Importance of Conserved Thr<sup>214</sup> in Domain A of the Na<sup>+</sup>,K<sup>+</sup>-ATPase for Stabilization of the Phosphoryl Transition State Complex in E<sub>2</sub>P Dephosphorylation*  

Mads Toustrup-Jensen and Bente Vilsen‡  
From the Department of Physiology, University of Aarhus, Ole Worms Allé 160, DK-8000 Aarhus C, Denmark  

Thr<sup>214</sup> of the highly conserved 214TGES sequence in domain A of the Na<sup>+</sup>,K<sup>+</sup>-ATPase was replaced with alanine, and the mutant was compared functionally with the previously characterized domain A mutant Glu<sup>263</sup> → Ala. Thr<sup>214</sup> → Ala displayed a conspicuous 150-fold reduction of the apparent vanadate affinity for inhibition of ATPase activity, which could not simply be explained by the observed shifts of the conformational equilibria in favor of E<sub>1</sub> and E<sub>P</sub>. The intrinsic vanadate affinity of the E<sub>2</sub> form and the effect on the apparent vanadate affinity of displacement of the E<sub>1</sub>→E<sub>2</sub> equilibrium were determined in a phosphorylation assay that allows the enzyme-vanadate complex to be formed under equilibrium conditions. When the E<sub>1</sub> form prevailed, Thr<sup>214</sup> → Ala retained a reduced vanadate affinity relative to wild type, whereas the affinity of Glu<sup>263</sup> → Ala became wild type-like. Thus, mutation of Thr<sup>214</sup> affected the intrinsic affinity of E<sub>2</sub> for vanadate. Furthermore, Thr<sup>214</sup> → Ala showed at least a 5-fold reduced E<sub>P</sub> dephosphorylation rate relative to wild type in the presence of saturating concentrations of K<sup>+</sup> and Mg<sup>2+</sup>. Because vanadate is a phosphoryl transition state analog, it is proposed that defective binding of the phosphorylation transition state complex (transition state destabilization) causes the inability to catalyze E<sub>P</sub> dephosphorylation properly. By contrast, the phosphorylation site in the E<sub>1</sub> form was unaffected in Thr<sup>214</sup> → Ala. Replacement of the glutamate, Glu<sup>216</sup>, of 214TGES with alanine was incompatible with cell viability, indicating a very low transport activity or expression level. Our results support the hypothesis that domain A is isolated in the E<sub>1</sub> form, but contributes to make up the catalytic site in the E<sub>2</sub> and E<sub>P</sub> conformations.

The Na<sup>+</sup>,K<sup>+</sup>-ATPase<sup>1</sup> maintains high Na<sup>+</sup> and K<sup>+</sup> gradients across the plasma membrane in all animal cells by mediating active transport of Na<sup>+</sup> out and K<sup>+</sup> into the cell at a stoichiometry of 3:2, using the energy derived from hydrolysis of ATP (1). The reaction steps linking ion transport to ATP hydrolysis through conformational changes are described by the consecutive “Post-Albers” or “E<sub>2</sub>→E<sub>1</sub>″ model depicted in Scheme 1 (2–4). The Na<sup>+</sup>,K<sup>+</sup>-ATPase is a member of the family of P-type ATPases, which include among others the Ca<sup>2+</sup>-ATPase and H<sup>+</sup>,K<sup>+</sup>-ATPase, and for which autophosphorylation by ATP of an aspartyl residue is a characteristic feature of the reaction cycle. In the Na<sup>+</sup>,K<sup>+</sup>-ATPase, the phosphorylation reaction is triggered by binding of three cytoplasmic Na<sup>+</sup> ions to the E<sub>1</sub> form of the enzyme, and dephosphorylation is activated by the binding of K<sup>+</sup> to extracellularly facing sites. The translocation of Na<sup>+</sup> occurs in connection with a conformational change in the phosphoenzyme, E<sub>1</sub>P(Na<sub>3</sub>) → E<sub>P</sub> transition. The counter-transport of K<sup>+</sup> is associated with the dephosphorylation of E<sub>P</sub> and another conformational change, E<sub>2</sub>(K<sub>2</sub>) → E<sub>1</sub>, which decludes K<sup>+</sup> at the cytoplasmic side and is accelerated by binding of ATP (Scheme 1). To understand the mechanism of cation transport, it is essential to elucidate the nature of the conformational changes and the catalytic events, as well as the links between them.

The high-resolution crystal structure of the Ca<sup>2+</sup>-ATPase in the E<sub>1</sub>Ca<sub>2</sub> state (5) shows that the protein is comprised by 10 membrane-spanning segments, M1–M10, and a cytoplasmic part consisting of three distinct main domains: A for actuator, N for nucleotide binding, and P for phosphorylation (i.e. containing the phosphorylated aspartate residue). The overall structure of the Na<sup>+</sup>,K<sup>+</sup>-ATPase α-subunit resembles that of Ca<sup>2+</sup>-ATPase (6, 7), and many residues, including those known to be involved in ion binding and catalysis (8–11), are highly conserved between these two pump proteins. The most conserved sequences among P-type pumps are 371DKTGT (containing the phosphorylated aspartate), 610MVTGD, and 710TG-DGVNDS, within domain P, and 214TGES in domain A (12, 13). A number of studies have suggested that 214TGES plays an important role in energy transduction. Proximity relations determined by specific affinity cleavage have indicated that 214TGES moves toward domain P during the E<sub>1</sub> → E<sub>P</sub>(K<sub>2</sub>) and E<sub>P</sub>(Na<sub>3</sub>) → E<sub>2</sub>P transitions, thereby displacing domain N from domain P (14). It was hypothesized that the E<sub>P</sub>(Na<sub>3</sub>) → E<sub>P</sub> conformational transition is associated with a change in Mg<sup>2+</sup>-ligation, and that the glutamic acid residue of 214TGES is involved in Mg<sup>2+</sup> coordination in E<sub>P</sub>, but not in E<sub>2</sub>P(Na<sub>3</sub>) (14). Previous mutagenesis analysis of the Ca<sup>2+</sup>-ATPase has implicated each of the residues TGE of the conserved TGES sequence in the E<sub>P</sub>(Ca<sub>2</sub>) → E<sub>P</sub> transition (15). Other residues in domain A have also been shown by mutagenesis to be involved in the E<sub>2</sub>(K<sub>2</sub>) → E<sub>1</sub> and E<sub>P</sub>(Na<sub>3</sub>) → E<sub>P</sub> transitions of the catalytic site in the E<sub>2</sub> and E<sub>P</sub> conformations.

