Mutation to the Glutamate in the Fourth Membrane Segment of Na^+,K^+-ATPase and Ca^{2+}-ATPase Affects Cation Binding from Both Sides of the Membrane and Destabilizes the Occluded Enzyme Forms†

Bente Vilsen* and Jens Peter Andersen*

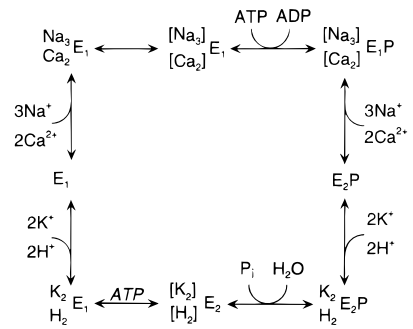
Department of Physiology, University of Aarhus, Ole Worms Allé 160, DK-8000 Aarhus C, Denmark

Received February 4, 1998; Revised Manuscript Received May 15, 1998

ABSTRACT: The functional consequences of mutations Glu329 → Gln in the Na^+,K^+-ATPase and Glu309 → Asp in the sarco(endo)plasmic reticulum Ca^{2+}-ATPase were analyzed and compared. Relative to the wild-type Na^+,K^+-ATPase, the Glu329 → Gln mutant exhibited a 20-fold reduction in the apparent K^+ affinity determined by titration of the rate of ATP hydrolysis at 50 μM ATP, and the rate of release of occluded K^+ or Rb^+ to the cytoplasmic side of the membrane was up to 30-fold enhanced by the mutation, as measured in kinetic studies of the phosphorylation by ATP of enzyme equilibrated with K^+ or Rb^+. The apparent affinity for extracellular K^+ was 12-fold reduced by the Glu329 → Gln mutation, as determined by K^+ titration of the dephosphorylation. The maximum rate of phosphorylation by ATP of the Na^+ form of the enzyme was reduced more than 2-fold by the mutation, but this effect could be counteracted by stabilizing Na^+ occlusion with oligomycin. Similar studies on the Glu309 → Asp mutant of the Ca^{2+}-ATPase showed that the maximum rate of phosphorylation of the Ca^{2+} form was 8−9-fold reduced relative to that of the wild-type Ca^{2+}-ATPase, and no Ca^{2+} occlusion could be detected in the mutant. Dephosphorylation of the phosphoenzyme intermediate formed with P_i was blocked in the Ca^{2+}-ATPase mutant. The sensitivity to inhibition by thapsigargin, which binds selectively to the putative proton-occluded form of the Ca^{2+}-ATPase, was reduced almost 300-fold in the mutant at neutral pH, but only 3−4-fold at pH 6.0. These data indicate that the mutations destabilize the occluded enzyme forms and interfere with cation binding from the extracytoplasmic side as well as with the gating process at the cytoplasmic entrance to the cation occlusion pocket.

The Na^+,K^+-ATPase[1] and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase mediate active transport of cations driven by the hydrolysis of ATP at stoichiometries of 3:2:1 (Na^+:K^+:ATP) and 2:1 (Ca^{2+}:ATP), respectively (*1*, *2*). As shown in Scheme 1, the ion translocation is brought about by a series of conformational changes coupled to the formation and breakdown of an aspartyl phosphorylated intermediate, the hallmark of the P-type ATPase family. The phosphorylation of the Na^+,K^+-ATPase by ATP is activated by the binding of three cytoplasmic sodium ions at the transport sites of the E_1 form. The analogous reaction in the Ca^{2+}-ATPase is activated by the binding of two cytoplasmic Ca^{2+}s. Following conformational changes in the phosphoenzyme, the translocated sodium or calcium ions are released on the extracytoplasmic side of the membrane. In the Na^+,K^+-ATPase, the ensuing dephosphorylation of the E_2P form is triggered by the binding of extracytoplasmic K^+ to be transported in the opposite direction. There is evidence that the Ca^{2+}-ATPase likewise countertransports H^+ (or H_3O^+) (*3*), and the dephosphorylation of the E_2P form of the Ca^{2+}-ATPase may in analogy with the Na^+,K^+-ATPase be accelerated by the binding of the counterion.

Scheme 1: Common Reaction Scheme for the Na^+,K^+-ATPase and the Ca^{2+}-ATPase[a]



[a] Ions transported by the Na^+,K^+-ATPase are indicated *above* those transported by the Ca^{2+}-ATPase. Occluded ions are shown in *brackets*. The *ATP* shown in italics indicates the modulatory effect of ATP on the reaction rate.

†This research was supported by grants from the Danish Medical Research Council, the NOVO Nordisk Foundation, and the Research Foundation of Aarhus University.

* Correspondence should be addressed to either author. Fax: 45 86 12 90 65. E-mail: bv@fi.au.dk (B.V.) or jpa@fi.au.dk (J.P.A.).

[1] Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; Ca^{2+}-ATPase, the sarco(endo)plasmic reticulum Ca^{2+}-transporting adenosine triphosphatase (EC 3.6.1.38); E_1 and E_2, different conformational states of the Na^+,K^+-ATPase or the Ca^{2+}-ATPase; EP, phosphorylated enzyme; $K_{0.5}$, ligand concentration giving half-maximum activation; M1-M10, putative membrane segments numbered from the NH_2-terminal end of the peptide chain; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Na^+,K^+-ATPase, the Na^+- and K^+-transporting adenosine triphosphatase (EC 3.6.1.37); S1−S5, stalk segments connecting M1−M5 with the cytoplasmic domains; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

S0006-2960(98)00292-X CCC: $15.00
Published on Web 07/16/1998

A central theme in the understanding of the mechanism of ion translocation is the occurrence of so-called "occluded" enzyme forms, in which the bound ions appear to have no immediate access to the medium on either side of the membrane (indicated by brackets in Scheme 1). These "occluded" ions seem to be separated from the medium by large energy barriers ("gates"), which must be broken down to allow the ions to dissociate. In the Na^+,K^+-ATPase, the K^+-induced dephosphorylation of E_2P normally leads to formation of a rather stable K^+-occluded $E_2(K)$ form,[2] and millimolar ATP is needed to activate deocclusion toward the cytoplasmic side (*1*, *4*). The occlusion of Na^+ in the E_1 form of Na^+,K^+-ATPase or of Ca^{2+} in the E_1 form of Ca^{2+}-ATPase is more transient, but can be demonstrated experimentally in the presence of agents such as oligomycin (*5*) or the nonphosphorylating substrate analogue CrATP (*6*, *7*) that stabilize the occluded forms.

To elucidate the molecular details of ion translocation, it is essential to identify the amino acid residues involved in the binding of the cations and in the opening and closing of the gates to the cation occlusion pocket(s). An important question is whether the residues taking part in the binding of the cations at the two sides of the membrane are the same or whether the cations migrate from one set of residues to another. Previous mutagenesis studies of the Ca^{2+}-ATPase have pointed to five residues with oxygen-containing side chains in membrane segments M4, M5, and M6 as being important in connection with the binding and occlusion of Ca^{2+} (*8*−*11*). These residues are conserved in the Na^+,K^+-ATPase, where they may play a role in Na^+ and/or K^+ binding (*12*−*17*). However, in neither of the two ATPases is the exact functional role of any of these residues very clear at present. An important example is the glutamate in M4. This residue is located in the cytoplasmic half of M4, which is linked through the S4 stalk segment to the phosphorylated aspartic acid residue in the cytoplasmic domain. In the Na^+,K^+-ATPase, substitution of the glutamate in M4 (Glu329)[3] with glutamine seems to be compatible with Na^+ and K^+ transport, albeit with reduced apparent affinity for the cations (*12*, *13*), whereas the transport function and possibly the structural integrity of the protein are lost upon substitution with aspartate or alanine (*14*, *15*). Substitution of the homologous residue Glu309[3] in the Ca^{2+}-ATPase with glutamine completely abolishes the occlusion and transport of Ca^{2+} as well as Ca^{2+} activated phosphorylation by ATP, but not Ca^{2+} inhibition of "backdoor" phosphorylation by P_i (*8*, *9*, *11*, *18*). Ca^{2+} transport is also abolished when Glu309 is replaced with aspartate, retaining the negative charge, but the Glu309 → Asp mutant can be phosphorylated by ATP in a Ca^{2+} activated reaction (*19*), suggesting that calcium binding is preserved. In the Ca^{2+}-ATPase, as well as the Na^+,K^+-ATPase, residues located close to the glutamate, near the boundary between M4 and S4, have been shown to be essential to the E_1P to E_2P conformational change (*20*, *21*).

