Interaction between the Catalytic Site and the A-M3 Linker Stabilizes $E_2/E_2P$ Conformational States of Na$^+$,K$^+$-ATPase*

Mads Toustrup-Jensen and Bente Vilsen‡

From the Department of Physiology, Institute of Physiology and Biophysics, University of Aarhus, Ole Worms Allé 160, DK-8000 Aarhus C, Denmark

The consequences of mutations Ile$^{265}$ → Ala, Thr$^{267}$ → Ala, Gly$^{271}$ → Ala, and Gly$^{274}$ → Ala for the partial reaction steps of the Na$^+$,K$^+$-ATPase transport cycle were analyzed. The mutated residues are part of the long loop (“A-M3 linker”) connecting the cytoplasmic A-domain with transmembrane segment M3. It was found that mutation Ile$^{265}$ → Ala displaces the $E_1/E_2$ and $E_1P/E_2P$ equilibria in favor of $E_1/E_1P$, whereas mutations Thr$^{267}$ → Ala, Gly$^{271}$ → Ala, and Gly$^{274}$ → Ala displace these conformational equilibria in favor of $E_2/E_2P$. The mutations affect both the rearrangement of the cytoplasmic domains (seen by changes in phosphoenzyme properties and apparent ATP/vanadate affinities) and the membrane sector (indicated by change in K$^+$/Rb$^+$ declucuclation rate). Destabilization of $E_2/E_2P$ in Ile$^{265}$ → Ala, as well as a direct effect on the intrinsic affinity of the $E_2$ form for vanadate, may be explained on the basis of the $E_2$ crystal structures of the Ca$^{2+}$-ATPase, showing interaction of the equivalent isoleucine with conserved residues near the catalytic region of the P-domain. The rate of phosphorylation from ATP was unaffected in Ile$^{265}$ → Ala, indicating a lack of interference with the catalytic function in $E_1/E_1P$. The effects of mutations Thr$^{267}$ → Ala, Gly$^{271}$ → Ala, and Gly$^{274}$ → Ala provide the first evidence in the literature of a relative stabilization of $E_2/E_2P$ resulting from perturbation of the A-M3 linker region. These mutations may lead to increased strain of the A-M3 linker in $E_1/E_1P$, increased stability of the A3 helix of the A-M3 linker in $E_2/E_2P$, and/or change of the orientation of the A3 helix, facilitating its interaction with the P-domain.

The Na$^+$,K$^+$-ATPase, located in the cell membrane, utilizes the free energy derived from the hydrolysis of ATP to import 2 K$^+$ in exchange for export of three Na$^+$ out of the cell (1). It is a member of the family of P-type ion-transporting ATPases (2) characterized by the transfer during the functional cycle of the γ-phosphoryl of ATP to a conserved aspartic acid residue (Asp$^{377}$ in rat kidney Na$^+$,K$^+$-ATPase)$^2$ located in the large cytoplasmic domain of the enzyme. The transport mechanism of the Na$^+$,K$^+$-ATPase is often described by the simplified reaction sequence $E_1Na_3 → E_1P(3Na_3) → E_1PK_2 → E_2(K_2)$. The cleaved enzyme retained the ability to hydrolyze ATP, taking place in the cytoplasmic part of the molecule some 40 Å away from the intramembranous ion binding sites (1–3). The Na$^+$,K$^+$-ATPase consists of ten transmembrane helices, M1–M10 (2, 4, 5), of which at least M4, M5, and M6 contribute to the cation transport pathway (2, 6–10). The transmembrane part is linked to a cytoplasmic head piece made up by three domains, denoted A- (actuator), N- (nucleotide-binding), and P- (phosphorylation) domains in the terminology based on the high resolution crystal structure of the closely related Ca$^{2+}$-ATPase (2, 5, 11). The A-domain consists of the N-terminal portion of the polypeptide chain in addition to the loop between M2 and M3, whereas the N- and P-domains are composed of the loop between M4 and M5 with the N-domain being inserted into the P-domain (11). Comparing the atomic structures of the Ca$^{2+}$-ATPase in Ca$^{2+}$-bound $E_1(\text{Ca}_3)$ (11) and $E_1(\text{Ca}_2)\cdot\text{Mg}\cdot\text{AlF}_4\cdot\text{ADP}$ (12) with those of the Ca$^{2+}$-free $E_2$ forms stabilized by thapsigargin ($E_2(\text{TG})$) (13) and $E_2(\text{TG})\cdot\text{Mg}\cdot\text{AlF}_4$ (14), it appears that the cytoplasmic domains undergo large rearrangements in relation to the $E_1/E_2$ conformational changes, and a key event in active transport may be the extensive rotation of the A-domain parallel to the membrane. Because the A-domain is linked to the transmembrane domain through long cytoplasmic extensions of M1, M2, and M3, it is likely that these linker segments play critical role in rotation of the A-domain and are essential for the interaction of the A-domain with the P-domain, with concomitant positioning of the conserved A-domain motif $^{214}\text{TGES}^{217}$ in the vicinity of the catalytic site (15–17) and long range communication with the ion binding sites in the membrane.

Long before the appearance of the Ca$^{2+}$-ATPase crystal structures, proteolytic cleavage experiments with Na$^+$,K$^+$-ATPase had demonstrated that cleavage sites in the cytoplasmic region linking M3 to the A-domain (“A-M3 linker”) are alternately exposed in relation to the $E_1/E_2$ and $E_1P/E_2P$ conformational transitions. Functional characterization of the chymotrypsin cleaved enzyme showed a block of the translocation of Na$^+$, due to stabilization of the $E_1P(3Na_3)$ intermediate (18, 19). Proteolytic cleavage in the corresponding region of the Ca$^{2+}$-ATPase with excision of a small five amino acid sequence ($^{220}\text{MAATE}^{224}$) likewise seems to affect the conformational changes (20). The cleaved enzyme retained the ability to bind Ca$^{2+}$ and react with ATP, but the transition from $E_1P$ to $E_2P$ was blocked (20). The functional consequences of mutation

* This work was supported by grants from the Danish Medical Research Council, the Novo Nordisk Foundation, Denmark, the Lundbeck Foundation, Denmark, and the Research Foundation of Aarhus University. 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. Tel.: 45-89-42-28-32; Fax: 45-86-12-90-65; E-mail: bvi@f.au.dk.