* This work was supported by grants from the Danish Medical Research Council, the Research Foundation of Aarhus University, the Novo Nordisk Foundation, Denmark, and the Lundbeck Foundation, Denmark. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Physiology, University of Aarhus, Ole Worms Allé 160, DK-8000 Aarhus C, Denmark. Fax: 45-86-12-90-45; E-mail: bv@f.au.dk.

1 The abbreviations used are: Na<sup>+</sup>,K<sup>+</sup>-ATPase, the Na<sup>+</sup>- and K<sup>+</sup>-transporting adenosine triphosphatase (EC 3.6.1.37); E<sub>1</sub> and E<sub>P</sub>, conformational states of the Na<sup>+</sup>,K<sup>+</sup>-ATPase; E<sub>P</sub> and E<sub>p</sub>, phosphoryl-ated conformational states; E<sub>a</sub>, ligand concentration giving half-maximum activation or inhibition; M1–M10, putative transmembrane segments numbered from the NH<sub>2</sub>-terminal end of the peptide chain.

2 All numbering of Na<sup>+</sup>,K<sup>+</sup>-ATPase residues in this article refers to the sequence of the rat α1 isof orm.
the Na\(^+\),K\(^+\)-ATPase. Hence, mutation of Glu\(^{233}\) in the Na\(^+\),K\(^+\)-ATPase accelerated the \(E_3(K_2) \rightarrow E_1\) transition of the dephospho-

ence (16), and mutation of Gly\(^{262}\) located in the loop

cleaving domain \(E\) connecting domain \(E\) and the mutant (95

domain \(E\) presented here add functional evidence to the hypothesis that

...membranes from the cell. Titration of the ouabain concentration dependence of the 

...E, leading to formation of a compact headpiece in the \(E_2\) state, 

...other catalytically important residues, see Fig. 1 (24). This could mean that the threonine plays a role in the catalytic function of \(E_2\) and \(E_2P\).

In the present study, the functional role of Thr\(^{214}\) of the Na\(^+\),K\(^+\)-ATPase was examined by replacement with alanine. To analyze both the conformational changes and the function of the catalytic site in the mutant, we have conducted rapid kinetic measurements of phosphorylation, as well as dephosphorylation. In addition, the affinity for the phosphate transition state analog vanadate was determined both during enzyme turnover and at equilibrium under conditions where the \(E_2\) form prevails. The results show that Thr\(^{214}\) is important for proper function of the catalytic site in \(E_2\) and \(E_2P\), whereas the catalytic site of the \(E_1\) form seems to function normally in the Thr\(^{214}\) \(\rightarrow\) Ala mutant. The effects of the Thr\(^{214}\) \(\rightarrow\) Ala mutation presented here add functional evidence to the hypothesis that domain \(A\) contributes substantially to make up the catalytic site in the \(E_2\) and \(E_2P\) conformations.

EXPERIMENTAL PROCEDURES

**Mutagenesis, Expression, and Basic Functional Characterization—**

Oligonucleotide-directed mutagenesis (25) of the cDNA encoding the ouabain-insensitive rat \(\alpha_1\)-isofrom of Na\(^+\),K\(^+\)-ATPase, expression of mutants and wild type in COS-1 cells, using 5 \(\mu\)m ouabain in the growth medium to select stable transfectants, and the isolation of crude plasma membranes from the cells were carried out as described previously (26, 27), and the concentration of total protein was determined using the dye binding method of Bradford (28).

ATPase activity was measured on sodium deoxycholate- or alamethi-
cin-treated leaky plasma membranes at 37 °C as described previously (26, 27). Titration of the ouabain concentration dependence of the ATPase activity gave similar \(K_{50}\) values for the wild type (132 ± 8 \(\mu\)M) and the mutant (95 ± 28 \(\mu\)M. The fraction of active Na\(^+\),K\(^+\)-ATPase molecules in the preparation contributed by the exogenous enzyme calculated as in Ref. 29 was 92% for the wild type and 95% for the mutant, indicating that only a minimal amount of endogenous COS-1 cell enzyme was present. To eliminate the contribution of the latter, 10 \(\mu\)M ouabain was added to all assays. The Na\(^+\),K\(^+\)-ATPase activity associated with the expressed exogenous enzyme was calculated by subtracting the ATPase activity measured at a concentration of ouabain (10 \(\mu\)M) that inhibits all Na\(^+\),K\(^+\)-ATPase activity from that measured at 10 \(\mu\)M ouabain.

Studies of the Na\(^+\) dependence of steady-state phosphorylation from \([\gamma\(^32\)P]ATP and the time course of ADP-dependent dephosphorylation, and the determination of the active site concentration by phosphorylation in the presence of 150 mM NaCl and oligomycin (20 \(\mu\)g/ml) to inhibit dephosphorylation were carried out at 0 °C as previously described (30). Deocclusion of K\(^+\) was generally studied in phosphorylation experiments at 10 °C, following formation of K\(^+\)-occluded enzyme at room temperature as previously described (17, 29, 31). To prevent dephosphorylation, oligomycin was added prior to initiation of phosphorylation with \([\gamma\(^32\)P]ATP (31). In addition, some deocclusion experiments were performed in the same way, but at 0 °C in the presence of final concentrations of 5 \(\mu\)M \([\gamma\(^32\)P]ATP, 5 mM MgCl\(_2\), 20 mM Tris (pH 7.5), 1 mM EGTA, 20 \(\mu\)g/ml oligomycin, and 10 \(\mu\)M ouabain, following incubation at 20 °C for 30 min with 8 mM RbCl, to mimic the conditions prevailing in the vanadate binding assay described below. In all cases, background phosphorylation was determined in the presence of 50 mM KCl without NaCl.

**Phosphorylation Assay for Determination of Vanadate Binding at Equilibrium—**

To examine the vanadate affinity under equilibrium conditions, 10 \(\mu\)g of deoxycholate-treated plasma membranes were incubated at 20 °C for 30 min in 40 \(\mu\)l of medium containing 20 mM Tris (pH 7.5), 5 mM MgCl\(_2\), 1 mM EGTA, 10 \(\mu\)M ouabain, and the indicated concentration of orthovanadate. To promote accumulation of the enzyme in the \(E_1\) form, 8 mM RbCl was added to the medium during the incubation with vanadate. The samples were then cooled to 0 °C to prevent dissociation of bound vanadate (32), and the enzyme fraction with no vanadate bound was determined by measuring the amount of phosphoenzyme formed during a 30-s incubation following the addition of 360 \(\mu\)l of ice-cold phosphorylation medium producing final concentrations of 50 mM NaCl, 50 mM choline chloride, 20 mM Tris (pH 7.5), 5 \(\mu\)M \([\gamma\(^32\)P]ATP, 5 mM MgCl\(_2\), 1 mM EGTA, and 20 \(\mu\)g/ml oligomycin. The data were fitted by assuming a simple one-site binding model, where only the enzyme fraction with no vanadate bound phosphorylates,

\[ EP = (EP_{max} - EP) \times (1 - [\text{vanadate}]K_{50} + [\text{vanadate}]) + EP \]  

(1) \(EP_{max}\) is the phosphorylation level obtained in the absence of vanadate, and \(EP\), corresponds to infinite vanadate concentration.