The present investigation has been undertaken to elucidate the functional roles of the glutamate in M4 by comparing the consequences of mutation of this residue in Na^+,K^+-ATPase and Ca^{2+}-ATPase. We have focused on the substitutions Glu329 → Gln in the Na^+,K^+-ATPase and Glu309 → Asp in the Ca^{2+}-ATPase, since these two minimally perturbed mutants retain the ability to be phosphorylated by ATP, allowing investigation of the effects of the mutations on the various partial reaction steps of the enzyme cycles. Because the events occurring in the phosphorylation site are closely linked to conformational changes in the cation-binding domain, changes in the cation-binding properties induced by the mutations are reflected as changes in the phosphorylation and dephosphorylation kinetics. The mutations seem to affect both the cytoplasmically and the extracytoplasmically facing cation sites, as well as the stability of the occluded enzyme intermediates.[4]

MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis of the cDNAs encoding the ouabain-insensitive rat α1-isoform of the Na^+,K^+-ATPase and the rabbit SERCA1 Ca^{2+}-ATPase isoform and the expression of mutants and wild-types in COS-1 cells was performed essentially as described in refs *10*, *12*, *14*, and *21*, using transient expression for the Ca^{2+}-ATPase and selection of stable transfectants in ouabain for the Na^+,K^+-ATPase. Membrane preparation, protein determination, and measurement of the molecular rate of ATP hydrolysis ("turnover number") as well as Ca^{2+} occlusion in the Ca^{2+}-ATPase have also been detailed previously (*9*, *10*, *20*, *21*). For the Na^+,K^+-ATPase, 10 μM ouabain was routinely included in all assays to inhibit the endogenous enzyme. The maximum phosphorylation level used for calculation of turnover number was determined in the presence of 150 mM Na^+ and 20 μg oligomycin/mL, using a filtration procedure (*20*).

Phosphorylation and Dephosphorylation Kinetics of the Na^+,K^+-ATPase. Phosphorylation of the Glu329 → Gln mutant or the wild-type Na^+,K^+-ATPase in E_1 form (Figure 4) was carried out on deoxycholate-treated leaky membranes incubated in 20 mM Tris buffer (pH 7.4), 3 mM $MgCl_2$, and 10 μM ouabain. The desired concentration of NaCl (or 50 mM KCl for determination of background) together with 1 mM EGTA, with or without 20 μg of oligomycin/mL, was added 10 min before the phosphorylation reaction was initiated with 2 μM [γ-^{32}P]ATP. The reaction mixture (100 μL) contained in an Eppendorf tube immersed in ice water was stirred continually by a vertically orientated tiny magnet bar (*20*). Acid quenching of the phosphoenzyme was performed at serial time intervals by addition of 1 mL of 1 M ice-cold phosphoric acid (pH 2.4).

To study the rate of deocclusion of K^+ or Rb^+ (Figure 2), a modified version of the procedure described in ref *22* was applied. Deoxycholate-treated microsomes were first incu-

[2] In the text, parentheses are used to indicate the presence of occluded ions without specifying their stoichiometry. Thus, for the wild-type Na^+,K^+-ATPase, $E_2(K)$ refers to the intermediate indicated as $[K_2]E_2$ in Scheme 1 and $E_1(Na)$ refers to $[Na_3]E_1$ etc.

[3] The glutamate residue in the fourth membrane segment of the rat α1-isoform of the Na^+,K^+-ATPase examined in the present study is Glu329. The equivalent residue of the sheep isoform is Glu327. In the rabbit SERCA1 Ca^{2+}-ATPase isoform, the homologous residue is Glu309.

[4] Part of this work was presented in preliminary form at the VIIIth International Conference on the Na/K-ATPase and related transport ATPases in Mar del Plata, Argentina, August 1996, and at the XXXIII International Congress of Physiological Sciences in St. Petersburg, Russia, June 1997.

bated at 37 °C for 10 min in 20 μM ouabain, 3 mM $MgCl_2$, and 20 mM Tris buffer (pH 7.5) or 20 mM MES/Tris buffer (pH 6.5). This was followed by incubation at room temperature for 1 h with the indicated K^+ or Rb^+ concentration. At the end of the incubation at room temperature, oligomycin was added at a concentration of 150 μg/mL, and 1 min later the sample, comprising 41 μL, was cooled to 10 or 0 °C. Deocclusion of K^+ or Rb^+ and phosphorylation of the deoccluded enzyme were initiated by addition of 360 μL of a phosphorylation solution (of the same temperature) to produce final concentrations of 100 mM NaCl, 1 μM [γ-^{32}P]-ATP, 1 mM $MgCl_2$, 1 mM EGTA, 20 mM Tris buffer (pH 7.5), or 20 mM MES/Tris buffer (pH 6.5) and dilute the K^+ or Rb^+ concentration to $^1/_{10}$ that originally present during the 1 h incubation. In several control experiments, we have verified that such low concentrations of K^+ or Rb^+ are without measurable influence on the rate of phosphorylation of enzyme preincubated in the presence of 100 mM Na^+ and oligomycin. To monitor the time course of deocclusion by the phosphorylation level, acid quenching of the phosphoenzyme was performed at serial time intervals following the dilution. The reaction mixture was stirred continually as described above, the temperature being further controlled by a thermostated water bath. For determination of maximum phosphorylation (representing fully deoccluded enzyme), the 1 h incubation was carried out in the presence of 50 mM Na^+ and absence of K^+ or Rb^+. For background phosphorylation, the K^+ concentration in the incubation medium was 50 mM, and no Na^+ was present in the phosphorylation solution.

To study the effect of ADP or K^+ on the rate of dephosphorylation (*20*), phosphorylation was first carried out for 10 s at 0 °C in 100 μL of reaction mixture containing 20 mM NaCl, 20 mM Tris buffer (pH 7.4), 130 mM choline chloride, 3 mM $MgCl_2$, 1 mM EGTA, and 2 μM [γ-^{32}P]-ATP. This was followed by the addition of 10 μL ice-cold chase solution producing a final concentration of 1 mM unlabeled ATP (to prevent further phosphorylation with [γ-^{32}P]ATP) together with 2.5 mM ADP (Figure 3A) or the indicated KCl concentration (Figure 3B), and acid quenching was performed at serial time intervals. The rate coefficients shown in Figure 3B were calculated from data points corresponding to 2 s dephosphorylation (cf. ref *20*).

In all of the kinetic experiments described above, quantification of the amount of ^{32}P incorporated in the acid-quenched Na^+,K^+-ATPase phosphoenzyme was carried out using a Packard InstantImager apparatus for electronic autoradiography of the 100 kDa band separated by acid gel electrophoresis (*10*, *12*, *20*).

Phosphorylation and Dephosphorylation of the Ca^{2+}-ATPase. Kinetic studies of phosphorylation of the E_1 form of Ca^{2+}-ATPase were carried out on 100 μL samples at 0 °C, using the same mixing system as described above. The phosphorylation reaction was initiated by addition of 2 μM [γ-^{32}P]ATP to microsomes suspended in 20 mM MOPS buffer (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, and the indicated Ca^{2+} concentration buffered with EGTA. Following acid quenching by the addition of 1 mL of ice-cold trichloroacetic acid containing 1 mM KH_2PO_4, ^{32}P incorporation was determined as described for the Na^+,K^+-ATPase.

To study dephosphorylation of the E_2P intermediate (Figure 8), phosphorylation with $^{32}P_i$ was first carried out at room temperature in 0.25 mM $^{32}P_i$, 100 mM MES/Tris buffer (pH 6.0), 20% (v/v) dimethyl sulfoxide (to increase the affinity for P_i, cf. ref *18*), 10 mM $MgCl_2$, and 2 mM EGTA. The sample was then cooled to 0 °C, and the phosphorylation by $^{32}P_i$ was terminated by a 20-fold dilution in ice-cold medium containing 50 mM TES/Tris buffer (pH 7.0), 10 mM $MgCl_2$, 2 mM EGTA, 100 mM KCl, and 5 mM KH_2PO_4, followed by acid quenching at serial time intervals to monitor the time course of dephosphorylation.

Thapsigargin inhibition of phosphorylation (Figure 9) was studied after 15 min incubation of microsomes corresponding to 10 nM Ca^{2+}-ATPase at 20 °C with various thapsigargin concentrations in 2 mM EGTA and 50 mM MOPS/Tris buffer (pH 7.0) or 50 mM MES/Tris buffer (pH 6.0). Phosphorylation with 2 μM [γ-^{32}P]ATP was carried out for 5 s at 20 °C, following the addition of 5 mM $MgCl_2$ and 3.5 mM $CaCl_2$ (producing approximately 1.5 mM free Ca^{2+}, i.e., more than sufficient to saturate the sites on the mutant or the wild-type). The enzyme concentration was determined by measurement of the maximum amount of phosphoenzyme formed by phosphorylation with $^{32}P_i$ as well as by a quantitative enzyme-linked immunosorbent assay using monoclonal antibody A52 (*21*).

