variants including Glu_{334} mutants are able to generate current. Maximal pump current is, however, reduced for both OS and OR Glu_{334} mutants and for the OR960 mutant. Comparison of the data presented in Fig. 2 and Fig. 3 reveals that the variations in pump current are similar to those in Rb^+ uptake, an indication that the transport stoichiometry is not altered.

3.3. ATPase activity of expressed pump molecules

To complete functional characterisation of the OS and OR pump mutants, we determined ATPase activities as measured by ouabain-sensitive formation of P_i for oocyte microsomes containing expressed pumps. Fig. 4 shows normalised ATPase activities for all pump variants. For OS pumps (Fig. 4(A)), ATPase activity was measured as a difference between activity without ouabain and with 10 mM of ouabain to block both expressed and endogenous pumps, and then the contribution of the endogenous *Xenopus* pumps has been subtracted. All OS pumps exhibit ATPase activity. P_i formation by OR wild type and OR959 mutant is at the level of the OS forms. In contrast, the Glu_{334} and Glu_{960} mutants exhibited a pronounced inhibition of ATPase activity (Fig. 4(B)), though they are expressed to the same extent (compare Fig. 1). The OR334 mutant is nearly inactive. This observation would be in line with published findings [13] that mutation of Glu_{329} to Ala results in an inactive enzyme. However, it would contradict the data in Fig. 2 and Fig. 3 showing that the mutants are active. The activity of ouabain-resistant pumps was determined as a difference of ATPase activities in presence of 10 μM ouabain to block the endogenous pumps and of 10 mM of ouabain to block both pumps. If after mutation of Glu_{334} and Glu_{960} the ATPase would have regained ouabain sensitivity, the reduced activity of OR344 and OR960 compared to the OS variants of mutants could be attributed to inhibition by the 10 μM ouabain. Therefore, we investigated ouabain dependence of ATPase and of transport activity in more detail.

Fig. 5 shows concentration-dependent inhibition of ATPase activity for a series of ouabain-sensitive (Xenopus, OS960) and ouabain-resistant pump species (OR, OR959, OR960). The data for RNA-injected oocytes represent the ATPase activity after subtraction of endogenous contribution. The OS ATPases have a similar sensitivity to the inhibitor as the endogenous pump as illustrated for OS960. The conclusion is based on the fact that the data for *Xenopus* and OS960 can be fitted by the same curve with a $K_{1/2}$ value for inhibition by ouabain of 0.39 ± 0.03 μM. For OR and OR959, the data could be fitted with a $K_{1/2}$ value of about 59 ± 5 μM and millimolar concentrations of ouabain are necessary for complete inhibition of their ATPase activity. This is obviously not the case for OR960 mutant, which can be inhibited by 12 ± 4 μM of ouabain by 50%. Therefore, the lower level of ATPase activity of OR960 compared to OS960 mediated transport activity (Figs. 3 and 4) can be attributed to the presence of 10 μM ouabain in the ATPase assay for OR pumps. Indeed, when ATPase activity for OR960 mutant was determined by the same assay as for the ouabain-sensitive pumps (data not shown), the values were higher and were comparable with those in Fig. 4(A). Interestingly, the dependency of ATPase activity on ouabain for the OR960 mutant exhibits a reduced slope, and looks like a two-component dependency. Since ATPase activity of endogenous pumps has already been subtracted for the data presented in Fig. 5, a contribution from *Xenopus* pumps can not be the reason. The best fit to the data of OR960 is obtained with a Hill coefficient of $n = 0.5$. A simple explanation would be a negative cooperativity between ouabain binding sites residing on the diprotomeric pump protein; binding of a first ouabain molecule to

the $\alpha\beta$ complex with high affinity ($K_{I1} = 0.8\,\mu M$) hinders the binding of the second ouabain molecule to the $(\alpha\beta)_2$ heterodimer ($K_{I2} = 130\,\mu M$).