**Rapid Kinetic Phosphorylation and Dephosphorylation Studies—**

To perform rapid kinetic phosphorylation and dephosphorylation experiments at 25 °C, a Bio-Logic quench-flow module QFM-5 (Bio-Logic Science Instruments, Claix, France) was used as previously described (17). The phosphorylation rate of enzyme present in the Na\(^+\)-saturated form was determined in single-mixing experiments carried out as previously described according to "Protocol 1" (17, 33). To monitor the rate of the \(E_1P \rightarrow E_2P\) transition or the dephosphorylation of \(E_2P\), a double-mixing procedure was used to study dephosphorylation of phosphoenzyme formed either in the presence of 600 mM NaCl to accumulate \(E_2P\).
Expression, Na⁺, K⁺, and ATP Dependence of Na⁺,K⁺-ATPase Activity, and Catalytic Turnover Rate—Thr²¹⁴ of the rat kidney Na⁺,K⁺-ATPase was replaced with alanine, and the resulting enzyme was expressed in COS-1 cells under ouabain selective pressure as previously (26, 27). The Thr²¹⁴ → Ala mutant was able to confer ouabain resistance to the cells, indicating that the mutant is able to transport Na⁺ and K⁺ at rates compatible with cell growth. Thr²¹⁴ → Ala was expressed at a level very similar to that of the wild-type enzyme (about 60 pmol/mg of total membrane protein determined as the maximum phosphoenzyme level, $E_P^{\text{max}}$, see below and Refs. 30 and 33), allowing the effects of this mutation on the overall and partial reaction steps of the enzyme cycle to be analyzed. Transfection was also carried out with Glu³¹⁶ → Ala, but this mutant failed to confer ouabain resistance to the cells, indicating that the transport activity or the expression level of this mutant is too low to maintain cell viability. Titrations of the Na⁺, K⁺, and ATP dependence of ATPase activity of Thr²¹⁴ → Ala showed a slightly increased apparent affinity for Na⁺ (1.5-fold), a slightly decreased apparent affinity for K⁺ (1.2-fold), and a 3.3-fold increase in apparent affinity for ATP, relative to wild type (Fig. 2 and Table I). Because the $E_1$ form binds Na⁺ and ATP with high affinity, but K⁺ with low affinity, and the $E_2$ form binds K⁺ with high affinity and ATP with low affinity, the effects of the mutation on the apparent Na⁺, K⁺, and ATP affinities are compatible with a shift of the $E_1$→$E_2$ conformational equilibrium of the dephosphoenzyme in favor of $E_1$.

The maximal catalytic turnover rate of the Na⁺,K⁺-ATPase was estimated as the ratio between $V_{\text{max}}$ for ATP hydrolysis and the active site concentration, determined as the maximum phosphoenzyme level, $E_P^{\text{max}}$, measured at 0 °C by phosphorylation with ATP in the presence of 150 mM NaCl and oligomycin to inhibit dephosphorylation. A value of $3225 \pm 262$ min⁻¹ was found for Thr²¹⁴ → Ala, i.e. about 38% of the turnover rate of 8474 ± 165 min⁻¹ determined for the wild-type (30, 33).

Vanadate Dependence of Na⁺, K⁺-ATPase Activity—Vanadate is considered an analog of the phosphoryl transition state and binds specifically to $E_2$ and $E_2(K_v)$, leading to an inhibited dead-end state that probably resembles the $E_2P$ complex from which phosphate is released (34). Fig. 3 shows titration of the vanadate inhibition of steady-state Na⁺,K⁺-ATPase activity in the presence of 20 mM K⁺, i.e. a condition facilitating vanadate inhibition in the wild type. Relative to the wild type, the mutant Thr²¹⁴ → Ala displayed a conspicuous 150-fold reduction of the apparent affinity for vanadate in the ATPase assay. This suggests either that the steady-state level of the vanadate-reactive $E_2$ conformation was depleted or the intrinsic affinity for vanadate reduced in the mutant.

Na⁺ Dependence of Phosphorylation—To reveal the Na⁺ affinity at the cytoplasmically facing Na⁺ sites without interference from K⁺, the Na⁺ dependence of phosphorylation from ATP was studied in the absence of K⁺ and presence of oligomycin to inhibit dephosphorylation (Fig. 4). Relative to wild type, the apparent affinity for Na⁺ was slightly increased (1.6-fold) in Thr²¹⁴ → Ala, in line with the above described increase in the apparent affinity for Na⁺ in measurement of Na⁺,K⁺-ATPase activity.

Time Course of Phosphorylation with [γ⁻³²P]ATP—To test the phosphorylation reaction $E_1Na_3 → E_1P(Na_3)$ and the ATP affinity of the $E_1Na_3$ form, the time course of phosphorylation with 2 μM [γ⁻³²P]ATP was studied in the presence of oligomycin (Fig. 5), using a quench-flow module QFM-5 (see “Experimental Procedures”). The observed rate constant, $k_{o bs}$, was 32 s⁻¹ for Thr²¹⁴ → Ala, i.e. very similar to that of the wild-type enzyme (29 s⁻¹). In the presence of 2 μM ATP, the phosphorylation reaction is less than half-saturated in the wild type (17), and, accordingly, any difference between mutant and wild type with respect to the affinity of $E_1Na_3$ for ATP should be reflected in the $k_{o bs}$ value. Hence, the result of the phosphorylation experiment suggests that in Thr²¹⁴ → Ala the phosphorylation reaction as well as the binding of ATP to $E_1Na_3$ is wild type-like.