Curve Fitting. Data were analyzed by nonlinear regression using the Sigmaplot program (Jandel Scientific), and the best fits are shown as curves in the figures. Ligand concentration dependencies of reaction rates were fitted to the Hill equation:

$$V = (V_{max} - V_0)[L]^n/(K_{0.5}^n + [L]^n) + V_0$$

The time dependence of phosphorylation of enzyme in the E_1 form was fitted to a monoexponential time course, whereas the time dependence of phosphorylation starting from a mixture of E_1 and the K^+- or Rb^+-occluded E_2 form was fitted to a biphasic time course:

$$EP = (EP_{max} - E_{occluded})\,(1 - e^{-ht}) + E_{occluded}\,(1 - e^{-kt})$$

$E_{occluded}$ represents the part of the enzyme that phosphorylates slowly because of its initial presence as the occluded E_2 form. $EP_{max} - E_{occluded}$ is the fraction of the enzyme that phosphorylates almost instantaneously, because it is present as the nonoccluded form. Good fits could be obtained for all values of $h > 1\ s^{-1}$, without any significant variation of the values being determined for $E_{occluded}$ and the rate constant k corresponding to the slow phase. Consequently, the term $(1 - e^{-ht})$ was set equal to one to obtain the curves in Figure 2.

The dependence of the phosphorylation level on the molar ratio (R) between the total thapsigargin concentration (free plus bound) and the enzyme concentration was fitted to the equation

$$EP = 100\%(1 - F_b)$$

where F_b is the fraction of the enzyme which has bound thapsigargin and therefore is inactive:

$$F_b = 1/2[R + R_0 + 1 - (R^2 + {R_0}^2 + 2RR_0 + 2R_0 - 2R + 1)^{1/2}]$$

To derive this equation, a simple one-site binding model is

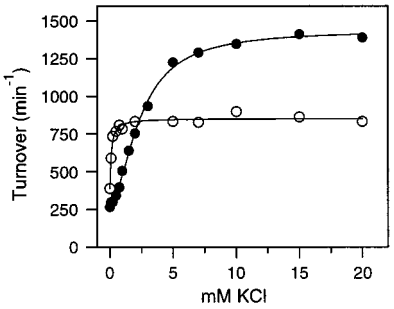


FIGURE 1: K^+ dependence of the molecular rate of ATP hydrolysis ("turnover number") of the Glu329 → Gln mutant (●) and the wild-type Na^+,K^+-ATPase (○) measured at 37 °C in the presence of 50 μM ATP, 40 mM NaCl, 3 mM $MgCl_2$, 30 mM histidine buffer (pH 7.4), 1 mM EGTA, 10 μM ouabain, and the indicated K^+ concentrations. The data points (average values corresponding to two experiments) were fitted to the Hill equation giving $K_{0.5}(K^+)$ values of 2.42 mM and 0.12 mM and V_{max} values of 1440 and 853 min^{-1} for the mutant and the wild-type, respectively.

considered. R_0 is the ratio between the dissociation constant for the enzyme−thapsigargin complex and the enzyme concentration. The reason for presenting the data as a function of *R* instead of the free concentration of thapsigargin is that the latter is unknown as a consequence of the extremely high affinity displayed by the enzyme toward thapsigargin (K_d in the nanomolar range).

RESULTS

K^+ Dependence of Na^+,K^+-ATPase Activity at Low ATP Concentration. Previous experiments with the Glu329 → Gln mutant demonstrated a 6-fold reduction of the apparent affinity for K^+ relative to the wild-type enzyme as determined in titrations of Na^+,K^+-ATPase activity at 3 mM ATP (*12*). It is well-known that in the wild-type Na^+,K^+-ATPase the binding of ATP with low affinity accelerates the $E_2(K)$ to E_1 conversion, thereby enhancing the rate of K^+ release to the cytoplasmic side (*1*, *4*, *23*). In this study, we therefore examined the apparent K^+ affinity at lower ATP concentrations, i.e., more favorable conditions for K^+ binding. Results obtained at 50 μM ATP are presented in Figure 1. Under these conditions, a spectacular difference between the apparent K^+ affinities of wild-type and mutant is revealed, amounting to as much as 20-fold.

Turnover Number for Na^+,K^+- and Na^+,Rb^+-ATPase Activities. The molecular rate of ATP hydrolysis ("turnover number") was determined as the ratio between the ATPase activity and the active-site concentration measured as the maximum oligomycin supported phosphorylation from ATP. Under optimum conditions for ATP hydrolysis at 3 mM ATP and saturating Na^+ and K^+ concentrations, the turnover number was found to be more than 3-fold reduced in the Glu329 → Gln mutant relative to the wild-type (Table 1). At low ATP concentrations (50 and 100 μM shown in Figure 1 and Table 1, respectively) and saturating Na^+ and K^+ concentrations, the turnover number for Na^+,K^+-ATPase activity was higher in the mutant than in the wild-type, consistent with the previously reported 15-fold increase in apparent affinity for ATP in the mutant relative to the wild-type (*12*). When K^+ was replaced by Rb^+, the turnover number decreased considerably in the wild-type, and more so at low than at high ATP concentration (Table 1). This is accounted for by the lower rate of the Rb^+-releasing $E_2(Rb)$ → E_1 transition relative to the K^+-releasing $E_2(K)$ → E_1 transition (*4*, *23*). As shown in Table 1, the Glu329 → Gln mutant behaved differently. Thus, the turnover number for the Na^+,Rb^+-ATPase activity of the mutant is seen to be almost unchanged, or even enhanced (at high ATP concentration), relative to the turnover number for Na^+,K^+-ATPase activity.

Table 1: Turnover Number for Na^+,K^+-ATPase Activity and the Effect of Replacing K^+ by Rb^+ [a]

	3 mM ATP		100 μM ATP	
	KCl	RbCl	KCl	RbCl
wild-type Na^+,K^+-ATPase	8500 ± 181	5584 ± 76	1570 ± 45	639 ± 34
mutant Glu329 → Gln	2530 ± 106	3553 ± 34	1762 ± 57	1857 ± 19

[a] The ATPase activity was measured at 37 °C in the presence of 130 mM NaCl, 20 mM KCl or RbCl, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, and the indicated concentrations of ATP. The turnover number in inverse minutes was calculated as the ratio between the ATPase activity and the active site concentration determined as the maximum amount of phosphoenzyme formed in the presence of oligomycin. Average values ± SEM corresponding to at least three experiments are shown.

Deoccluding $E_2(K)$ to E_1 Transition of the Na^+,K^+-ATPase. The above-described observations suggest that the $E_2(K)$ → E_1 and $E_2(Rb)$ → E_1 transitions may be less rate limiting for the overall reaction in the Glu329 → Gln mutant than in the wild-type. To examine K^+ and Rb^+ occlusion and the rate of release of the occluded K^+ or Rb^+ at low ATP concentration, the method described by Blostein and co-workers (*22*) was adapted. The enzyme was first equilibrated with K^+ or Rb^+ in the absence of Na^+, to form the occluded $E_2(K)$ or $E_2(Rb)$ intermediate. The time dependence of phosphorylation was then studied upon a 10-fold dilution of the K^+- or Rb^+-equilibrated enzyme into 100 mM Na^+ and 1 μM [γ-^{32}P]ATP (Figure 2). Under these conditions, the phosphorylation of $E_2(K)$ proceeds through the steps $E_2(K)$ → E_1 → $E_1P(Na)$, in which the release of occluded K^+ is followed by the binding and occlusion of Na^+, triggering phosphoryl transfer from ATP. Oligomycin was added at the end of the equilibration with K^+ or Rb^+ to ensure a rapid phosphorylation of the E_1 form (see below), so that the slow release of K^+ or Rb^+ was rate limiting for the phosphorylation of the enzyme fraction being present in the occluded E_2 form. In addition to a slow phase reflecting release of occluded K^+ or Rb^+, the time course of phosphorylation contains a rapid phase corresponding to nonoccluded enzyme which is able to bind Na^+ instantaneously and phosphorylate within the first 5 s after the addition of ATP. The extents of the slow and rapid phases depend on the K^+ or Rb^+ concentration and the equilibrium constant for the interconversion of occluded and nonoccluded enzyme forms. Following equilibration with 8 mM K^+ at pH 7.5, the wild-type Na^+,K^+-ATPase exhibited almost no rapid phase of phosphorylation, reflecting a close to 100% saturation of the K^+ occlusion sites. The extent of the rapid phase was approximately 50% for the Glu329 → Gln mutant under the same conditions (Figure 2A). At 10 °C, the rate constant characterizing the slow phase of phosphorylation was 30-fold higher in the Glu329 → Gln mutant compared with the wild-type. When the temperature was reduced to 0 °C during the phosphorylation, the slow component was characterized