‡ All numbering of Na$^+$,K$^+$-ATPase residues in this report refers to the sequence of the rat α1 isofrom.
in the Na\textsuperscript{+},K\textsuperscript{+}-ATPase of Gly\textsuperscript{274} next to the proteolytic cleavage point, or mutation of the homologous counterpart, Gly\textsuperscript{233} in the Ca\textsuperscript{2+}-ATPase, are consistent with effects on the $E_1-E_2$, and $E_1P-E_2P$ conformational equilibria (21, 22). It is therefore very interesting that the crystal structures of the Ca\textsuperscript{2+}-ATPase show large structural differences in this region. In the $E_2$ forms (13, 14), the whole region connecting domain A with M3 has moved from a peripheral to a more central position closer to domain P, and the rearrangements include the formation of an α-helix in the $E_2$ form, involving residues immediately C-terminal to Gly\textsuperscript{233} (Gly\textsuperscript{263} of Na\textsuperscript{+},K\textsuperscript{+}-ATPase).

In this report we have investigated the functional consequences of mutation in this region further. Ile\textsuperscript{265}, with its bulky hydrophobic side chain, and Thr\textsuperscript{267} were both replaced with alanine. To examine the role of the conformation of the peptide backbone, we furthermore replaced the two glycines Gly\textsuperscript{271} and Gly\textsuperscript{274} with alanine. Neither of the residues selected for the present study, or their equivalents in other P-type ATPases, have previously been studied by mutagenesis. The isoleucine is particularly well conserved, being present in Na\textsuperscript{+},K\textsuperscript{+}-ATPases, sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPases, and H\textsuperscript{+},K\textsuperscript{+}-ATPases. To examine the functional importance of these residues in Na\textsuperscript{+},K\textsuperscript{+}-ATPase, we have studied the effects of the mutations on the overall and partial reactions of the enzyme, using steady-state and transient kinetic measurements.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Enzyme Preparation—**Site-directed mutagenesis of the ouabain-resistant rat $\alpha_1$-isoform of Na\textsuperscript{+},K\textsuperscript{+}-ATPase was carried out by the Kunkel method (23) or by using the QuikChange site-directed mutagenesis kit (Stratagene) to introduce the desired mutations directly into full-length cDNA. The mutant or wild-type cDNA was expressed in COS-1 cells, using 5 μl ouabain in the growth medium to select stable transfectants, and a crude plasma membrane fraction was isolated from the cells and made leaky by treatment with sodium deoxycholate or alamethicin (8, 24, 25).

**Functional Analysis—**To eliminate the contribution of the endogenous ouabain-sensitive COS-1 cell enzyme (~10% of the total Na\textsuperscript{+},K\textsuperscript{+}-ATPase content of the preparation), 10 μl ouabain was added to all assays except for the one in which the affinity for ouabain was determined by variation of the ouabain concentration. For the phosphorylation assays, the enzyme was moreover preincubated in the presence of ouabain.

ATP hydrolysis was measured at 37 °C as described previously (24, 25). The Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity associated with the background ATPase activity measured in 50 μl ouabain from the ATPase activity measured at 10 μl ouabain.

Studies of the Na\textsuperscript{+} dependence of steady-state phosphorylation from \([\gamma\textsuperscript{32}P]ATP\) and the time course of dephosphorylation, as well as the determination of the active site concentration by phosphorylation in the presence of 150 μM NaCl and oligomycin (20 μg/ml) to inhibit dephosphorylation, were carried out at 0 °C (26). Deconclusion of K\textsuperscript{+} or Rb\textsuperscript{+} was studied in phosphorylation experiments at 10 °C, following formation of K\textsuperscript{+}- or Rb\textsuperscript{+}-occluded enzyme at room temperature as described previously (21, 27, 28). Rapid kinetic phosphorylation experiments at 25 °C with enzyme present in the $E_1N_a$ form were performed using a BioLogic quench-flow module according to the previously described “Protocol 1” (21, 29). In all the above-described phosphorylation experiments, background phosphorylation was determined in the presence of 50 μM KCl without NaCl.

To examine the vanadate affinity under equilibrium conditions, 10 μM of deoxycholate-treated plasma membranes was incubated at 20 °C for 30 min in 40 μl of medium containing 20 mM Tris (pH 7.5), 5 mM MgCl\textsubscript{2}, 1 mM EGTA, 10 μM ouabain, and the indicated concentration of orthovanadate. To promote accumulation of the enzyme in the $E_1$ form, 8 mM RfCl was added to the medium during the incubation with vanadate. The samples were then cooled to 0 °C to prevent dissociation of bound vanadate (30), 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 μl of ice-cold phosphorylation medium producing final concentrations of 50 mM NaCl, 50 mM choline chloride, 20 mM Tris (pH 7.5), 5 μM [$\gamma\textsuperscript{32}P$]ATP, 5 mM MgCl\textsubscript{2}, 1 mM EGTA, and 20 μg/ml oligomycin. The data were fitted by assuming a simple one-site binding model where only the enzyme fraction with no vanadate bound phosphorylates, 

$$ EP = (EP_{\text{max}} - EP_{\beta})(1 - \frac{[\text{vanadate}]/(K_{\text{Hill}} + [\text{vanadate}])}{1 + [\text{vanadate}]/(K_{\text{Hill}} + [\text{vanadate}]}) + EP_{\beta},$$

(Eq. 1)

where $EP_{\text{max}}$ is the phosphorylation level obtained in the absence of vanadate, and $EP_{\beta}$ is the phosphorylation level corresponding to infinite vanadate concentration.