Occlusion and Deocclusion of K⁺—To examine the amount of K⁺-occluded enzyme at equilibrium and the rate of the K⁺ deocclusion reaction $E_2(K_v) → E_2 + 2K^+$, the previously described phosphorylation assay was applied (Fig. 6) (17, 29, 31). Following formation of the K⁺-occluded complex in the presence of 8 mM K⁺, the phosphorylation was monitored upon a 10-fold dilution of the enzyme in a solution containing 1 μM
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| Mutation | $K_{d}(Na^{+})$ | $K_{d}(K^{+})$ | $K_{d}(ATP)$ | $E_{i}/K_{d}$ | Deocclusion rate | $E_{i}P/E_{o}P$ | $E_{i}P \rightarrow E_{o}P$ rate |
|----------|----------------|---------------|---------------|--------------|-----------------|---------------|----------------|
| Wild type | 8.8\textsuperscript{a} | 0.66\textsuperscript{a} | 0.36\textsuperscript{a} | 96\textsuperscript{a} | 0.008\textsuperscript{a} | 20/80\textsuperscript{a} | 138\textsuperscript{a} |
| Thr\textsuperscript{214} → Ala | 5.9\textsuperscript{b} | 0.80\textsuperscript{b} | 0.11\textsuperscript{b} | 82\textsuperscript{b} | 0.032\textsuperscript{b} | 40/60\textsuperscript{b} | 57\textsuperscript{b} |
| Gly\textsuperscript{263} → Ala' | 5.1 | 0.86 | 0.06 | 46 | 0.187 | 75/25 | 28 |

\* From Fig. 2.  
\* From Fig. 6.  
\* From Fig. 8.  
\* From Ref. 10.  
\* From Ref. 17, obtained under the same conditions as used for the present paper.

Fig. 3. Vanadate dependence of Na\textsuperscript{+,K\textsuperscript{+}}-ATPase activity. The rate of ATP hydrolysis was determined for the expressed wild-type Na\textsuperscript{+,K\textsuperscript{+}}-ATPase (filled circles) and mutant Thr\textsuperscript{214} → Ala (open triangles) at 37 °C in the presence of 150 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM MgCl\textsubscript{2}, 30 mM histidine buffer (pH 7.4), 1 mM EGTA, 10 μM ouabain, and the indicated concentrations of vanadate. Average values corresponding to four to nine independent titrations are shown as percentage of the Na\textsuperscript{+,K\textsuperscript{+}}-ATPase activity measured in the absence of vanadate. Error bars can be seen when larger than the size of the symbol. Each line shows the best fit of the Hill equation. The corresponding $K_{d}$ values are 2.5 and 374 μM for the wild type and the Thr\textsuperscript{214} → Ala mutant, respectively.

Fig. 4. Na\textsuperscript{+} dependence of phosphorylation from ATP. Phosphorylation was carried out with the wild-type Na\textsuperscript{+,K\textsuperscript{+}}-ATPase (filled circles) and mutant Thr\textsuperscript{214} → Ala (open triangles) for 15 s at 0 °C in the presence of 20 mM Tris (pH 7.5), 3 mM MgCl\textsubscript{2}, 1 mM EGTA, 10 μM ouabain, oligomycin (20 μg/ml), and 0.5 mM vanadate. The data points are average values corresponding to four to six independent titrations. Error bars can be seen when larger than the size of the symbol. Each line shows the best fit of the Hill equation. The corresponding $K_{o,5}$ values are 0.55 and 0.34 mM for the wild type and Thr\textsuperscript{214} → Ala mutant, respectively.

Time course of phosphorylation by [γ\textsuperscript{32P}ATP at 25 °C. Rapid kinetic measurements at 25 °C of the time course of phosphorylation were performed with the wild-type Na\textsuperscript{+,K\textsuperscript{+}}-ATPase (filled circles) and the mutant Thr\textsuperscript{214} → Ala (open triangles) in the presence of 100 mM NaCl, 40 mM Tris (pH 7.5), 3 mM MgCl\textsubscript{2}, 1 mM EGTA, 10 μM ouabain, oligomycin (20 μg/ml), and 2 μM [γ\textsuperscript{32P}ATP, using the QFM-5 module according to Protocol 1 (17). The data points are average values of three experiments. Error bars can be seen when larger than the size of the symbol. Each line shows the best fit of a monoexponential time function. The rate constants are 29 and 32 s\textsuperscript{-1}, and the maximum level of phosphorylation reached, indicated as 100%, constituted 96, and 94% of the active site concentration, for the wild type and the Thr\textsuperscript{214} → Ala mutant, respectively.

Vanadate Binding at Equilibrium—The 150-fold reduction of the apparent affinity for vanadate determined in the ATPase assay may in principle arise from a direct effect of the mutation on the vanadate-binding site (a change to the “intrinsic” affinity for vanadate) or be secondary to changes in the rates of reactions in the enzyme cycle preceding and subsequent to the vanadate-reactive $E_{d}$ conformation, which would affect the accumulation of $E_{d}$ at steady state and, thereby, the sensitivity to inhibition by vanadate. To be able to distinguish between these possibilities, we have adopted a phosphorylation assay that allows the enzyme-vanadate complex to be formed under equilibrium conditions, where $E_{d}$ is not being continuously produced by dephosphorylation of $E_{d}P$ as during enzyme cycling. The enzyme is equilibrated with various concentrations of vanadate at 20 °C in the absence of ATP, and the vanadate-free enzyme fraction is determined by its ability to form a phosphoenzyme with [γ\textsuperscript{32P}ATP after cooling to 0 °C. Vanadate binding is competitive with phosphorylation, and because the dissociation of vanadate is very slow at 0 °C (32), the amount of phosphoenzyme formed in this assay reflects the equilibrium between the free and vanadate-bound enzyme forms existing during the equilibration with vanadate at 20 °C before the addition of [γ\textsuperscript{32P}ATP. 

[γ\textsuperscript{32P}ATP and 100 mM Na\textsuperscript{+}. As previously described for the wild type and other mutants (17, 29, 31), the time course of phosphorylation can be analyzed as a biphasic time function, in which the component corresponding to the rapid phase is at maximum from the beginning, because it reflects the nonocluded $E_{1}$ enzyme pool that binds Na\textsuperscript{+} and phosphorylates within 5 s. The slow phase (shown by the lines in Fig. 6) reflects the phosphorylation of $E_{d}(K_{d})$ through the steps $E_{d}(K_{d}) \rightarrow E_{1} \rightarrow E_{1}Na_{3} \rightarrow E_{i}P(Na_{3})$, where the release of occluded K\textsuperscript{+} is rate-limiting. The fitting procedure allows extraction of the rate constant corresponding to the slow phase and the amplitude of the slow phase (100% minus the ordinate intercept), corresponding to the relative amount of enzyme initially present as $E_{i}/(K_{d})$. These parameters are shown in Table I. Relative to wild type, the Thr\textsuperscript{214} → Ala mutant showed a 4-fold increase of the rate of release of occluded K\textsuperscript{+}, and the relative amount of $E_{i}/(K_{d})$ was 82% in the mutant versus 96% in the wild type.
Fig. 6. **Time course of K⁺ deocclusion.** Measurements were performed with the wild-type Na⁺,K⁺-ATPase (filled circles) and the mutant Thr²¹⁴ → Ala (open triangles). Enzyme treated with 20 μM ouabain, 3 mM MgCl₂, and 20 mM Tris buffer (pH 7.5) was equilibrated for 1 h at room temperature with 8 mM K⁺. Oligomycin (150 μg/ml) was then added, and the solution was cooled to 10 °C and diluted 10-fold by addition of a phosphorylation solution of the same temperature, producing final concentrations of 1 μM [γ⁻³²P]ATP, 100 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, and 20 mM Tris (pH 7.5). The phosphorylation was monitored by acid quenching at various time intervals. For determination of the 100% value representing the fully deoccluded enzyme, the 1-h incubation was carried out in the presence of 50 mM Na⁺ and absence of K⁺. Average values corresponding to two to seven independent experiments were analyzed by fitting a biphasic time function as previously described (17, 31). The slow component is indicated by the line, and the extracted rate constant, reflecting K⁺ deocclusion, and its amplitude, corresponding to the relative amount of enzyme initially present as E₁(Kᵢ), are listed in Table I. Error bars can be seen when larger than the size of the symbol.