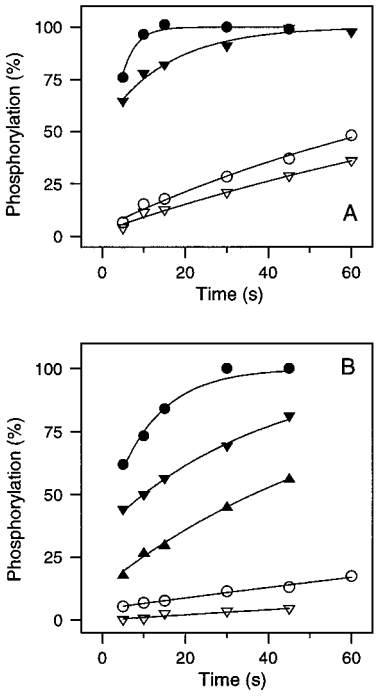


FIGURE 2: Time course of phosphorylation with [γ-^{32}P]ATP of the Glu329 → Gln mutant (●, ▲, ▼) and the wild-type Na^+,K^+-ATPase (○, ▽) in the presence of 100 mM Na^+ after 1 h incubation with K^+ at pH 7.5 or with Rb^+ at pH 6.5. (Panel A) Incubation with 8 mM K^+ at pH 7.5 was followed by phosphorylation at pH 7.5 and 10 °C (●, ○) or 0 °C (▼, ▽). (Panel B) Incubation at pH 6.5 with 0.5 mM Rb^+ (●, ○), 2 mM Rb^+ (▼, ▽), or 8 mM Rb^+ (▲) was followed by phosphorylation at pH 6.5 and 0 °C. The background phosphorylation determined in the presence of 50 mM K^+ without Na^+ (less than 10% of maximum phosphorylation) was subtracted and the resulting data points (average values corresponding to two or three experiments) were fitted to a biphasic time course as described in the Materials and Methods. The curves show the fits corresponding to times of ≥5 s. The extracted rate constants corresponding to the slow phase are as follows. (Panel A) (●) 0.30 s^{-1}, (○) 0.010 s^{-1}, (▼) 0.066 s^{-1}, (▽) 0.0070 s^{-1}. (Panel B) (●) 0.093 s^{-1}, (○) 0.0024 s^{-1}, (▼) 0.026 s^{-1}, (▽) 0.0010 s^{-1}, (▲) 0.015 s^{-1}.

by a rate constant some 10-fold higher than that seen for the wild-type (Figure 2A).

The occluded form of the wild-type Na^+,K^+-ATPase is known to be further stabilized at low pH and with Rb^+ as substitute for K^+ (*23*). When experiments similar to those described above were carried out at pH 6.5 and following equilibration with 8 mM Rb^+ in place of K^+, the extent of the slow phase of phosphorylation was found to constitute as much as 87% in the mutant, and the corresponding rate constant was more than 4 times lower than with K^+ at pH 7.5. When various Rb^+ concentrations were tested, the rate constant for the slow phase of phosphorylation was always 20−30-fold higher in the mutant relative to the wild-type (Figure 2B). The extent of the slow phase varied with the Rb^+ concentration with half saturation of the occlusion sites being reached between 0.5 and 1 mM Rb^+ in the mutant and at approximately 50 μM Rb^+ in the wild-type, corresponding to a more than 10-fold difference in apparent affinity.

Because the phosphorylated Na^+,K^+-ATPase catalytic subunit was separated by gel electrophoresis from most other proteins before quantification, the background phosphorylation (subtracted in Figure 2) constituted less than 10% of the maximum phosphorylation (cf. also ref *20*), and hence the background cannot in any way account for the rapid phase observed in the above-described experiments.

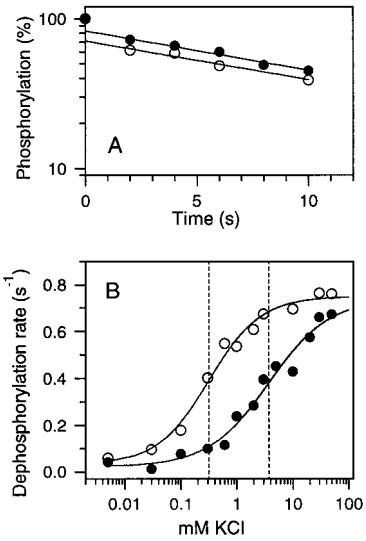


FIGURE 3: ADP sensitivity of the phosphoenzyme and K^+ dependence of dephosphorylation of the Glu329 → Gln mutant (●) and the wild-type Na^+,K^+-ATPase (○) in the presence of 20 mM NaCl at 0 °C. (Panel A) The phosphoenzyme was chased with 1 mM unlabeled ATP and 2.5 mM ADP. The data points (average values corresponding to two experiments) were fitted to a biexponential time course, the slow phase, reflecting the ADP-insensitive phosphoenzyme, being indicated by a line with ordinate intercept at 83% for the mutant and 71% for the wild-type. Note the logarithmic scale. (Panel B) The phosphoenzyme was chased with 1 mM unlabeled ATP and the indicated KCl concentrations. The ordinate shows the rate constants for the dephosphorylation occurring within 2 s. The data points (average values corresponding to two or three experiments) were fitted to the Hill equation giving $K_{0.5}(K^+)$ values of 3.74 and 0.32 mM (indicated by dashed lines) and V_{max} values of 0.74 and 0.75 s^{-1} for the mutant and the wild-type, respectively.

ADP Sensitivity and K^+-Induced Dephosphorylation of Na^+,K^+-ATPase. As indicated in Scheme 1, the phosphorylated Na^+,K^+-ATPase is often considered to exist in two major forms, E_1P, which is ADP sensitive (i.e., able to donate the phosphoryl group back to ADP forming ATP) and K^+ insensitive, and E_2P, which is insensitive to ADP but sensitive to K^+ (i.e., undergoes hydrolysis liberating P_i upon the binding of K^+ from the external side). In the wild-type Na^+,K^+-ATPase, the ADP-insensitive and K^+-sensitive E_2P intermediate accumulates, when the enzyme is phosphorylated by ATP in the presence of a low Na^+ concentration, such as 20 mM, without K^+, due to the rate limitation imposed by the dephosphorylation of E_2P under these conditions. To compare the fractions of phosphoenzyme accumulated as E_2P in the Glu329 → Gln mutant and the wild-type, the time course of dephosphorylation was studied following the addition of ADP to rapidly dephosphorylate E_1P (Figure 3A). As indicated by the extrapolated ordinate intercepts of the slow phases of the dephosphorylation, the ADP-insensitive fractions of the wild-type and mutant phosphoenzymes are rather similar, amounting to 70−80 and 80−90%, respectively, at 20 mM Na^+. The slight increase in the level of E_2P in the mutant may be caused either by an increased rate of the E_1P to E_2P conversion or by a reduced rate of the K^+-independent dephosphorylation of E_2P. Because the rate coefficients of the slow phases, reflecting

the dephosphorylation of E_2P, are virtually identical in the wild-type and the mutant, the latter possibility seems to be excluded.

The rather high fractions of phosphoenzyme accumulated as E_2P in the wild-type and the mutant in the absence of K^+ allowed us to compare the apparent affinities for K^+ at the extracellularly facing sites on E_2P by studying the K^+ dependence of the rate coefficient for dephosphorylation as done previously for other mutants (*20*). Thus, following the phosphorylation in the presence of 20 mM Na^+ without K^+, various concentrations of K^+ were added, and the rate coefficient for the dephosphorylation occurring within 2 s was measured in each case. As seen in Figure 3B, the apparent affinity for K^+ determined for the mutant in this way is 12-fold lower than that of the wild-type. The maximum rate coefficients for dephosphorylation, corresponding to saturating K^+ concentrations, are, on the other hand, rather similar in the wild-type and the mutant.

Na^+-Dependent Phosphorylation of Na^+,K^+-ATPase in the E_1 Form and the Effect of Oligomycin. The low maximum turnover number for Na^+- and K^+-stimulated ATP hydrolysis displayed by the Glu329 → Gln mutant indicates that at least one partial reaction in the enzyme cycle must be considerably slowed in the mutant relative to the wild-type. The above-described findings seem to exclude the $E_2(K)$ to E_1 and E_1P to E_2P conversions, as well as the K^+-induced dephosphorylation, as candidates for the slow step in the mutant. A possibility would therefore be that the rate limitation in the mutant is imposed by the formation of the E_1P phosphoenzyme from E_1.

Figure 4 shows results of experiments in which we measured the time dependence of phosphorylation of the wild-type and mutant enzymes with [γ-^{32}P]ATP at 0 °C, following preincubation in the presence of various concentrations of Na^+ with and without oligomycin. The data could be fitted to a monoexponential rise in the phosphorylation level. Table 2 shows the rate coefficients determined for the various conditions tested and the $K_{0.5}(Na^+)$ values extracted by fitting the dependence of the rate coefficients on Na^+ concentration to the Hill equation. In the absence of oligomycin, the maximum rate of phosphorylation is more than 2-fold higher in the wild-type compared with the mutant. Because the time resolution is limited with our mixing system, the rate coefficients close to 2.5 s^{-1} determined for the wild-type should be considered minimum estimates. Hence, the true $K_{0.5}(Na^+)$ value of the wild-type may be higher than that shown in Table 2. This means that the actual difference between the $K_{0.5}(Na^+)$ values of the wild-type and the mutant in the absence of oligomycin could be smaller than the 4−5-fold indicated by the data in the table.

When oligomycin, which is known to promote occlusion of Na^+ (*5*), was included during preincubation with Na^+ and during phosphorylation, the maximum phosphorylation rate of the mutant increased close to 2-fold. The rates at nonsaturating Na^+ concentrations increased even more in the presence of oligomycin (Figure 4 and Table 2), resulting in a decrease in the $K_{0.5}(Na^+)$ value from 6.4 to 3.2 mM.