Fig. 6 shows ouabain sensitivity of $^{86}Rb^+$ uptake in oocytes with expressed OR334 mutant in comparison to the ouabain-sensitive endogenous pump. In the RNA-injected oocytes about 65% of Rb^+ uptake is mediated by the OR334 pumps (see inset). Both sets of oocytes can be described by the same ouabain dependency with a $K_{1/2}$ value of $0.12 \pm 0.02\,\mu M$. If the OR334 mutant would have a considerably lower sensitivity for ouabain compared to the *Xenopus* pump, a difference in ouabain dependence should have been visible with a contribution of the OR334 of 2/3 to the total of Rb^+ uptake. Obviously, the ouabain-resistant pumps have, indeed, regained ouabain sensitivity after the mutation of Glu_{334} to Ala. As a consequence, nearly complete inhibition of the ATPase activity occurs in presence of $10\,\mu M$ ouabain (Fig. 4(B)).

In summary, the measurements of flux, current and ATPase activity performed under conditions of saturating ligand concentrations, clearly show that the mutants containing substitution of glutamic acid residues at the positions 334, 959 and 960 in both OS and OR forms of *Torpedo* pumps are equally expressed in the oocytes, and all of them exhibit transport and ATPase activity.

3.4. Sensitivity to external K^+

To answer the question whether transport is effected at non-saturating K^+ concentrations, we determined the voltage dependence of the apparent K_m values for pump stimulation by external K^+ by the procedure described previously [21,22,27]. Measurements were performed at external pH 7.8 to minimise activation of an additional, unusual inward current generated by the pump in the absence of external Na^+ and K^+ at pH 7.2 [23,24]. An example for voltage dependence of steady-state K^+-activated currents of OS pumps measured in the potential range between -150 and $+30$ mV for different K^+ concentrations ranging from 0.05 to 5 or 10 mM are shown in the inset to Fig. 7. The dependencies of K^+-activated currents on external K^+ concentration were analysed in terms of Michaelis-Menten kinetics (Eq. (1)) for each potential (as indicated e.g. for -50 and 0 mV by the vertical lines). Calculated K_m values as a function of membrane potential for OS and OR pumps are presented in Fig. 7. As it was demonstrated before [22], the voltage dependence of the K_m values for OS *Torpedo* pump can be described by the sum of two exponentials:

$$K_m = \sum_{i=1,2} K_{mi}(0)\exp(z_i\, EF/RT) \qquad (2)$$

and can be interpreted by successive voltage-dependent binding of the two K^+ ion into an access channel. In terms of this interpretation, the effective valencies z_i represent an apparent dielectric length of the channel for the respective K^+ ion, with the larger value for the first ion entering the channel. For the OS pumps (Fig. 7(A)) the data for three pump species (OS, OS959, OS960) could roughly be fitted by the same set of parameters (Table 1.); the mutations of Glu_{959} and Glu_{960} to Ala have practically no effect on pump stimulation by external K^+. There may be only a tendency to an increase in voltage dependence for OS959 and OS960 mutants.

Interestingly, substitution of the two bordering residues in the first ectodomain to convert the pump to ouabain-insensitive reduces the K_m values, which is due to a reduction of K_m at 0 mV and of the effective valencies z_i (compare Fig. 7(A) and (B), fit to the filled squares and Table 1). In terms of potential-dependent access to the binding sites, the change in z means reduction of the apparent dielectric length of the channel. For the OR960 mutant K_m values were indistinguishable from those for the OR, while those of the OR959 mutant were higher within the whole potential range. The fit with Eq. (2) reveals that the increase in $K_m(0)$ is the reason for the elevation while the z_i values are even reduced (Table 1).