**Data Analysis and Statistics—**Data normalization, averaging, and nonlinear regression analysis were carried out as previously (21). The lines in the figures show the best fit to the complete set of normalized data, and the extracted parameters with standard errors are indicated in the tables. ATP and vanadate dependences of the ATPase activity and the Na\textsuperscript{+} dependence of phosphorylation were analyzed using the Hill equation. In the analysis of the ouabain dependence of ATPase activity, the ouabain-inhibited enzyme was represented by the sum of two Hill functions, one corresponding to the exogenous rat enzyme and one corresponding to the endogenous COS-1 cell Na\textsuperscript{+},K\textsuperscript{+}-ATPase. The time course of K\textsuperscript{+} deocclusion was analyzed using the biphasic time function described previously (21, 27) in which the component corresponding to the rapid phase is at maximum from the beginning. The time dependence of phosphorylation of enzyme in the $E_1N_a$ form was fitted by using a mono-exponential function, and the dephosphorylation time courses following chase with ATP or ADP were fitted by using a bi-exponential function.

**RESULTS**

**Expression, Ouabain Sensitivity, and Catalytic Turnover Rate—**The amino acid substitutions Ile\textsuperscript{265} → Ala, Thr\textsuperscript{267} → Ala, Gly\textsuperscript{271} → Ala, and Gly\textsuperscript{274} → Ala were introduced into the ouabain-resistant rat $\alpha_1$-isoform of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, and the mutant enzymes were expressed in COS-1 cells as previously (8, 24, 25). As in our earlier studies, we used a culture medium containing 5 μM ouabain for selection of the stable COS-1 cell lines expressing the mutant $\alpha_1$ enzymes. This is feasible, provided the mutant enzyme is functional, because the rat kidney Na\textsuperscript{+},K\textsuperscript{+}-ATPase is less sensitive to ouabain (K\textsubscript{Hill} for ouabain inhibition > 100 μM, cf. Table I) than the endogenous COS-1 cell Na\textsuperscript{+},K\textsuperscript{+}-ATPase (K\textsubscript{Hill} 0.3–0.8 μM) (24, 25). For all mutants the Na\textsuperscript{+},K\textsuperscript{+}-transport rate was high enough to support cell growth. The mutants were expressed to an active site concentration (determined by phosphorylation with oligomycin included to maximally stabilize the phosphoenzyme) similar to that determined for the wild-type Na\textsuperscript{+},K\textsuperscript{+}-ATPase, ranging between 30 and 60 pmol/mg of total membrane protein. This high expression level enabled us to characterize the functional properties of the mutants by measuring the overall function and by using various phosphorylation and dephosphorylation protocols to provide kinetic information on the individual steps in the catalytic cycle.

The ouabain concentration dependence of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity showed that the K\textsubscript{0.5} value for ouabain inhibition of mutants Thr\textsuperscript{267} → Ala, Gly\textsuperscript{271} → Ala, and Gly\textsuperscript{274} → Ala was very similar to that of the wild type, whereas mutant Ile\textsuperscript{265} → Ala displayed a 2.7-fold increased K\textsubscript{0.5} value (Table I). To avoid any contribution from the endogenous COS-1 cell enzyme, all measurements of ATPase activity and phosphoenzyme described below were carried out in the presence of 10 μM ouabain.

The catalytic turnover rate was calculated as the ratio between the maximum Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity (determined at 37 °C and saturating substrate concentrations) and the active site concentration. As seen in Table I, the catalytic turnover rate was reduced to 61% in mutant Ile\textsuperscript{265} → Ala, and to 80 and 86%, relative to wild type, in Thr\textsuperscript{267} → Ala and Gly\textsuperscript{271} → Ala, respectively, whereas it was wild type-like in Gly\textsuperscript{274} → Ala.

**ATP Dependence of Na\textsuperscript{+},K\textsuperscript{+}-ATPase Activity—**Fig. 1 shows results of experiments in which the ATP dependence of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity was studied. The K\textsubscript{0.5} value for ATP extracted from the data is listed in Table I. Relative to the wild
that an increase of ATP concentration from 10 nM to 300 μM enhances the turnover rate 37-fold in the wild type, whereas the turnover rate enhancement is only 8.5-fold in Ile265 → Ala, Gly271 → Ala, and Gly274 → Ala. At this low ATP concentration, K subscript 0.5 (ATP) for NaATPase activity was fitted to the data points (average values corresponding to at least three experiments). The K subscript 0.5 value for the enzyme and the K subscript 0.5 value of the expressed exogenous enzyme is listed here.

The catalytic turnover rate calculated as the ratio between the maximal NaATPase activity (measured in the presence of 3 mM ATP and 100 mM NaCl) and 3 mM ATP, 3 mM MgCl2, 30 mM histidine buffer (pH 7.4), 1 mM EGTA, and various concentrations of ouabain. A function containing 100 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM MgCl2, 30 mM histidine buffer (pH 7.4), 1 mM EGTA, and various concentrations of ouabain. A function presenting the ordinate intercept reflects the non-occluded enzyme. This procedure allows estimation of the level of the K 2(κ2) initially present, as well as the rate constant for the K 2(κ2) deocclusion reaction E (K 2) → E + 2K+.