The steady-state phosphorylation level formed by the mutant was maximal and constant between 100 and 200 mM Na^+ in the presence of oligomycin (results not shown). As shown in Figure 4, the addition of oligomycin increased the steady-state phosphoenzyme level of the mutant from 20 to 36% of maximum at 1 mM Na^+ and from 52 to 91% of maximum at 40 mM Na^+. When the Na^+ concentration was increased to 150 mM, the steady-state phosphorylation level determined for the mutant in the absence of oligomycin decreased to less than one-third of the maximal level determined in the presence of oligomycin, whereas for the wild-type, the steady-state phosphorylation level determined at 150 mM Na^+ in the absence of oligomycin was higher than 80% of the maximum value (results not shown). The reason for the decrease of the steady-state phosphorylation level of the mutant at high Na^+ concentrations in the absence

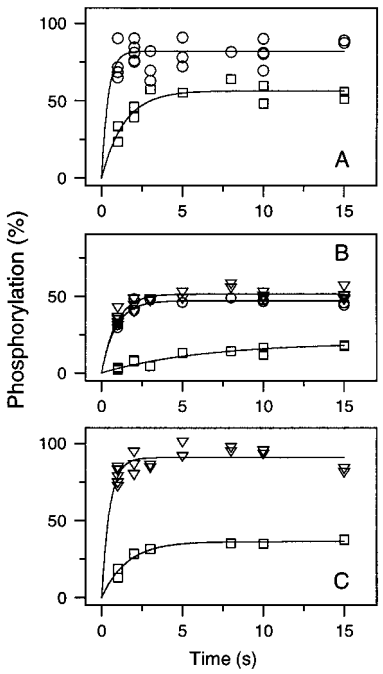


FIGURE 4: Time course of phosphorylation with [γ-^{32}P]ATP of the Glu329 → Gln mutant and the wild-type Na^+,K^+-ATPase at 0 °C after preincubation in the presence of Na^+ with or without oligomycin. (Panel A) Wild-type Na^+,K^+-ATPase at 1 mM Na^+ (□) and 20 mM Na^+ (○), no oligomycin present. (Panel B) Mutant at 1 mM Na^+ (□), 20 mM Na^+ (○), and 40 mM Na^+ (▽), no oligomycin present. (Panel C) Mutant at 1 mM Na^+ (□) and 40 mM Na^+ (▽) with 20 μg of oligomycin/mL present during preincubation and phosphorylation. In all three panels, the phosphorylation levels are shown as percentage of the maximal level determined after phosphorylation for 10 s at 150 mM Na^+ in the presence of oligomycin. Each data point represents one experiment. The data were fitted to a monoexponential rise in phosphorylation level, giving the rate constants shown in Table 2.

Table 2: Na^+ Concentration Dependence of the Rate of Phosphorylation at 0 °C of the Wild-Type Na^+,K^+-ATPase and the Glu329 → Gln Mutant[a]

	rate constant (s^{-1}) Na^+ concn (mM)				
	1	5	20	40	$K_{0.5}$ (mM)
wild-type	0.72	2.49[b]	2.59[b]	2.51[b]	1.38
mutant	0.16	0.45	1.13	1.10	6.35
mutant with oligomycin	0.64	1.21	1.99	1.99	3.21

[a] The rate constants were determined from monoexponential fits to experimental data of which examples are shown in Figure 4. The $K_{0.5}$ values were estimated by fitting the dependence of the rate constants on Na^+ concentration to the Hill equation. [b] Minimum estimate, as the time resolution does not permit the measurement of higher values than ~2.5 s^{-1}.

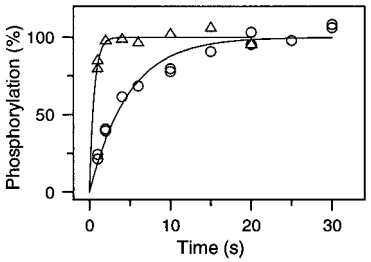


FIGURE 5: Time course of phosphorylation with [γ-^{32}P]ATP of the Glu309 → Asp mutant (○) and the wild-type Ca^{2+}-ATPase (△) at 100 μM Ca^{2+} and 0 °C. The data were fitted to a monoexponential rise in phosphorylation level, giving the rate constants shown in Figure 6. The 100% values corresponding to the steady-state phosphorylation levels constitute 80−90% of the site concentrations determined by phosphorylation with $^{32}P_i$.

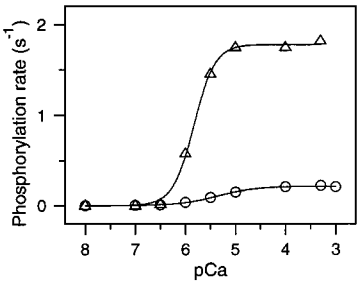


FIGURE 6: Ca^{2+} dependence of the rate constants for phosphorylation of the Glu309 → Asp mutant (○) and the wild-type Ca^{2+}-ATPase (△) determined in experiments similar to those shown in Figure 5. The data points were fitted to the Hill equation giving $K_{0.5}$ values of 4.5 and 1.5 μM, Hill numbers of 1.11 and 2.05, and V_{max} values of 0.22 and 1.78 s^{-1} for the mutant and the wild-type, respectively.

of oligomycin is probably the combination of the reduced phosphorylation rate, described above, with the normal acceleration of the dephosphorylation of E_2P induced by Na^+ binding with low affinity at the extracellularly facing sites on E_2P (*4*). By stabilizing the Na^+-occluded E_1 and E_1P forms, oligomycin counteracts both effects.

Ca^{2+}-Dependent Phosphorylation of the Ca^{2+}-ATPase. Substitution of the glutamic acid residue Glu309 of the Ca^{2+}-ATPase (homologous to Glu329 of the Na^+,K^+-ATPase) with glutamine results in a mutant that does not form detectable amounts of phosphoenzyme in the presence of ATP and Ca^{2+} concentrations up to at least 25 mM (*11*). So far, Glu309 → Asp is the only Ca^{2+}-ATPase mutant with alteration to Glu309 for which phosphorylation from ATP has been demonstrated at Ca^{2+} concentrations in the micromolar range (*19*). The phosphorylation rates of the Glu309 → Asp mutant and the wild-type Ca^{2+}-ATPase were studied in experiments analogous to those described above for the Na^+,K^+-ATPase. The phosphorylation followed a monoexponential time course at all Ca^{2+} concentrations tested. As an example, Figure 5 shows the data corresponding to 100 μM Ca^{2+}. At this Ca^{2+} concentration, the steady-state phosphorylation level of the mutant is indistinguishable from that of the wild-type, but the phosphorylation rates differ. The dependence of the extracted rate constants on the Ca^{2+} concentration is shown in Figure 6, from which it appears that 100 μM Ca^{2+} is saturating for the wild-type as well as the mutant. The phosphorylation rate rises with the Ca^{2+} concentration with a $K_{0.5}(Ca^{2+})$ which is 3-fold higher for the mutant compared with the wild-type. There is a marked difference between the maximum phosphorylation rates of wild-type and mutant,

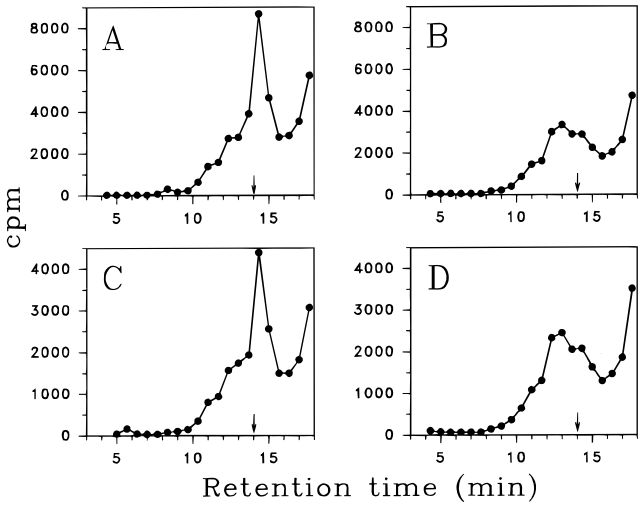


FIGURE 7: HPLC measurement of CrATP-supported Ca^{2+} occlusion. Microsomal membranes containing equivalent amounts of the expressed wild-type Ca^{2+}-ATPase (A and C) and the Glu309 → Asp mutant (B and D) were incubated for 1 h at 37 °C in the presence of 40 μM (A and B) or 400 μM (C and D) $^{45}Ca^{2+}$, together with 1 mM CrATP, 5 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tes/Tris buffer (pH 7.0). Following solubilization of the membranes by addition of 5 mg of $C_{12}E_8$/mL and removal of insoluble material by centrifugation, the supernatant was subjected to size fractionation in a TSK G 4000 SW HPLC column at 20 °C. The eluant contained 1.5 mM $^{40}CaCl_2$ (to replace exchangeable nonoccluded $^{45}Ca^{2+}$), 5 mg of $C_{12}E_8$/mL, 100 mM NaCl, 50 mM TES/Tris buffer (pH 7.0), 1.0 mM EGTA, and 10 mM $MgCl_2$. The ordinate shows radioactivity in collected fractions. The specific radioactivity of $^{45}Ca^{2+}$ differed between the experiments performed with 40 μM and 400 μM $^{45}Ca^{2+}$ The *arrow* indicates the elution position corresponding to Ca^{2+}-ATPase purified from rabbit skeletal muscle.

amounting to as much as 8−9-fold. This is analogous to the behavior of the above-described Na^+,K^+-ATPase mutant. Furthermore, the apparent cooperativity in Ca^{2+} activation indicated by the Hill number is reduced from approximately two in the wild-type to a value close to one in the mutant (see legend to Figure 6).