Results obtained with this assay are shown in Fig. 7 and summarized in Table II. Generally, the vanadate affinity reported here is higher than that determined during enzyme turnover with ATP. This is probably because of the lack of competition from ATP binding and phosphorylation during the equilibration with vanadate.

Fig. 7A shows results obtained following equilibration with vanadate in the absence of any of the transported cations. Under these conditions, Thr²¹⁴ → Ala exhibited 8-fold reduction of the apparent affinity for vanadate relative to wild type. A change in this direction could in principle arise from a mutational effect shifting the E₁-E₂ conformational equilibrium of the dephosphoenzyme in favor of E₁, and, indeed, there is evidence for such a shift from the changes to the apparent affinities for Na⁺, K⁺, and ATP, as well as the rate of the K⁺ deocclusion reaction and the equilibrium level of E₂(K₂) presented above, although the changes to these parameters were relatively small (see Table I). For comparison, Fig. 7 also shows vanadate inhibition results obtained with the previously characterized mutant Gly²⁶³ → Ala (17) that exhibited more pronounced changes than Thr²¹⁴ → Ala with respect to the parameters characterizing the conformational equilibrium (cf. Table I). As seen in Fig. 1, Gly²⁶³ is located in the loop connecting domain A with M3. The corresponding glycine in the Ca²⁺-ATPase undergoes a large movement in relation to the E₁-E₂ transition, but unlike the threonine does not approach the phosphorylated aspartate. In accordance with a more pronounced shift of the conformational equilibrium in favor of E₁ in Gly²⁶³ → Ala as compared with Thr²¹⁴ → Ala, the apparent affinity for vanadate observed in the absence of the transported cations (Fig. 7A) was also reduced more in Gly²⁶³ → Ala (22-fold reduction relative to wild type) than in Thr²¹⁴ → Ala.

An important question was, however, whether the relatively small change to the E₁-E₂ conformational equilibrium of Thr²¹⁴ → Ala indicated by the above mentioned parameters (Table I) is sufficient to account for the 8-fold reduction in apparent vanadate affinity observed in the equilibrium binding assay. To eliminate the influence of the shift of E₁-E₂ equilib-
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The wild-type Na$^+$.K$^+$.-ATPase (filled circles) and mutant Thr$^{214} \rightarrow$ Ala (open triangles) were phosphorylated for 10 s at 0$^\circ$C in the presence of 2 $\mu$M [γ-32P]ATP, 20 mM NaCl, 130 mM choline chloride, 20 mM Tris (pH 7.5), 3 mM MgCl$_2$, 1 mM EGTA, and 10 $\mu$M ouabain. Dephosphorylation was monitored by addition of a chase solution producing a final concentration of 1 mM unlabeled ATP and 2.5 mM ADP, followed by acid quenching at the indicated time intervals. The data points are average values corresponding to five to eight independent experiments, calculated following normalization to the 100% value obtained by quenching after 10 s of phosphorylation without dephosphorylation. Error bars can be seen when larger than the size of the symbol. 

**Fig. 8. Time course of ADP-dependent dephosphorylation at 0$^\circ$C.** The wild-type Na$^+$.K$^+$.-ATPase (filled circles) and mutant Thr$^{214} \rightarrow$ Ala (open triangles) were phosphorylated for 10 s at 0$^\circ$C in the presence of 2 $\mu$M [γ-32P]ATP, 20 mM NaCl, 130 mM choline chloride, 20 mM Tris (pH 7.5), 3 mM MgCl$_2$, 1 mM EGTA, and 10 $\mu$M ouabain. Dephosphorylation was monitored by addition of a chase solution producing a final concentration of 1 mM unlabeled ATP and 2.5 mM ADP, followed by acid quenching at the indicated time intervals. The data points are average values corresponding to five to eight independent experiments, calculated following normalization to the 100% value obtained by quenching after 10 s of phosphorylation without dephosphorylation. Error bars can be seen when larger than the size of the symbol.}

**The lines show the best fit of a biexponential function. Note the logarithmic ordinate scale. The dotted lines show the extrapolation of the slow decay component corresponding to E$_2$P back to the ordinate intercept to show its initial value. The extents of the rapid and slow decay components corresponding to the initial values of E$_2$P and E$_3$P, respectively, are given in Table I. The rate constant corresponding to the slow decay component of E$_3$P hydrolysis is 0.034 s$^{-1}$ for wild type, and 0.018 s$^{-1}$ for Thr$^{214} \rightarrow$ Ala.**

"Conformational mutant" in which the intrinsic affinity of E$_2$P for vanadate is unaffected by the mutation. By contrast, Thr$^{214} \rightarrow$ Ala displayed a 4-fold reduced affinity for vanadate relative to wild type, even under conditions where the enzyme was 100% on the E$_2$(Rb$_2$)$_2$ form, indicating that this mutation affected the intrinsic affinity for vanadate.