From the data in Fig. 1, it can be calculated that an increase of ATP concentration from 10 μM to 3 mM enhances the turnover rate 37-fold in the wild type, whereas the enhancement is only 8.5-fold in Ile265 → Ala. At 10 μM ATP, the turnover rate is 230 and 606 min⁻¹ for the wild type and Ile265 → Ala, respectively. At this low ATP concentration, K 0.5, deocclusion, E (K 2) → E + 2K+, is rate-limiting for the overall ATPase reaction. Therefore, the increased turnover rate of Ile265 → Ala, relative to wild type, determined under these conditions suggests that the rate of K+ deocclusion is increased in the mutant.

To examine the rate of K+ deocclusion directly, the previously described phosphorylation assay was applied (21, 27, 28). Briefly, the enzyme is equilibrated with K+ in the absence of Na+ and ATP to form E (K 2). The phosphorylation kinetics is then determined following initiation of the phosphorylation reaction by a 10-fold dilution of the enzyme in a solution containing 100 mM Na+ and 1 μM [γ-32P]ATP. Oligomycin is added just prior to phosphorylation to prevent decay of the phosphoenzyme. This procedure allows estimation of the level of the E (K 2) initially present, as well as the rate constant for the K+ deocclusion reaction E (K 2) → E + 2K+. The data presented in Fig. 2 were analyzed by fitting a biphasic time function (21, 27), in which the ordinate intercept reflects the non-occluded enzyme pool ready to bind Na+ and phosphorylate immediately, whereas the exponential part (shown by the line in Fig. 2) corresponds to deocclusion of K+ from E (K 2). The fraction of enzyme initially present as E (K 2) equals 100% minus the ordinate intercept. The parameters determined by this procedure are listed in Table II for all mutants. As is seen in Table II (upper panel) and Table II, Ile265 → Ala differed significantly from the other mutants and wild type with respect to both the deocclusion rate (9-fold enhanced, relative to wild type) and the level of the K+-occluded intermediate initially present (65%
the phosphorylation site of the $E_2$ and $E_2(K_2)$ conformations, thereby forming a stable dead-end state, probably resembling the $E_2P_i$ complex from which phosphate normally is released (32). To obtain information about the steady-state concentration of $E_2/E_2(K_2)$ accumulated during ATP hydrolysis, we investigated the vanadate dependence of Na$^+$/K$^+$-ATPase activity. As shown in Fig. 3 and Table I, Ile$^{265} \rightarrow$ Ala was found ~23-fold less sensitive to vanadate inhibition than wild-type Na$^+$/K$^+$-ATPase. By contrast, the other mutants showed higher sensitivity to vanadate inhibition than the wild type, 2-fold for Thr$^{267} \rightarrow$ Ala and Gly$^{274} \rightarrow$ Ala and about 1.4-fold for Gly$^{271} \rightarrow$ Ala. This result is consistent with an increased accumulation of $E_2(K_2)$, caused by the decrease in the K$^+$ deocclusion rate described above for these mutants. The reduced sensitivity of Ile$^{265} \rightarrow$ Ala to vanadate inhibition is consistent with the depletion of the $E_2(K_2)$ intermediate during enzyme cycling caused by the increase in the K$^+$ deocclusion rate. Another contributing factor could be a direct effect of the mutation on the vanadate-binding site, i.e. a change of the intrinsic affinity for vanadate. This was examined in the experiments described in the next section.

Vanadate Binding at Equilibrium—To gain further insight into the effect of the Ile$^{265} \rightarrow$ Ala mutation on the vanadate-binding properties of the $E_2$ form and the equilibrium between $E_2$ and $E_1$, we determined the vanadate affinity in a phosphorylation assay allowing the enzyme-vanadate complex to be formed under equilibrium conditions without enzyme cycling (16). The enzyme is incubated at 20 °C with various concentrations of vanadate in the absence of Na$^+$ and ATP and for an extended period of time to attain equilibrium. Subsequently phosphorylation from ATP is carried out in the presence of Na$^+$. The rationale is that no high affinity ATP binding can take place when vanadate is bound to the enzyme. Consequently, the phosphorylation level equals the enzyme fraction that has not reacted with vanadate during the preincubation period and, thus, reflects the equilibrium between the free and vanadate-bound enzyme forms existing before initiation of phosphorylation. Care is taken to minimize dissociation of vanadate from the preformed enzyme-vanadate complex during the phosphorylation reaction by reducing the temperature to 0 °C and keeping the phosphorylation time short, yet long enough to ensure full phosphorylation of all vanadate-free enzyme.

Such experiments were performed with Ile$^{265} \rightarrow$ Ala and wild type (Fig. 4), and the parameters, determined assuming a simple one-site binding model (see “Experimental Procedures”), are summarized in Table III. The equilibrium binding affinity of wild type for vanadate was found about 30-fold higher than the apparent affinity determined during enzyme cycling with ATP present. This is presumably due to the lack of competition from ATP binding and phosphorylation during the equilibration with vanadate. Fig. 4 (upper panel) shows the results obtained following equilibration with vanadate in the absence of any of the transported cations. Under these conditions, Ile$^{265} \rightarrow$ Ala exhibited a 28-fold reduction of the apparent affinity for vanadate relative to wild type. As seen in Fig. 4 (lower panel) the vanadate affinity was also determined in the presence of 8 mM Rb$^+$, i.e. conditions where 90% of the enzyme is $E_2(Rb_2)$ as revealed in the measurements of occlusion and deocclusion of Rb$^+$ discussed above (Fig. 2, lower panel), thereby eliminating the influence of the shift of the $E_1$-$E_2$ equilibrium induced by the mutation. Under these conditions mutant Ile$^{265} \rightarrow$ Ala still displayed a 5-fold reduced affinity for vanadate relative to wild type. This indicates that the Ile$^{265} \rightarrow$ Ala mutation affects not only the $E_2(E_2)$ conformational equilibrium, but also the intrinsic affinity of $E_2$ for vanadate.