Ca^{2+} Occlusion in the Ca^{2+}-ATPase. Because the phosphorylation of the E_1 forms of Na^+,K^+- and Ca^{2+}-ATPase is closely linked to the occlusion of Na^+ and Ca^{2+}, one possible explanation of the reduced rate of phosphorylation described above would be a defect in the occlusion process. Ca^{2+} occlusion in the Glu309 → Asp mutant and the wild-type Ca^{2+}-ATPase was measured using the previously developed HPLC technique (*9*, *10*), following incubation of the enzyme with $^{45}Ca^{2+}$ in the presence of CrATP to stabilize the occluded form (Figure 7). Whereas the HPLC elution profile of the wild-type contained a large peak of occluded $^{45}Ca^{2+}$ eluting at the position of the Ca^{2+}-ATPase (indicated by the *arrow*), there was no such peak for the mutant, when the incubation was carried out in the presence of 40 μM $^{45}Ca^{2+}$ or 400 μM $^{45}Ca^{2+}$. At least the latter Ca^{2+} concentration should fully saturate the Ca^{2+}-binding sites according to the phosphorylation data presented above. The data in Figure 7 indicate that the equilibrium between occluded and nonoccluded Ca^{2+}-bound E_1 forms of the Glu309 → Asp mutant is displaced in favor of the nonoccluded form, or that both the occlusion and the deocclusion occur much more rapidly in the mutant than in the wild-type, so that the bound $^{45}Ca^{2+}$ is exchanged for nonradioactive Ca^{2+} in the eluant within the 15 min it lasts to elute the enzyme. In the wild-

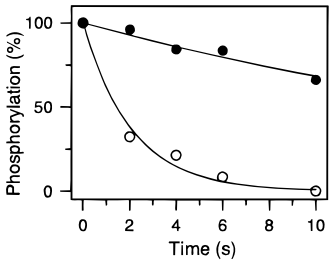


FIGURE 8: Dephosphorylation at pH 7.0 and 0 °C of the E_2P phosphoenzyme intermediate formed by phosphorylation with $^{32}P_i$ in the Glu309 → Asp mutant (●) and the wild-type Ca^{2+}-ATPase (○). The data points (average values corresponding to three to four experiments) were fitted to a monoexponential decay giving rate coefficients of 0.038 and 0.48 s^{-1} for the mutant and the wild-type, respectively.

type Ca^{2+}-ATPase, exchange of 90% of the occluded $^{45}Ca^{2+}$ in the enzyme complex with CrATP requires several hours (*7*). Like the wild-type Ca^{2+}-ATPase, the mutant enzyme was able to withstand the solubilization in detergent without undergoing rapid denaturation, since we could demonstrate that the amount of phosphoenzyme formed in the presence of [γ-^{32}P]ATP remained high (>80%) 15 min after the solubilization (result not shown).

Dephosphorylation of E_2P of the Ca^{2+}-ATPase. Just as the K^+-induced dephosphorylation of the Na^+,K^+-ATPase phosphoenzyme reflects the binding of K^+ at the extracellularly facing transport sites (Figure 3B), the dephosphorylation of the E_2P phosphoenzyme of the Ca^{2+}-ATPase may depend on the binding at extracytoplasmically facing sites of H^+ (or H_3O^+) to be countertransported in exchange for Ca^{2+} (*24*). Figure 8 depicts the results of experiments in which the time course of dephosphorylation of the E_2P intermediate was examined at pH 7.0, following termination of phosphorylation from $^{32}P_i$ by dilution of the phosphorylated enzyme in a medium containing nonradioactive P_i. While the wild-type Ca^{2+}-ATPase dephosphorylated rapidly, the half-life being 1−2 s, the phosphoenzyme of the Glu309 → Asp mutant dephosphorylated very slowly with a half-life close to 20 s. This finding is in accordance with the hypothesis that the binding of H^+ (or H_3O^+) ions at extracytoplasmically facing sites responsible for activation of dephosphorylation in the Ca^{2+}-ATPase is deficient in the Glu309 → Asp mutant. The block of dephosphorylation in the mutant could not be overcome by decreasing the pH to 6.0 (results not shown), which is about the lowest pH value for which it is meaningful to carry out such studies of the dephosphorylation rate, as acidic conditions cause a general stabilization of the aspartyl phosphoryl bond.

Thapsigargin Inhibition of Ca^{2+}-ATPase. Thapsigargin is a specific inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}-ATPases that binds selectively and with very high affinity to $E_2(H)$ or to an "E_2-like" conformation, thereby interfering with the conformational transition to E_1 and, thus, with Ca^{2+} binding and with the phosphorylation reaction (*25*−*28*). Thapsigargin inhibition of the Ca^{2+}-ATPase is therefore dependent on the $E_2(H) \leftrightarrow E_1$ equilibrium. The data presented in Figure 9 show that, depending on the pH, the sensitivity to thapsigargin inhibition may be strongly affected by the Glu309 → Asp mutation. The mutant and wild-type Ca^{2+}-ATPases were incubated with thapsigargin at pH 7.0 or 6.0 in the absence of Ca^{2+} (presence of EGTA),

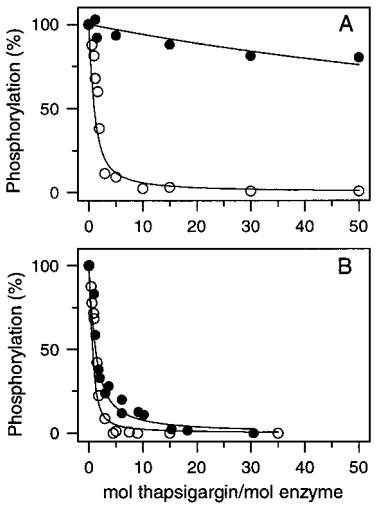


FIGURE 9: Thapsigargin inhibition of the Ca^{2+} dependent phosphorylation from ATP in the Glu309 → Asp mutant (●) and the wild-type Ca^{2+}-ATPase (○) at pH 7.0 (panel A) and pH 6.0 (panel B). The abscissa indicates the molar ratio between the total thapsigargin concentration (free plus bound) and the enzyme concentration (10 nM). The data points (average values corresponding to four experiments) were fitted to the equation for a simple one-site binding model (see Materials and Methods), giving the following values for R_0 (the ratio between the dissociation constant for the enzyme−thapsigargin complex and the enzyme concentration). (Panel A) (●) 158.4; (○) 0.54. (Panel B) (●) 0.76; (○) 0.22.

and then $CaCl_2$, $MgCl_2$, and [γ-^{32}P]ATP were added to test the Ca^{2+}-activated phosphorylation by ATP at the respective pH values. At pH 7.0, the apparent affinity with which thapsigargin inhibits the phosphorylation was found to be almost 300-fold lower in the Glu309 → Asp mutant relative to the wild-type Ca^{2+}-ATPase (Figure 9A). However, as seen in Figure 9B, when the pH was changed from 7.0 to 6.0, the apparent thapsigargin affinity of the mutant was only 3−4-fold lower than that of the wild-type. Because the effect of the mutation on the thapsigargin sensitivity could be overcome to this extent by lowering the pH, the binding site for thapsigargin in the $E_2(H)$ form is probably not disturbed by the mutation, but the $E_2(H) \leftrightarrow E_1$ conformational equilibrium controlling thapsigargin binding seems to be displaced in favor of E_1. The effect on thapsigargin sensitivity of changing the pH was seen independently of whether the temperature during phosphorylation was 0 or 20 °C and of whether the free Ca^{2+} concentration was just saturating for the phosphorylation reaction or Ca^{2+} was present in large excess during the phosphorylation as was the case in the experiments shown in Figure 9.

DISCUSSION

Understanding the function of the highly conserved glutamic acid residue in the fourth membrane segment of P-type ATPases has for a long time presented a challenge. The present study of minimally perturbed mutants retaining the ability to phosphorylate allows evaluation of the central role played by this residue in multiple partial reaction steps in the enzymatic cycle and reveals a high degree of homology between the functional perturbations induced in the Na^+,K^+-ATPase and Ca^{2+}-ATPase mutants.