Table 1
Parameters of Eq. (2) fitted to the data presented in Fig. 7

Pump type	$K_{m1}(0)$ (mM)	$K_{m2}(0)$ (mM)	$z1$	$z2$
OS				
OS959	0.32	0.32	0.91	0.20
OS960				
OR	0.11	0.20	0.81	0.10
OR959	0.59	0.18	0.69	0.02
OR960	0.11	0.20	0.81	0.10

4. Discussion

Carboxyl oxygens of the glutamic acid residues Glu_{327}, Glu_{953} and Glu_{954} (numbering for sheep $\alpha 1$) in the putative intramembraneous segments IV and IX of the α subunit have been considered as possible candidates for the coordination of translocated cations [3,6,28–30]. In our investigation of their functional significance, the homologous residues in *Torpedo* α subunits Glu_{334}, Glu_{959} and Glu_{960} were substituted by Ala to exclude any contribution of oxygen-containing groups at these positions. All of these mutants are expressed to equal extend in *Xenopus* oocytes as judged by [^{3}H]ouabain binding or immunoprecipitation with specific anti-α antibodies (Fig. 1) and have characteristics essential for Na^+,K^+-transporting ATPases. We can conclude, therefore, that carboxyl oxygens at none of these positions are critical for the formation of cation binding sites. Comparative analysis of transport characteristics, ATPase activity, sensitivity to ouabain and voltage dependence of apparent K_m values for external K^+ reveal, however, alterations.

4.1. OS pumps

Being expressed to an equal extend in the oocytes, the investigated OS mutants exhibit different maximal transport activity. Mutation of the neighbouring Glu_{959} and Glu_{960} results in only slight reduction of $^{86}Rb^+$ uptake and of pump-generated current (Fig. 2(A) and 3(A)) while mutation of Glu_{334} leads to a clear inhibition of about 50% compared to the values for the OS pump. The reduction of $^{86}Rb^+$ uptake paralleled by a reduction of pump current suggests that the transport stoichiometry of 3 Na^+/2 K^+ is not affected. On the other hand, comparison of the relative changes in ATPase activity (Fig. 4(A)) with those in transport activity (Fig. 2(A) and 3(A)) reveals for the Glu_{334} and Glu_{960} mutants a more pronounced reduction for the hydrolytic activity. A possible explanation for the mismatch of transport and ATPase activity may be attributed to the fact that the respective measurements were performed under different ionic conditions. In transport measurements the ATPase molecules face different ion composition in the E1 and E2 conformation. In E1 the cation binding sites face the cytoplasm with optimised ion composition of 100 mM [Na^+] and 5 mM [K^+], as determined by ion-selective microelectrodes ([27], and S. Elsner unpubl.), which is compatible with the concentrations for Na^+ and K^+ in the ATPase assay (140 mM [Na^+] and 14 mM [K^+]). In contrast, the extracellular medium does not contain Na^+ in our transport measurements. Measurements of ATPase activity were performed with microsomal membrane fragments, which means identical concentrations of Na^+ and K^+ on both sides of the membrane. External Na^+ inhibits the pump by interacting competitively to K^+ with the E2 form [27]. Hence, an increased affinity of E2 for Na^+ could account for the apparently reduced ATPase activity of the Glu_{334} and Glu_{960} mutants that was determined in presence of 140 mM Na^+.

4.2. OR pumps

In contrast to the observations for the OS variants, OR960 has a clearly reduced maximum transport activity compared to OR and the OR959 mutant (Fig. 2(B) and 3(B)). This reduction cannot be attributed to an increase of the apparent K_m value for external K^+, the parameters are identical to those of OR (see Table 1). This indicates that steps in the reaction cycle other than interaction with extracellular K^+ may be involved in the inhibition of maximal pump turnover in the OR960 mutant. Oppositely, mutation of Glu_{959} leads to higher K_m values for external K^+ over the entire potential range (Fig. 7(B)). This increase, however, does not effect the measurements of maximal $^{86}Rb^+$ uptake and pump current performed in presence of 5 mM of external Rb^+ or K^+, respectively, which is much higher than K_m.