$K^+$ and Na$^+$ Affinities—The mutational effects on $K^+$ bind-
Because only the E' rate of ATP hydrolysis was determined for the expressed wild-type of phosphorylation from ATP (in the absence of K\textsuperscript{+} (pH 7.4), and the indicated concentrations of vanadate. The average values corresponding to at least four independent experiments), giving the K\textsubscript{d} and Hill numbers listed.

* Determined by Na\textsuperscript{+} titration of phosphorylation by \([\gamma\textsuperscript{32P}]\)ATP. Phosphorylation was carried out for 15 s at 0 °C in a medium containing 2 \(\mu\text{M} \gamma\textsuperscript{32P}\)ATP, 20 mM Tris (pH 7.5), 3 mM MgCl\textsubscript{2}, 1 mM EGTA, 10 \(\mu\text{M}\) ouabain, oligomycin (20 \(\mu\text{M}\)/ml), and various concentrations of NaCl and N-methyl-D-glucamine (keeping the ionic strength constant at 150 mM). A Hill function was fitted to the data points (average values corresponding to at least two independent experiments), giving the K\textsubscript{d} and Hill numbers listed.

* Determined in phosphorylation experiments at 25 °C, using a Bio-Logic quench-flow module. Phosphorylation was carried out in the presence of 2 \(\mu\text{M} \gamma\textsuperscript{32P}\)ATP and oligomycin according to “Protocol 1” previously described (21, 29). The enzyme was preincubated in a medium containing 100 mM NaCl, 20 mM Tris (pH 7.5), 3 mM MgCl\textsubscript{2}, 1 mM EGTA, 10 \(\mu\text{M}\) ouabain, and oligomycin (20 \(\mu\text{M}\)/ml), and mixed with an equal volume of the same buffer containing 4 \(\mu\text{M} \gamma\textsuperscript{32P}\)ATP, followed by acid quenching at various time intervals. A mono-exponential function was fitted to the data points (average values corresponding to at least two independent experiments), giving the rate constants listed.

* Ratio between phosphoenzyme levels obtained without and with oligomycin (20 \(\mu\text{M}\)/ml) following incubation with 2 \(\mu\text{M} \gamma\textsuperscript{32P}\)ATP for 15 s at 0 °C in the presence of 150 mM NaCl, 20 mM Tris (pH 7.5), 3 mM MgCl\textsubscript{2}, 1 mM EGTA, and 10 \(\mu\text{M}\) ouabain.

* From Fig. 2.
* From Fig. 2, rate constant corresponding to the slow phase indicated by the fitted line. Data obtained with Rb\textsuperscript{+} are shown in parentheses.
* From Table II more...
Mutations in the A-M3 linker of Na\(^+\),K\(^+\)-ATPase

In the present study, we have examined the functional consequences of alanine substitution of either of the residues Ile\(^{265}\), Thr\(^{267}\), Gly\(^{271}\), and Gly\(^{274}\) in the A-M3 linker segment of the Na\(^+\),K\(^+\)-ATPase. Our results demonstrate that Ile\(^{265}\) → Ala constant corresponding to the slow phase, reflecting the \(E_2P\) dephosphorylation, was increased 1.6- to 1.7-fold in Thr\(^{267}\) → Ala and Gly\(^{274}\) → Ala, whereas in Gly\(^{271}\) → Ala it was wild type-like (for rate constants, see legend to Fig. 5).

**Kinetics of \(E_1P\) Phosphoenzyme Turnover.**—Because the above-described findings reveal a shift in the distribution between \(E_1P\) and \(E_2P\) fractions, respectively, are listed in Table II. The rate constant of the slow component, corresponding to the rate of \(E_2P\) hydrolysis, is as follows: wild type, 0.030 s\(^{-1}\); Ile\(^{265}\) → Ala, 0.025 s\(^{-1}\); Thr\(^{267}\) → Ala, 0.048 s\(^{-1}\); Gly\(^{271}\) → Ala, 0.031 s\(^{-1}\); and Gly\(^{274}\) → Ala, 0.050 s\(^{-1}\).

FIG. 5. Dephosphorylation in the presence of ADP. The wild-type Na\(^+\),K\(^+\)-ATPase (filled circles) and the mutants Ile\(^{265}\) → Ala (open circles), Thr\(^{267}\) → Ala (open squares), Gly\(^{271}\) → Ala (open triangles pointing upwards), and Gly\(^{274}\) → Ala (open triangles pointing downwards) were phosphorylated for 10 s at 0 °C in the presence of 2 μM \([\gamma\text{-}^{32}P]ATP\), 20 mM NaCl, 130 mM choline chloride, 3 mM MgCl\(_2\), 1 mM EGTA, 20 mM Tris (pH 7.5), and 10 μM ouabain. Dephosphorylation was initiated by addition of a chase solution producing final concentrations of 2.5 mM ATP and 1 mM unlabeled ATP, followed by acid quenching at the indicated time intervals. The data points are average values of four to seven determinations, shown as the percentage of the phosphorylation level obtained after 10 s of phosphorylation without dephosphorylation. The lines show the best fit of a bi-exponential time function. The extents of the rapid and slow components, representing the initial \(E_1P\) and \(E_2P\) fractions, respectively, are listed in Table II. The rate constant of the slow component, corresponding to the rate of \(E_2P\) hydrolysis, is as follows: wild type, 0.030 s\(^{-1}\); Ile\(^{265}\) → Ala, 0.025 s\(^{-1}\); Thr\(^{267}\) → Ala, 0.048 s\(^{-1}\); Gly\(^{271}\) → Ala, 0.031 s\(^{-1}\); and Gly\(^{274}\) → Ala, 0.050 s\(^{-1}\).