Destabilization of the Occluded E_2 Form. In the normal reaction cycle of the Na^+,K^+-ATPase, the K^+-occluded E_2 form $E_2(K)$ is rather stable at low ATP concentration, and

release of K^+ to the cytoplasmic side of the membrane is coupled to the slow conformational transition to E_1 (Scheme 1). Our findings clearly show that the Glu329 → Gln mutation destabilizes the occluded E_2 form, and the rate constant characterizing K^+ or Rb^+ release was found to be up to 30-fold higher in the mutant compared with the wild-type. This could in principle imply that the bound K^+ or Rb^+ is able to dissociate directly to the medium on the cytoplasmic or the extracytoplasmic side of the membrane without the need for a conformational change, i.e., that no occlusion occurs in the mutant enzyme. However, since it was possible under selected conditions to demonstrate a large component (close to 90% following incubation in 8 mM Rb^+ at pH 6.5) that phosphorylated more slowly than what would be expected for nonoccluded enzyme, it seems likely that a "truly" occluded complex is formed in the mutant, despite its relative instability.

Nielsen et al. (*29*) recently reported that occlusion of the K^+ congeners $^{86}Rb^+$ and $^{204}Tl^+$ measured "at equilibrium" is abolished by the equivalent Glu327 → Gln mutation of sheep Na^+,K^+-ATPase.[3] Their data are fully consistent with our finding of an enhanced rate of deocclusion of K^+ and Rb^+ in the mutant, since actually their measurements were not performed at equilibrium, but after the removal of free $^{86}Rb^+$ or $^{204}Tl^+$ by ionic exchange chromatography—a procedure which requires time, thereby allowing release of the occluded ions in the mutant.

The enhanced rate of the $E_2(K)$ → E_1 conversion in the Glu329 → Gln mutant also explains the very high apparent affinity of the mutant for ATP observed in titrations of Na^+,K^+-ATPase activity (*12*), since the much lower apparent ATP affinity of the wild-type reflects the enhancement by millimolar ATP of the $E_2(K)$ → E_1 conversion. In the mutant, the $E_2(K)$ → E_1 conversion rate is too high to be rate determining for the overall ATP hydrolysis reaction, even at low ATP concentrations, and under these conditions, the ATP dependence of the overall reaction reflects the high affinity of the E_1 form for ATP. The acceleration of the $E_2(Rb)$ → E_1 conversion likewise accounts for the finding that no inhibition of ATP hydrolysis was observed in the Glu329 → Gln mutant, when Rb^+ replaced K^+ (Table 1), because the inhibition of the overall reaction observed in the wild-type under these circumstances depends on the rate limitation imposed by Rb^+ release (*23*). Moreover, the instability of the $E_2(K)$ form explains the finding that K^+ is unable to prevent ouabain binding (lack of K^+-ouabain antagonism) in the Glu327 → Gln mutant of the ouabain-sensitive sheep isoform (*15*).[3]

In the Ca^{2+}-ATPase, the very slow phosphorylation of the E_1 form of the Glu309 → Asp mutant precludes studies of the $E_2(H)$ → E_1 transition by techniques similar to those used for the Na^+,K^+-ATPase. Information about the $E_2(H)$ ↔ E_1 equilibrium could instead be obtained by use of thapsigargin, which appears to bind selectively to $E_2(H)$ (*26*, *27*). At pH 7.0, thapsigargin sensitivity was reduced dramatically in the Glu309 → Asp mutant relative to the wild-type Ca^{2+}-ATPase. Ca^{2+} competes with protons and stabilizes the $E_1(Ca)$ form, whereas protons stabilize $E_2(H)$ (*30*, *31*). The effect of the Glu309 → Asp mutation on thapsigargin sensitivity at pH 7.0 resembles the effect of increasing pH to alkaline values in the wild-type. The major reason for the loss of thapsigargin sensitivity in the mutant does not appear to be a defect in the thapsigargin-binding site, since at pH 6.0 the apparent affinity of the mutant for thapsigargin was rather close to normal. Therefore, it seems that the Glu309 → Asp mutation destabilizes the $E_2(H)$ form of the Ca^{2+}-ATPase in analogy with the destabilization of the $E_2(K)$ form of the Na^+,K^+-ATPase Glu329 → Gln mutant.

Defective Occlusion of Na^+ and Ca^{2+} at the Cytoplasmically Facing Sites in the E_1 Form. In the Na^+,K^+-ATPase mutant as well as the Ca^{2+}-ATPase mutant, the maximum rate of phosphorylation by ATP was found to be significantly reduced compared with the wild-type enzyme, and in the Na^+,K^+-ATPase mutant, this effect was counteracted by the addition of oligomycin, which is known to stabilize the occluded form (*5*, *32*). As discussed by Tanford et al. (*33*) and Skou (*32*), a close relationship exists between the occlusion of Ca^{2+} or Na^+ and the structural change in the ATP binding/phosphorylation domain that confers the ability to accept the γ-phosphoryl group of ATP, and the occlusion process is likely to be rate limiting in formation of the phosphoenzyme. The low maximum rate of phosphorylation observed with the mutants may, therefore, be accounted for by a displacement of the equilibrium between occluded and nonoccluded E_1 forms in favor of the nonoccluded form (cf. Scheme 1). The HPLC experiment in Figure 7 provides a direct confirmation of the defective occlusion of Ca^{2+} in the Glu309 → Asp mutant of the Ca^{2+}-ATPase. It is noteworthy in this connection that, despite the lack of Ca^{2+} occlusion in the Glu309 → Asp mutant, titration of the rate of phosphorylation provided an apparent Ca^{2+} affinity only 3-fold lower than that of the wild-type (Figure 6). In the Na^+,K^+-ATPase, the difference between the apparent Na^+ affinities of the Glu329 → Gln mutant and the wild-type amounts to maximally 4−5-fold (Table 2). Hence, there seems to be only a modest change in binding affinity associated with the defective occlusion of the cations in the E_1 form.

It is interesting to note that the apparent cooperativity of Ca^{2+} binding indicated by the Hill number is reduced in the Glu309 → Asp mutant. Tanford et al. (*33*) have derived the equations for the dependence of the Hill number of the Ca^{2+}-ATPase on the binding affinities for the two calcium ions and the equilibrium constants for the conformational changes associated with Ca^{2+} binding in an expanded version of Scheme 1. The decrease in Hill number could in principle be brought about by the observed displacement of the $E_2(H)$ ↔ E_1 equilibrium in favor of E_1, by the displacement of the equilibrium between occluded and nonoccluded E_1 forms in favor of the nonoccluded form, and/or by a reduced binding affinity of E_1 for the second calcium ion, assuming a consecutive binding mechanism for the two ions. Another possibility that could account for the reduced Hill number would be that the binding stoichiometry is reduced in the mutant, with the binding of only one calcium ion being sufficient to activate the phosphorylation by ATP, very unlike the situation for the wild-type enzyme where the binding of two Ca^{2+} is required for phosphorylation.

Cation Binding at Extracytoplasmically Facing Sites. While the above-discussed findings seem to demonstrate a central role of Glu329 in connection with the release of K^+ and occlusion of Na^+ at the intracellularly facing cation-binding sites of the Na^+,K^+-ATPase, the interaction with K^+ that binds from the extracellular side was evaluated by titrating the K^+-dependence of dephosphorylation of the E_2P

phosphoenzyme. The 12-fold reduced apparent affinity for K^+ observed for the Glu329 → Gln mutant suggests that Glu329 is involved in the binding of K^+ at the extracellularly facing sites or in controlling the access of extracellular K^+ to the binding pocket. For the Ca^{2+}-ATPase, there is presently no direct evidence proving an obligatory requirement for binding of the countertransported protons in order to activate dephosphorylation of E_2P, but in light of the present observation of a strongly reduced dephosphorylation rate in the Glu309 → Asp mutant of the Ca^{2+}-ATPase, it is tempting to hypothesize that the mechanistic features of the Na^+,K^+-ATPase and the Ca^{2+}-ATPase dephosphorylations are similar, and, hence, that the block of dephosphorylation in the Ca^{2+}-ATPase mutant is due to involvement of Glu309 in the binding of extracytoplasmic protons (or H_3O^+) to be countertransported in exchange for Ca^{2+}. An apparent p*K* value of 7.7 has previously been assigned to the extracytoplasmically facing proton transport sites on the E_2P form of the Ca^{2+}-ATPase (*31*). Our data would suggest that the Glu309 → Asp mutation reduces the proton affinity of the extracytoplasmically facing sites on E_2P by at least 1 order of magnitude.