4.3. Ouabain sensitivity

The most striking consequence of the mutations performed in this work is the complete restoration of sensitivity to ouabain in the OR334 mutant, which was originally assumed to be ouabain-insensitive. A partial restoration of ouabain-sensitivity was obtained for the OR960 mutant. Since the assay for determination of ATPase activity for OR pump variants was performed in the presence of 10 μM of ouabain to block the endogenous contribution, OR960 and OR334 mutants looked as if they are nearly inactive

(Fig. 4(B)). This demonstrates that evaluation of the importance of the mutations in ouabain-insensitive pumps using ouabain selection methodology [14,31] may lead to a misinterpretation. An essential role of the Glu_{334} (Glu_{329}) and of residues with long side chains at this position in formation of a gate to the occlusion sites [13] is not supported by our findings.

The presence of charged residues bordering the first ectodomain has been considered as a necessary attribute of ouabain-resistant pump isoforms ([32], for review see [1]). Indeed, mutation of Glu-118 to Arg and Asn-129 to Asp confers ouabain resistance also to the otherwise ouabain-sensitive *Torpedo* pump (see Fig. 5). Interestingly, if in addition Glu_{334} is substituted by Ala these mutations become ineffective. The residue homologous to Glu_{334} is conserved in all known pump isoforms and in other P-type ATPases, the Ca^{2+}-ATPase and H^+,K^+-ATPase [29,33–35]. Substitution of the corresponding Glu_{329} of rat $\alpha 1$ or rat $\alpha 2^*$ forms by residues with longer side chains (Gln or Leu) does not effect affinity for ouabain [12,31]. This may suggest that the length of the side chain of the amino acid residue at position 334 is more important for adaptation of the pump to ouabain binding than for cation interaction. Substitution of this Glu neighbouring the Pro_{333} by a residue with a short side chain may increase local perturbation within the IV putative transmembrane domain optimising access of ouabain to the receptor site of the molecule.

4.4. Potential dependence of K_m for external K^+

Due to the electrogenic nature of the Na^+,K^+-ATPase, transport activity is controlled by membrane potential. The fact that the potential dependency can be completely eliminated in absence of extracellular Na^+ and at high K^+ suggests that binding of external cations to the protein in its E2P form may be a dominating voltage-dependent steps in the reaction cycle [21,22,27,36–38]. Measurements of pump-generated current at different potentials and concentrations of the activator K^+ revealed an exponential dependency of the apparent K_m value for external K^+. This finding formed the basis for the hypothesis that extracellular cations have a potential-dependent access to their binding sites by passing through ''high-field'' access channel or ion well [21,22,39,40] so that they sense part of the electrical field in the membrane. The apparent dielectric length of the channel is represented by the effective valency z (see Eq. (2)). Additional evidence supporting this idea was obtained later by measurements of transient currents [41–43] and of $^{22}Na^+$ tracer fluxes [37]. In *Torpedo* pump, the voltage dependence of K_m for external K^+ is described by a two-exponential function, which was interpreted by sequential binding of the two K^+ ions that move in single file into the access channel [22].

Determination of the $K_m(V)$ for stimulation of the pump by external K^+ for mutated pumps was the most time-consuming part of this investigation. To analyse the current of the expressed pumps, the current generated by endogenous pumps was subtracted from the total signal. As demonstrated previously, these results were indistinguishable from those where the endogenous component was blocked by 1 μM ouabain and current generated by the expressed *Torpedo* OR pumps could, therefore, be measured directly [10]. The dependencies of $K_m(V)$ for OS, OS959 and OS960 pumps could be fitted by the same two-exponential function within the experimental error (see fitted parameters in Table 1). The absence of considerable changes in K_m values for OS959 and OS960 mutants argues against their direct contribution in formation of the cation-translocation pathway in OS variants. Only a tendency of an increase in steepness of voltage dependence was observed in OS959 and OS960 (Fig. 7(A)). We have reported previously that mutation of Glu_{960} in the OS form led to a much more pronounced alteration in the potential dependence [10]. These measurements were performed at pH 7.2 (in our present investigation at pH 7.8) suggesting that the effect of this mutations is pH-dependent. Taking into account that the pK of free glutamic acid is 4.3, one could expect that at pH 7.2 the carboxylate group is fully deprotonated and no additional effects should be observed by further increase of pH. However, electrostatic interactions within the pump protein and particularly shielding by the neighbouring negatively charged residues may increase the apparent pK value, so that the difference in pH 7.2 and 7.8 may still influence protonation of carboxylate groups. The effects of pH on the Glu_{959} and Glu_{960} mutants are currently investigated in more detail. In our previous investigations at pH 7.2 [10],