**FIG. 4. Vanadate binding at equilibrium in the absence or presence of Rb\(^{+}\).** The wild-type Na\(^+\),K\(^+\)-ATPase (filled circles) or the mutant Ile\(^{265}\) → Ala (open circles) was initially equilibrated at room temperature for 30 min in the presence of the indicated concentrations of vanadate in the absence (upper panel) or presence (lower panel) of 8 mM RbCl. After cooling of the samples to 0 °C, the vanadate-free enzyme fraction was determined by its ability to form a phosphoenzyme upon dilution in a phosphorylation buffer containing Na\(^+\) and \([\gamma\text{-}^{32}P]ATP\) as described under “Experimental Procedures.” The data points are average values of four to six determinations, normalized to the maximal phosphorylation obtained in the absence of vanadate. Error bars represent standard errors. The lines show the best fit of the Hill equation to the complete set of normalized data, giving the \(K_{0.5}\) values indicated in Table III.

**TABLE III**
**Equilibrium binding affinities for vanadate determined in phosphorylation experiments**

| Mutation       | \(K_{0.5}\) (vanadate) | \(n M\) |
|----------------|------------------------|--------|
| Wild type      |                        |        |
| Ile\(^{265}\) → Ala | 99 ± 16\(^a\) | 16 ± 4\(^b\) |
|                | 2804 ± 395\(^a\)      | 82 ± 19\(^b\) |

\(^a\) From Fig. 4, upper panel.
\(^b\) From Fig. 4, lower panel.

mediate in the wild type (presence of 20 mM Na\(^+\) and absence of K\(^+\)), and the time course of dephosphorylation was followed upon addition of ADP. The extents of the rapid and slow decay components, which reflect the initial amounts of the ADP-sensitive \(E_1P\) and the ADP-insensitive \(E_2P\), respectively, were estimated by fitting a bi-exponential function to the data points as described previously (21, 29). The results of this analysis are listed in Table II for all mutants. It is seen that for wild-type Na\(^+\),K\(^+\)-ATPase 30% of the phosphoenzyme was ADP-sensitive \(E_1P\). For Ile\(^{265}\) → Ala, the \(E_1P\) level was significantly higher, 89%, i.e., a 3-fold increase relative to wild type. For Thr\(^{267}\) → Ala, Gly\(^{271}\) → Ala, and Gly\(^{274}\) → Ala, on the other hand, the \(E_1P\) level was only 14, 14, and 19%, respectively (Table II), indicative of a shift in the distribution between \(E_1P\) and \(E_2P\) in favor of \(E_2P\). It is furthermore interesting to note that the rate
Mutations in the A-M3 linker of Na\textsuperscript{+},K\textsuperscript{+}-ATPase

FIG. 6. Dephosphorylation of phosphoenzyme formed at 600 mm NaCl. Dephosphorylation was carried out for 10 s at 0 °C in the presence of 2 μM [γ\textsuperscript{32}P]ATP, 600 mm NaCl (to accumulate E\textsubscript{3}P), 5 mm MgCl\textsubscript{2}, 1 mm EGTA, 20 mm Tris (pH 7.5), and 10 μM ouabain. Dephosphorylation was initiated by diluting the phosphorylated enzyme in a solution producing final concentrations of 200 mM NaCl (upper panel) or 600 mM NaCl (lower panel), 1 mM unlabeled ATP, and 20 mM KCl (to ensure rapid dephosphorylation of E\textsubscript{3}P), followed by acid quenching at the indicated time intervals. The data points are average values of four to nine determinations, calculated as the percentage of the phosphorylation obtained after 10 s of phosphorylation without dephosphorylation. The half-life (t\textsubscript{1/2}) of the phosphoenzyme along with standard error is shown in Table II. The symbols represent wild-type Na\textsuperscript{+},K\textsuperscript{+}-ATPase (filled circles), Ile\textsuperscript{265} → Ala (open circles), Thr\textsuperscript{267} → Ala (open squares), Gly\textsuperscript{271} → Ala (open triangles pointing upwards), and Gly\textsuperscript{274} → Ala (open triangles pointing downwards).

Ala displaces the E\textsubscript{1}E\textsubscript{2} and E\textsubscript{1}P-E\textsubscript{2} phosphoenzyme equilibria in favor of E\textsubscript{3}E\textsubscript{2}P, whereas Thr\textsuperscript{267} → Ala, Gly\textsuperscript{271} → Ala, and Gly\textsuperscript{274} → Ala displace these conformational equilibria in the opposite direction, in favor of E\textsubscript{3}E\textsubscript{2}P. The mutations affect both the rearrangement of the cytoplasmic domains (seen by changes in phosphoenzyme properties and apparent ATP/vanadate affinities) and the conformational change of the membrane sector (indicated by the change of K\textsuperscript{+}/Rb\textsuperscript{+} deocclusion rate).

Hence, Ile\textsuperscript{265} → Ala led to increased apparent affinity for ATP, reduced apparent affinity for vanadate, reduced equilibrium concentration of the occluded E\textsubscript{3}P form even in the absence of ATP, increased K\textsuperscript{+} deocclusion rate, and increased apparent affinity for Na\textsuperscript{+} in activation of phosphoenzyme, i.e. properties consistent with a destabilization of the E\textsubscript{4} form relative to E\textsubscript{1}. Moreover, the increased fractional amount of the ADP-sensitive E\textsubscript{1}P phosphoenzyme intermediate and the 10-fold reduced rate of decay of E\textsubscript{1}P imply that the E\textsubscript{1}P-E\textsubscript{2}P equilibrium is displaced in favor of E\textsubscript{1}P in Ile\textsuperscript{265} → Ala. The reduced apparent affinity for ouabain seen for Ile\textsuperscript{265} → Ala may accordingly be interpreted in terms of a reduced level of E\textsubscript{2}P, because ouabain is thought to bind preferentially to E\textsubscript{2}P (34).