**ADP Sensitivity of the Phosphoenzyme—**The phosphoenzyme is usually considered to consist of two major pools, E$_2$P and E$_3$P, which can be distinguished by their different reactivities toward ADP and K$^+$. The pool referred to as E$_2$P is K$^+$-insensitive but ADP-sensitive, i.e., rapidly dephosphorylated by ADP because of its ability to donate the phosphoryl group back to ADP forming ATP, whereas E$_3$P is ADP-insensitive and K$^+$-responsive, i.e., rapidly hydrolyzed in the presence of K$^+$, binding at extracellularly facing sites. The partition of the phosphoenzyme between these two pools at steady state can be analyzed by studying the ADP sensitivity at 0$^\circ$C, where the equilibration of the two pools is slow. Fig. 8 presents results of experiments in which the time course of dephosphorylation was monitored following addition of 2.5 mM ADP together with 1 mM nonradioactive ATP to phosphoenzyme formed in the presence of [γ-32P]ATP. The phosphoenzyme as well as the dephosphorylation was carried out in the presence of 20 mM Na$^+$ and absence of K$^+$. Under these conditions, E$_3$P dephosphorylates only very slowly, whereas E$_2$P dephosphorylates rapidly because of the reaction with ADP. Thus, the data points representing the time course of dephosphorylation could be fitted by a biexponential function where the extents of the rapid and slow decay components reflect the initial amounts of the ADP-sensitive E$_2$P and ADP-insensitive E$_3$P, respectively. The dotted lines in Fig. 8 show the extrapolation of the slow decay component back to the ordinate intercept to indicate the amount of E$_3$P. For the wild type, the ordinate intercept yielded about 80% E$_2$P, indicating that the steady-state distribution of the phosphoenzyme intermediates between the E$_2$P and E$_3$P forms favored the ADP-insensitive E$_2$P form. For Thr$^{214} \rightarrow$ Ala, the amount of E$_2$P was 60%. This is considerably more than the 25% previously observed for Gly$^{263} \rightarrow$ Ala (Table I). Hence, in contrast to Gly$^{263} \rightarrow$ Ala, the distribution of E$_2$P and E$_3$P in Thr$^{214} \rightarrow$ Ala was only slightly more in favor of E$_2$P than in the wild type, in line with the observation described above that the E$_1$-E$_2$P conformational equilibrium of the dephosphoenzyme was only slightly shifted in favor of E$_1$. From Fig. 8 it can, moreover, be seen that the rate constant corresponding to the slow phase, reflecting the dephosphorylation of E$_2$P was reduced in Thr$^{214} \rightarrow$ Ala relative to wild type. This reduction amounted to about 2-fold relative to the wild-type enzyme (see legend to Fig. 8).

**Dephosphorylation Kinetics at 25$^\circ$C of Phosphoenzyme Formed at 20 mM Na$^+$—**The 2-fold reduction of the E$_2$P dephosphorylation rate seen in Fig. 8 raised the question whether a slow dephosphorylation of E$_2$P is a general characteristic of Thr$^{214} \rightarrow$ Ala, observable also at a more physiological temperature and in the presence of K$^+$ to activate the dephosphorylation. Consequently the E$_2$P dephosphorylation was studied at 25 instead of 0$^\circ$C, using the quench-flow module as previously described (17, 33) and phosphorylation conditions otherwise similar to the ADP sensitivity experiments to promote the accumulation of E$_2$P (presence of 20 mM NaCl with 130 mM choline chloride and without K$^+$, cf. Fig. 8). Fig. 9 shows that at 3 different sets of dephosphorylation conditions: a Na$^+$ concentration of 200 mM without K$^+$ (Fig. 9A), a nonsaturating K$^+$ concentration of 1 mM (Fig. 9B), and a saturating K$^+$ concentration of 20 mM (Fig. 9C), the dephosphorylation rate was significantly reduced in the Thr$^{214} \rightarrow$ Ala mutant relative to wild type. The reduction amounted to 1.8- (Fig. 9A), 2.1- (Fig. 9B), and at least 4.8-fold (Fig. 9C). Because the dephosphorylation rate of the wild type at 20 mM K$^+$ was too high to measure accurately (>300 s$^{-1}$, Fig. 9C), the reduction seen for the mutant in this condition may actually amount to more than 4.8-fold. It is noteworthy that for all three conditions a monoequivalent function could be fitted satisfactorily to the data, indicating that at the beginning of the dephosphorylation the major part of the phosphoenzyme was present as E$_2$P with very little admixture of E$_3$P. The reason that E$_2$P accumulated to such an extent is that at 25$^\circ$C in the presence of only 20 mM Na$^+$ without K$^+$ (i.e., the conditions prevailing during formation of the phosphoenzyme) the rate of E$_2$P dephosphorylation is more than 50-fold lower than the rates of phosphorylation and E$_2$P → E$_3$P interconversion, both for wild type and mutant (cf. Fig. 9A, where a rather low rate of E$_2$P dephosphorylation is seen even at 200 mM Na$^+$ because of the absence of K$^+$).

Fig. 9 shows that a saturating K$^+$ concentration of 20 mM enhanced the dephosphorylation rate 184- and 69-fold in the wild type and the Thr$^{214} \rightarrow$ Ala mutant, respectively, relative to the rate in the mere presence of Na$^+$. Hence, the difference between the mutant and the wild type increased upon binding of K$^+$. For the Thr$^{214} \rightarrow$ Ala mutant, the dephosphorylation rate was more than half-maximal at 1 mM K$^+$ (compare the rate constants of 38 and 69 s$^{-1}$ obtained at 1 and 20 mM K$^+$, respectively), whereas for the wild type the dephosphorylation rate at 1 mM K$^+$ was maximally 24% of that corresponding to 20 mM K$^+$, showing that the reduced rate of E$_2$P dephosphorylation in Thr$^{214} \rightarrow$ Ala is not caused by a decrease in the affinity for K$^+$. Fig. 9C, furthermore, shows that the inhibition of the dephosphorylation rate in Thr$^{214} \rightarrow$ Ala could not be overcome by increasing the Mg$^{2+}$ concentration from 3 to 15 mM. For the wild type, the Mg$^{2+}$ concentration of 3 mM is saturating with respect to activation of dephosphorylation. Likewise, the data obtained for the mutant at 15 mM Mg$^{2+}$ were indistinguishable
from those obtained at 3 mM Mg$^{2+}$, indicating that the inhibition is not caused by a reduction of Mg$^{2+}$ affinity.

Dephosphorylation Kinetics at 25 °C of Phosphoenzyme Formed at 600 mM NaCl—Finally, the $E_1P \rightarrow E_2P$ interconversion was studied at 25 °C (Fig. 10). To accumulate the $E_1P$ form, phosphatase was carried out in the presence of a high NaCl concentration of 600 mM, which shifts the steady-state distribution of phosphoenzyme intermediates in favor of $E_1P$, as previously described (17, 33). To observe the $E_1P \rightarrow E_2P$ conversion, dephosphorylation was initiated by a downward jump in the Na$^+$ concentration to 200 mM, and simultaneously 1 mM unlabeled ATP was added together with 20 mM K$^+$, followed by acid quenching at various time intervals. The dephosphorylation involves the steps $E_1P \rightarrow E_2P \rightarrow E_2$. In the presence of a saturating K$^+$ concentration of 20 mM, $E_1P \rightarrow E_2$ is much faster than $E_1P \rightarrow E_2P$ in the wild-type enzyme and does not contribute significantly to rate limitation. Therefore, the wild-type data could be satisfactorily fitted by a monoexponential decay function (rate constant 111 s$^{-1}$, fit not shown).