Overall Reaction of the Na^+,K^+-ATPase Mutant. The overall ATP hydrolysis reaction of the Glu329 → Gln mutant was characterized by a reduced maximum turnover number and an increased $K_{0.5}$ for K^+ activation. Both effects can be understood in light of the observed changes in the partial reaction steps discussed above. The reduced rate of phosphorylation of the Glu329 → Gln mutant makes the phosphorylation a good candidate for the partial reaction step limiting the maximum ATP hydrolysis rate of the mutant to one-third that of the wild-type. The present analysis has not revealed any other partial reaction step showing a reduced rate in the mutant relative to the wild-type. Previously, it was concluded that the turnover numbers of wild-type and mutant are similar (*12*). This discrepancy with the present results is accounted for by the fact that the previous calculation of turnover number was based on the phosphorylation level determined at 150 mM Na^+ in the absence of oligomycin. As described in the Results, the phosphorylation level of the mutant is less than one-third of the maximum under these conditions. Furthermore, as described in ref *20*, technical improvements have now allowed us to determine the phosphorylation level more accurately than in ref *12*.

Because the measurement of ATP hydrolysis is carried out on leaky vesicles with K^+ having access to both membrane sides, two factors may contribute to the reduced apparent affinity for K observed in the K^+ titrations of ATPase activity. First, the 12-fold reduced affinity for K^+ at the extracellularly facing high-affinity sites involved in activation of dephosphorylation of E_2P must play a role, since the dephosphorylation is one of the major rate-limiting steps for the overall reaction in the absence of activation by K^+. In addition, K^+ normally exerts an inhibitory effect by binding with lower affinity at the cytoplasmically facing sites on the E_1 form in competition with Na^+, thereby driving the $E_2(K) \rightarrow E_1$ transition backward. By relieving this inhibition, the observed instability of the $E_2(K)$ form of the mutant changes the K^+ dependency of ATP hydrolysis in the mutant, so that the $K_{0.5}(K^+)$ value increases. Because the acceleration of the $E_2(K) \rightarrow E_1$ transition by high ATP concentrations provides some relief of the K^+ inhibition even in the wild-type, the effect of the mutation on apparent K^+ affinity should be most pronounced at low ATP concentration, in line with the actual finding reported in Figure 1. The relief of the K^+-inhibition induced by the mutation likewise explains that the turnover number of the mutant can become higher than that of the wild-type at low ATP concentration (Figure 1 and Table 1).

Concluding Remarks. A common feature of the functional changes described is that they can be traced to partial reaction steps associated with the binding/occlusion or release of the transported cations. Both the cytoplasmically and the extracytoplasmically facing cation-binding sites, as well as the occluded sites, seem to be affected by the mutations to the glutamate in M4. A unifying concept would therefore be that this residue is part of a common cation occlusion pocket that can open consecutively to either side of the membrane, binding the ions from the cytoplasmic side in the E_1 form and from the extracytoplasmic side in the E_2P form. The carboxylate group of the glutamate side chain may serve directly as a coordinating ligand for K^+/H^+ in the E_2P form of the enzyme as well as in the occluded E_2 state. The increased rate of the $E_2(K) \rightarrow E_1$ conversion in the Na^+,K^+-ATPase mutant and the analogous displacement of the $E_2(H) \leftrightarrow E_1$ equilibrium in favor of E_1 in the Ca^{2+}-ATPase mutant may reflect a reduced height of the energy barrier to be traversed in connection with the deocclusion toward the cytoplasmic side. The fact that only a modest change in binding affinity is associated with the defective occlusion of Ca^{2+}/Na^+ in the E_1 form seems to argue in favor of a role for the glutamate in the gating process at the cytoplasmic entrance to the occlusion pocket, much as described for the acidic residue at the ninth position of the Ca^{2+}-binding loop in EF-hand proteins (*34*, *35*). Mutations changing the length of the side chain of this residue altered the rate constant for dissociation independently of affinity, thus apparently tuning the frequency of gate opening. From a structural point of view, the position of the glutamate of M4 in the cytoplasmic half of the membrane makes it a good candidate for a gateway residue at the cytoplasmic entrance to the occlusion pocket. The participation of the glutamate also as a liganding residue for cations binding from the extracytoplasmic side in the E_2P form may be made feasible by a conformational rearrangement in relation to the E_1P to E_2P transition. There is evidence for a pivotal role of the neighboring residues near the M4S4 boundary and in S4 in this transition (*20*, *21*, *36*, *37*). In addition, electrophysiological measurements have provided evidence for the existence of a relatively long access channel connecting the extracytoplasmic space to a binding site near the cytoplasmic surface of the membrane (*1*, *38*).

ACKNOWLEDGMENT

Janne Petersen, Lene Jacobsen, Karin Kracht, and Jytte Jørgensen are thanked for expert technical assistance, and Dr. R. J. Kaufman, Genetics Institute, Boston, MA, for the expression vector pMT2.

REFERENCES

1. Glynn, I. M. (1993) *J. Physiol. 462*, 1−30.
2. Inesi, G., Sumbilla, C., and Kirtley, M. E. (1990) *Physiol. Rev. 70*, 749−760.

3. Levy, D., Seigneuret, M., Bluzat, A., and Rigaud, J.-L. (1990) *J. Biol. Chem. 265*, 19524−19534.
4. Post, R. L., Hegyvary, C., and Kume, S. (1972) *J. Biol. Chem. 247*, 6530−6540.
5. Esmann, M., and Skou, J. C. (1985) *Biochem. Biophys. Res. Commun. 127*, 857−863.
6. Vilsen, B., Andersen, J. P., Petersen, J., and Jørgensen, P. L. (1987) *J. Biol. Chem. 262*, 10511−10517.
7. Vilsen, B., and Andersen, J. P. (1992) *J. Biol. Chem. 267*, 3539−3550.
8. Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan, D. H. (1989) *Nature 339*, 476−478.
9. Vilsen, B., and Andersen, J. P. (1992) *J. Biol. Chem. 267*, 25739−25743.
10. Andersen, J. P., and Vilsen, B. (1994) *J. Biol. Chem. 269*, 15931−15936.
11. Andersen, J. P. (1995) *Biosci. Rep. 15*, 243−261.
12. Vilsen, B. (1993) *Biochemistry 32*, 13340−13349.
13. Jewell-Motz, E. A., and Lingrel, J. B. (1993) *Biochemistry 32*, 13523−13530.
14. Vilsen, B. (1995) *Biochemistry 34*, 1455−1463.
15. Kuntzweiler, T. A., Wallick, E. T., Johnson, C. L., and Lingrel, J. B. (1995) *J. Biol. Chem. 270*, 2993−3000.
16. Kuntzweiler, T. A., Argüello, J. M., and Lingrel, J. B. (1996) *J. Biol. Chem. 271*, 29682−29687.
17. Pedersen, P. A., Rasmussen, J. H., Nielsen, J. M., and Jorgensen, P. L. (1997) *FEBS Lett. 400*, 206−210.
18. Andersen, J. P., and Vilsen, B. (1992) *J. Biol. Chem. 267*, 19383−19387.
19. Clarke, D. M., Loo, T. W., and MacLennan, D. H. (1990) *J. Biol. Chem. 265*, 6262−6267.
20. Vilsen, B. (1997) *Biochemistry 36*, 13312−13324.
21. Vilsen, B., Andersen, J. P., Clarke, D. M., and MacLennan, D. H. (1989) *J. Biol. Chem. 264*, 21024−21030.
22. Daly, S. E., Lane, L. K., and Blostein, R. (1996) *J. Biol. Chem. 271*, 23683−23689.
23. Forbush, B., III (1987) *J. Biol. Chem. 262*, 11104−11115.
24. Andersen, J. P. (1994) *FEBS Lett. 354*, 93−96.
25. Lytton, J., Westlin, M., and Hanley, M. R. (1991) *J. Biol. Chem. 266*, 17067−17071.
26. Sagara, Y., Fernandez-Belda, F., De Meis, L., and Inesi, G. (1992) *J. Biol. Chem. 267*, 12606−12613.
27. Wictome, M., Henderson, I., Lee, A. G., and East, J. M. (1992) *Biochem. J. 283*, 525−529.
28. Nørregaard, A., Vilsen, B., and Andersen, J. P. (1994) *J. Biol. Chem. 269*, 26598−26601.
29. Nielsen, J. M., Pedersen, P. A., Karlish, S. J. D., and Jørgensen, P. L. (1998) *Biochemistry 37*, 1961−1968.
30. Forge, V., Mintz, E., and Guillain, F. (1993) *J. Biol. Chem. 268*, 10953−10960.
31. Yu, X., Hao, L., and Inesi, G. (1994) *J. Biol. Chem. 269*, 16656−16661.
32. Skou, J. C. (1991) in *The Sodium Pump: Recent Developments* (Kaplan, J. H., and De Weer, P., Eds.) pp 317−319, The Rockefeller University Press, New York.
33. Tanford, C., Reynolds, J. A., and Johnson E. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7094−7098.
34. Renner, M., Danielson, M. A., and Falke, J. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6493−6497.
35. Drake, S. K., and Falke, J. J. (1996) *Biochemistry 35*, 1753−1760.
36. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) *J. Biol. Chem. 266*, 18839−18845.
37. Garnett, C., Sumbilla, C., Belda, F. F., Chen, L., and Inesi, G. (1996) *Biochemistry*, 11019−11025.
38. Gadsby, D. C., Rakowski, R. F., and De Weer, P. (1993) *Science 260*, 100−103.

BI9802925