By contrast, the Thr\textsuperscript{267}, Gly\textsuperscript{271}, and Gly\textsuperscript{274} mutations led to decreased apparent affinity for ATP, increased apparent affinity for vanadate, increased equilibrium level of the occluded E\textsubscript{3}E\textsubscript{2}P form even in the absence of ATP, and decreased K\textsuperscript{+} deocclusion rate, i.e. properties consistent with a stabilization of the E\textsubscript{4} form relative to E\textsubscript{1}. These mutations furthermore increased the relative amount of the ADP-insensitive E\textsubscript{1}P phosphoenzyme intermediate and enhanced the rate of decay of E\textsubscript{1}P, indicating destabilization of E\textsubscript{1}P. Notably, this is the first evidence in the literature of a displacement of the E\textsubscript{1}E\textsubscript{2} and E\textsubscript{1}P-E\textsubscript{2}P equilibria in favor of E\textsubscript{1}E\textsubscript{2}P resulting from perturbation of the A-M3 linker region. Previously reported effects of proteolytic cleavage or mutation in this region are all consistent with an accumulation of E\textsubscript{1}E\textsubscript{2}P similar to that seen here for Ile\textsuperscript{265} → Ala (18–22).

The apparent affinity of Ile\textsuperscript{265} → Ala for the inhibitor vanadate, which binds preferentially to the E\textsubscript{2} form (32), was found 28-fold decreased, relative to wild type, under equilibrium conditions where the enzyme is not cycling. This effect is more pronounced than the 9- to 15-fold increase of the rate of E\textsubscript{2} → E\textsubscript{1} determined in the K\textsuperscript{+} and Rb\textsuperscript{+} deocclusion experiments. Moreover, the affinity for vanadate was still found 5-fold reduced, relative to wild type, when determined in the presence of a high concentration of Rb\textsuperscript{+}, forcing the enzyme into the E\textsubscript{2} form and, thereby, eliminating the influence of the shift of the E\textsubscript{1}E\textsubscript{2} equilibrium induced by the mutation. This behavior differs from that of the previously described Gly\textsuperscript{263} → Ala mutant, which showed normal vanadate affinity upon the addition of Rb\textsuperscript{+} (16), as expected for a mutation affecting only the conformational equilibrium and not the intrinsic affinity. The retention of a significant reduction of vanadate affinity in Ile\textsuperscript{265} → Ala, even after the enzyme had been forced into the E\textsubscript{2} form, suggests that part of the interference of the mutation with vanadate binding owes to a direct effect on the intrinsic affinity of the E\textsubscript{2} form for vanadate, similar to the effect of mutation of Thr\textsuperscript{214} in the highly conserved TGES loop that becomes an integral part of the catalytic site in the E\textsubscript{1}E\textsubscript{2}P forms (16). Hence, it may possibly be inferred that Ile\textsuperscript{265} is close to the catalytic site in the E\textsubscript{1}E\textsubscript{2}P forms. On the other hand, the normal rate of phosphorylation from ATP indicates that the Ile\textsuperscript{265} → Ala mutation does not interfere with the function of the catalytic site in E\textsubscript{1}E\textsubscript{2}P.

Topology analysis, using site-directed chemical labeling and other techniques, has provided considerable evidence that the 30% identity and 65% similarity in amino acid sequence of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+},K\textsuperscript{+}-ATPase results in similar overall folds of these proteins (2, 5). Hence, although caution clearly is needed when interpreting the Na\textsuperscript{+},K\textsuperscript{+}-ATPase in light of the Ca\textsuperscript{2+}-ATPase crystal structures, the Ca\textsuperscript{2+}-ATPase crystal structures may provide useful provisional models for understanding Na\textsuperscript{+},K\textsuperscript{+}-ATPase structure-function relationship. Because Ile\textsuperscript{265} is conserved between Na\textsuperscript{+},K\textsuperscript{+}-ATPase and sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase, the relations of this residue in the Ca\textsuperscript{2+}-ATPase crystal structures are particularly relevant to understanding the present findings. The other residues studied here are less well conserved. Thr\textsuperscript{267} is replaced either by the closely related serine or by aspartate in the various sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase isoforms, and the Ca\textsuperscript{2+}-ATPase counterparts of Gly\textsuperscript{271} and Gly\textsuperscript{274} are alanine and glutamine/proline, respectively.

Fig. 7 shows the structural models of the crystalline E\textsubscript{1} and E\textsubscript{2} forms of Ca\textsuperscript{2+}-ATPase (11–14) with the Na\textsuperscript{+},K\textsuperscript{+}-ATPase residues Ile\textsuperscript{265}, Thr\textsuperscript{267}, Gly\textsuperscript{271}, and Gly\textsuperscript{274} mutated in the present study indicated in parentheses following the corresponding Ca\textsuperscript{2+}-ATPase residues (shown in yellow). Interestingly, in the E\textsubscript{2} forms (Fig. 7, lower panels) the isoleucine side chain is located within 3–4 Å of the valine equivalent to Val\textsuperscript{274} in

Phosphorylation (%)
FIG. 7. $E_1$ (upper panels) and $E_2$ (lower panels) crystal structures of Ca$^{2+}$-ATPase. The Ca$^{2+}$-ATPase structures with Protein Data Bank accession codes 1SU4 (upper left panel) (11), 1T5T (upper right panel) (12), 1IWO (lower left panel) (13), and 1WPG (lower right panel) (14) are shown. In the left panels, Ca$^{2+}$-ATPase residue numbering is shown with the numbers of the corresponding Na$^+$/K$^+$-ATPase residues in parentheses. Residues equivalent to the Na$^+$/K$^+$-ATPase residues mutated in the present study are highlighted in yellow. Asn$^{706}$ of the catalytic site, which is equivalent to Asn$^{715}$ in Na$^+$/K$^+$-ATPase, is highlighted in purple. Val$^{705}$, equivalent to Val$^{714}$ in Na$^+$/K$^+$-ATPase, is highlighted in blue. The white residues in the transmembrane part are those thought to coordinate the transported ions. The dashed ellipse indicates the catalytic site, including the AlF$_4^-$ (green molecule) mimicking the phosphoryl transition state. In the lower right panel, MgF$_4^-$, mimicking P$_i$, is shown with the Mg atom red and the fluorine atoms green.
Different in the and M5) leads to rearrangement of the transmembrane sector. Be the initial event in the conformational rearrangement, which might influence the 