This was obviously not the case for the mutant data (Fig. 10), the reason being that the $E_1P \rightarrow E_2P$ transition in the mutant is followed by a relatively slow $E_2P \rightarrow E_2$ reaction. By fitting Equation 2 under “Experimental Procedures,” describing two consecutive first-order reactions, with the rate constants for the dephosphorylation of $E_2P$ set at 69 and 500 s$^{-1}$ for Thr$^{214} \rightarrow$ Ala and the wild type, respectively, in accordance with the data described above for Fig. 9C, the respective rate constants corresponding to the $E_1P \rightarrow E_2P$ transition were found to be 57 and 39 s$^{-1}$, indicating that the $E_1P \rightarrow E_2P$ conversion rate is reduced 2.4-fold in Thr$^{214} \rightarrow$ Ala relative to wild type.

**DISCUSSION**

Thr$^{214} \rightarrow$ Ala is the first Na$^+$.K$^+$-ATPase mutant for which a change to the intrinsic affinity of the $E_1$ form for vanadate and a, presumably related, inhibitory effect of the mutation on the $V_{max}$ for dephosphorylation have been demonstrated. These effects together with effects on $E_1 \rightarrow E_2$ and $E_2P \rightarrow E_2P$ conformational equilibria all seem to contribute to the observed change in sensitivity to vanadate inhibition of ATPase activity induced by the mutation.

**Conformational Equilibria—**As summarized in Table I, titration of the Na$^+$.K$^+$, and ATP dependence of ATPase activity in Thr$^{214} \rightarrow$ Ala showed changes relative to the wild type compatible with a shift of the $E_1 \rightarrow E_2$ conformational equilibrium of the phosphoenzyme in favor of $E_1$. This interpretation was further supported by the K$^+$-deoccluding analysis, demonstrating an increase of the rate of the K$^+$-deoccluding $E_2(K_2) \rightarrow E_1$ step relative to wild type as well as a reduction of the level of the $E_2(K_2)$ intermediate in the absence of Na$^+$ and ATP. Studies of the phosphoenzyme showed a change in the steady-state distribution of ADP-sensitive $E_1P$ and ADP-insen-
sitive $E_P$ in favor of $E_3P$, and the rate constant characterizing the $E_3P \rightarrow E_2P$ conversion was reduced in Thr$^{214} \rightarrow$ Ala relative to wild type. Hence, it may be concluded that mutation Thr$^{214} \rightarrow$ Ala displaced the $E_3P$ and $E_3P-E_2P$ conformational equilibria in parallel in favor of the $E_1$ and $E_1P$ forms, respectively.

**Affinity for Vanadate**—A most remarkable feature displayed by mutant Thr$^{214} \rightarrow$ Ala was the conspicuous 150-fold reduction of the apparent affinity for vanadate inhibition determined in the ATPase assay (Fig. 3). The amount of enzyme-vanadate complex formed depends on the concentration of the vanadate-reactive $E_1$ intermediate accumulated at steady state and the intrinsic affinity of $E_3$ for vanadate. The displacement of the conformational equilibria in favor of the $E_1$ and $E_1P$ forms must, therefore, have contributed to lower the apparent affinity for vanadate through reduction of the steady-state concentration of $E_3$. It is noteworthy, however, that the previously studied mutation Gly$^{263} \rightarrow$ Ala in the loop connecting domain A with membrane segment M3 (see Fig. 1) exerted only a 7-fold reduction in the apparent vanadate affinity relative to the wild type under the conditions prevailing during the ATPase activity measurement (17), even though the displacement of the conformational equilibria in favor of $E_1$ and $E_1P$ was more pronounced than for Thr$^{214} \rightarrow$ Ala as judged on the basis of the apparent affinities for Na$^+$, K$^+$, and ATP and the analysis of K$^+$ deocclusion and of the phosphoenzyme intermediates (Table I). Consequently, the conspicuous reduction of the apparent vanadate affinity observed for Thr$^{214} \rightarrow$ Ala cannot be attributed solely to the change of conformational equilibrium, and there must be additional contributing factors in this case.

Importantly, we were able to examine the intrinsic vanadate affinity of the $E_3$ form and the effect on the apparent affinity for vanadate of displacement of the $E_3-E_2$ equilibrium, using for the first time a phosphorylation assay that determines the vanadate-free enzyme fraction under equilibrium conditions (i.e. in the absence of enzyme turnover producing $E_2$ from $E_3P$) (Fig. 7). For Gly$^{263} \rightarrow$ Ala, the vanadate affinity determined by this assay became wild type-like when the enzyme was forced into the $E_3$ form by the presence of the K$^+$ congener Rb$^+$, thus demonstrating that the intrinsic vanadate affinity of $E_3$ is normal in this mutant. By contrast, Thr$^{214} \rightarrow$ Ala retained a reduced affinity for vanadate in the $E_3P$ form accumulated in the presence of Rb$^+$, indicating that Thr$^{214} \rightarrow$ Ala manifests true low affinity for vanadate.

**Defective Catalysis of $E_3P$ Dephosphorylation**—In dephosphorylation experiments with phosphoenzyme accumulated under conditions favoring the $E_3P$ form, Thr$^{214} \rightarrow$ Ala showed a reduced dephosphorylation rate relative to wild type. This was seen independently of whether dephosphorylation was activated by Na$^+$ or by nonsaturating or saturating K$^+$ concentrations (Fig. 9). Normally, K$^+$ acting at extracellularly facing sites far away from the catalytic site activates the dephosphorylation, and the reduced dephosphorylation rate in Thr$^{214} \rightarrow$ Ala could possibly involve defective K$^+$ binding. However, the difference between Thr$^{214} \rightarrow$ Ala and the wild type with respect to the dephosphorylation rate increased when the K$^+$ concentration was increased from 1 to 20 mM, and the dephosphorylation rate of the mutant was more than half-maximal at 1 mM K$^+$, whereas the dephosphorylation rate displayed by the wild type at this K$^+$ concentration was maximally 24% of the rate at 20 mM K$^+$. Therefore, the inhibition of $E_3P$ dephosphorylation in Thr$^{214} \rightarrow$ Ala must be brought about by a reduction in $V_{\text{max}}$ for dephosphorylation rather than by a decrease in K$^+$ affinity at the activating $E_3P$ sites. Such an inhibitory effect on the $V_{\text{max}}$ for dephosphorylation has not previously been reported for any Na$^+,K^+\text{-ATPase}$ mutant. Together with the 2.4-fold reduction of the $E_3P \rightarrow E_3P$ conversion rate, the reduction of the $E_3P$ dephosphorylation rate, corresponding to at least 4.8-fold at 20 mM K$^+$, can account for the low turnover rate displayed by the mutant (38% of wild type). It is, furthermore, likely that the reduced rate of production of $E_2$ caused by the inhibition of dephosphorylation contributes to the striking reduction of the apparent vanadate affinity observed in the ATPase assay, through reduction of the steady-state concentration of $E_2$.