we also analysed K_m-E dependencies for OS334 and found a slight reduction in voltage dependence (reduced z values). Because of the relatively low degree of expression compared to the wild-type and to the Glu_{959} and Glu_{960} mutants (see Fig. 3) a detailed analysis of I–V dependencies at low $[K^+]$ becomes questionable and is not presented in this investigation.

Interestingly, transformation of OS to the OR by mutation of Gln_{118} to Arg and Asn_{129} to Asp results in a decrease of apparent K_m values, which can be attributed to the simultaneous reductions of K_m at 0 mV and of the effective valencies z. (Table 1). On the basis of competition between ouabain and K^+ for binding to the E2P conformation one could, indeed, expect an increase in apparent affinity for external K^+ for a less ouabain-sensitive pump. One may speculate, therefore, that mutation of Gln_{118} and Asn_{129} bordering the membrane segments to charged residues modulates the passage of the cations to their occlusion site. In view of the identity in ionic selectivity to different K^+ congeners [26] one may expect also structure-functional similarities in the K^+ binding in the K^+ channels and in the sodium pump. In cation-selective channels, the so-called H5 segment connecting the S5 and S6 transmembrane segment (P loop) contributes to the formation of the pore region, as was first suggested for the Na^+ channel [44], with carbonyl oxygens facing the pore [45]. In addition, the bordering region of S5 with negatively charged residue was also suggested to give an important contribution [46]. Another explanation of changes in K_m values would be an indirect allosteric influence on the cation-conducting pathway. Allosteric effects have recently been demonstrated to occur as a result of structural modifications in the domains of the α subunit that are obviously distant from the cation-binding sites [20,47].

In contrast to a predicted critical role of the glutamic acid residues on basis of chemical modification [3], the mutations of the glutamic acid residues in the IXth putative membrane segment leads to only slight alterations in the effective valencies associated with K^+ binding, leaving the pump active. Only the mutation of Glu_{959} in OR form leads to an increased apparent K_m values. The observed difference in K_m values in the Glu_{959} mutants of OR and OS forms suggests that Glu_{959} may be differently exposed with respect to cation translocating pathways so that its contribution is more pronounced in the ouabain-insensitive variant of the protein.

The carboxylate-containing residues Glu_{779} in intramembraneous segment V [48–50] and Asp_{804} and Asp_{808} in segment VI [51,52] were investigated as possible candidates for the cation binding structure. Glu_{779} was initially suggested to be critical for cation coordination on the basis of chemical modification [48,53]. However, mutations of this residue turned out to leave the enzyme active [49,50]. The site-directed mutagenesis of Asp_{804} and Asp_{808}, on the other hand, revealed that the mutated pumps no longer bind K^+ and Na^+ suggesting their critical role in the cation binding structure.

In conclusion, the effects of mutations of the glutamic acid residues 334, 959, and 960 to Ala excludes the possibility that the carboxylate or carbonyl oxygens at these positions directly participate in the formation of cation binding sites. At most indirect participance of the residues in regulation of passage of the cations to the occlusion sites may be stipulated. However, the effects of the mutations on ouabain sensitivity indicate the important role of Glu_{334} and Glu_{960} in preserving the ouabain-resistant conformation of the pump protein.

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