10218

rotation of the A-domain (14). The mutational effects on the E1-E2 and E1-P-E2-P equilibria observed in the present study are consistent with this hypothesis. The interaction of the side chain of the isoleucine of the A3 linker with P-domain residues in close to the catalytic site seen in the E2 crystal structures of Ca2+-ATPase, together with the other contacts between the A3 helix and the P6 helix, could be critical for controlling the A-domain rotation and, thus, correct insertion of the TGES loop in place of the leaving ADP molecule. Following dissociation of ADP from the catalytic site, the P-domain residues can come into contact with the isoleucine of the A3 linker. This interaction might influence the position of the P-domain, which inclines downward in the E2 structure, thereby relieving the strain of the A3 linker and enforcing movements of the transmembrane segments. The interaction of the hydrophilic side chain of Thr267 with the water phase, and the presence of the two glycines Gly271 and Gly274, imposing flexibility, could be important for the conformation and direction of the backbone of the A3 linker in Na+,K+-ATPase. The displacement of the E1-E2 and E1-P-E2-P equilibria in favor of E1-E2-P induced by substitution of either of these residues with alanine might be a consequence of increased strain of the A3 linker in E1/P-E2/P, increased stability of the A3 helix in E1/E2-P, and/or a change of the orientation of the A3 helix, facilitating its interaction with the P6 helix.

Acknowledgments—We thank 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.

REFERENCES

1. Glynn, I. M. (1985) in The Enzymes of Biological Membranes, Vol. 3 (Maron, A. N., ed), pp. 35–114, Plenum Publishing Corp., New York.

2. Kaplan, J. H. (2002) Annu. Rev. Biochem. 71, 511–535.

3. Post, R. L., Hegavey, C., and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540.

4. Hu, Y.-K., and Kaplan, J. H. (2000) J. Biol. Chem. 275, 19185–19191.

5. Sweadner, K. J., and Donnet, C. (2001) Biochem. J. 356, 685–704.

6. Arquello, J. M., and Kaplan, J. H. (1994) J. Biol. Chem. 269, 6892–6899.

7. Lingle, J. B., and Kuntzweiler, T. A. (1994) J. Biol. Chem. 269, 19659–19662.

8. Vilsen, B. (1995) Biochemistry 34, 1455–1463.

9. Petersen, P. A., Nielsen, J. M., Rasmussen, J. H., and Jørgensen, P. L. (1998) Biochemistry 37, 17818–17827.

10. Ogawa, H., and Toyoshima, C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15977–15982.

11. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655.

12. Sorensen, T. L. M., Moller, J. V., and Nissen, P. (2004) Science 304, 1672–1675.

13. Toyoshima, C., and Nomura, H. (2002) J. Biol. Chem. 278, 605–611.

14. Toyoshima, C., and Nomura, H., and Tanda, T. (2004) Nature 432, 361–368.

15. Patchornik, G., Goldshleger, R., and Karlish, S. J. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11954–11959.

16. Toustrup-Jensen, M., and Vilsen, B. (2003) J. Biol. Chem. 278, 11402–11410.

17. Clausen, J. D., Vilsen, B., McIntosh, D. B., Einholm, A. P., and Andersen, J. P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2776–2781.

18. Jørgensen, P. L., and Petersen, J. (1988) Biochim. Biophys. Acta 921, 319–333.

19. Jørgensen, P. L., and Andersen, J. P. (1988) J. Membr. Biol. 103, 95–129.

20. Moller, J. V., Lenoir, G., Marchand, C., Montigny, C., le Maire, M., Toyoshima, C., Juul, B. S., and Champel, P. (2002) J. Biol. Chem. 277, 38467–38456.

21. Toustrup-Jensen, M., Hauge, M., and Vilsen, B. (2001) Biochemistry 40, 5521–5532.

22. Andersen, J. P., Vilsen, B., Leberer, E., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 21018–21020.

23. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3314–3318.

24. Vilsen, B. (1992) FEBS Lett. 314, 301–307.

25. Toustrup-Jensen, M., and Vilsen, B. (2002) J. Biol. Chem. 277, 38607–38617.

26. Vilsen, B. (1997) Biochemistry 36, 13312–13324.

27. Vilsen, B., and Andersen, J. P. (1998) Biochemistry 37, 10961–10971.

28. Vilsen, B. (1999) Biochemistry 38, 11389–11400.

29. Vilsen, B. (2003) J. Biol. Chem. 278, 38653–38664.

30. Smith, R. L., Zinn, K., and Cantley, L. C. (1980) J. Biol. Chem. 255, 9852–9859.

31. Forbush, B., III (1987) J. Biol. Chem. 262, 11014–11115.

32. Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978) J. Biol. Chem. 253, 685–7068.

33. Post, R. L., Kume, S., Tobin, T., Orcutt, B., and Sen, A. K. (1969) J. Gen. Physiol. 54, 3066–3266.

34. Hansen, O. (1984) Pharmacol. Rev. 36, 143–163.

35. Wang, W., Cho, H. S., Kim, R., Jancarik, J., Yokota, H., Nguyen, H. H., Grigoriev, I. V., Wemmer, D. E., and Kim, S.-H. (2002) J. Mol. Biol. 319, 421–431.

36. Creighton, T. E. (1993) Proteins, Structures and Molecular Properties, 2nd Ed., pp. 1–507, W. H. Freeman and Company, New York.