Our observations can be explained according to the classic concept that the catalytic rate (in this case the rate of $E_3P$ dephosphorylation) depends on the ability of the enzyme to bind the activated complex (transition state) tightly, thereby lowering the energy barrier that has to be traversed during the reaction (35, 36). Hence, the catalytic rate increases with an increase of the affinity of the enzyme for the transition state complex, and decreases in cases where the transition state complex is bound less tightly. Because vanadate is considered an analog of the pentacoordinated transition state of the phosphoryl group (34), it is likely that the reduced intrinsic affinity of the $E_2$ form for vanadate in Thr$^{214} \rightarrow$ Ala is directly related to the inhibition of $E_3P$ dephosphorylation, implying that defective binding of the phosphoryl transition state complex (transition state destabilization) causes the inability to catalyze $E_3P$ dephosphorylation properly.

The catalytic site performs very different functions in $E_3P/E_3P$ as compared with $E_1P/E_1P$ ("phosphatase" and "kinase" functions, respectively). In $E_3P$, a nucleophilic attack by water on the aspartyl phosphate bond is facilitated. It is notable that in Thr$^{214} \rightarrow$ Ala catalytic mechanism is defective only in $E_1P/E_1P$, the phosphorolysis of $E_1$ from ATP being wild-type-like (Fig. 5). This supports a mechanism where the conserved Thr$^{214}$TGES of domain A is isolated in the $E_1$ form, but makes contact with the phosphorylation site and becomes catalytically important in the $E_1P/E_1P$ conformations (cf. Fig. 1). It is interesting to note the analogy to the non-ATPase members of the family of aspartyl-phosphate-utilizing phosphohydrolases/phosphotransferases such as phosphoserine phosphatase, which recently was studied by x-ray crystallography in several intermediates (37). These enzymes have in common with the P-type ATPases several highly conserved residues of the Rossman fold corresponding to domain P, but lack domain A with the TGES sequence present in P-type ATPases. Nevertheless, the catalytic function of phosphoserine phosphatase requires participation of residues such as Glu$^{20}$ outside the Rossmann fold, to assist the nucleophilic attack and stabilize the transition state (37). In the P-type ATPases, the corresponding requirement for domain A residues for proper catalysis of $E_1P$ hydrolysis, and the ability of domain A to move as shown in Fig. 1, thereby initiating conformational rearrangements in the membrane, may constitute an important element in the coupling of substrate utilization with cation transport.

The defective catalysis of $E_3P$ dephosphorylation in Thr$^{214} \rightarrow$ Ala would be in accordance with a role of the side chain hydroxyl of Thr$^{214}$ in the positioning of the attacking water molecule or in direct interaction with the phosphate in the transition state, as well as a more indirect role in optimization of the structure of the catalytic site in the $E_3P$ form through hydrogen bonding to other residues. Hydrogen bond formation with residues in domain P could also explain the importance of Thr$^{214}$ in the $E_1P$-$E_2$ and $E_3P$-$E_2P$ conformational changes, where domain A is thought to dock into domain P (cf. Fig. 1). The side chain interactions could, furthermore, affect the orientation of the main chain carbonyl group corresponding to Thr$^{214}$, which in the crystal structure of the Ca$^{2+}$-ATPase in the $E_2$ form is rather close to other catalytically important residues such as...
Importance of Thr$^{214}$ of the Na$^+$/K$^+$-ATPase

Ap$^703$ (Asp$^{712}$ in Na$^+$/K$^+$-ATPase$^2$).

Mg$^{2+}$ or another divalent cation such as Fe$^{2+}$ is required as cofactor for the phosphorylation of $E_1$ by ATP and for the hydrolysis of $E_2P$ (1). On the basis of detection of Fe$^{2+}$-catalyzed oxidative cleavage near the TGES sequence in $E_2$ and $E_2P$, but not in the $E_1$ form, Karlish and co-workers (38) have proposed that the glutamate, Glu$^{216}$, of TGES contributes to ligation of Mg$^{2+}$ in $E_2$ and $E_2P$, whereas in $E_1$ and $E_1P$ the coordination has changed so that all Mg$^{2+}$ coordinating groups now come from the P and N domains. In analogy with phosphoserine phosphatase (37), Mg$^{2+}$ ligands might be contributed by P-domain residues Asp$^{371}$, Thr$^{373}$, and Asp$^{712}$ (39) (numbering corresponding to Na$^+$-ATPase$^2$). In phosphoserine phosphatase, the remaining three Mg$^{2+}$ ligands are a phosphate oxygen and two water molecules (37). It is possible that in P-type ATPases one or both water molecules are substituted by protein groups of domain A in $E_2$ and $E_2P$, but not in $E_1$ and $E_1P$, or that protein groups of domain A serve to position the Mg$^{2+}$ liganding water molecules in $E_2$ and $E_2P$. In accordance with a crucial function of the glutamate of TGES, we found that the Na$^+$/K$^+$-ATPase mutant Glu$^{216}$ $\rightarrow$ Ala is unable to maintain cell viability. This indicates that the transport rate or the expression level is very low in Glu$^{216}$ $\rightarrow$ Ala. The advantage with the Thr$^{214}$ $\rightarrow$ Ala mutant was that although the function of this mutant clearly was defective, the activity and expression levels were sufficiently high to allow the detailed investigation of both the overall and the partial reactions. It is presently not known whether substitution of Thr$^{214}$ with residues other than alanine would lead to similar or quite different functional consequences as observed for Thr$^{214}$ $\rightarrow$ Ala. In principle, the mechanism underlying the inhibition of $E_2P$ dephosphorylation in Thr$^{214}$ $\rightarrow$ Ala could involve a defect in the ligation of the catalytic Mg$^{2+}$ ion. The catalytic ability of mutant Thr$^{214}$ $\rightarrow$ Ala was, however, not improved by a 5-fold increase of the Mg$^{2+}$ concentration (Fig. 9C). Thus, if Mg$^{2+}$ binding is defective in the mutant, it must be the positioning of the bound Mg$^{2+}$ ion and not simply the affinity, which is changed.

Acknowledgments—We thank Dr. Jens Peter Andersen for discussion and many helpful suggestions, Jytte Jørgensen, Janne Petersen, and Kirsten Lykke Pedersen for expert technical assistance, and Dr. R. J. Kaufman, Genetics Institute, Boston, MA, for the expression vector pMT2.

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