Mutations Ile\textsuperscript{279} → Ala, Ile\textsuperscript{283} → Ala, Glu\textsuperscript{284} → Ala, His\textsuperscript{285} → Ala, His\textsuperscript{285} → Lys, His\textsuperscript{285} → Glu, Phe\textsuperscript{286} → Ala, and His\textsuperscript{288} → Ala in transmembrane helix M3 of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase were studied. Except for His\textsuperscript{285} → Ala, these mutations were compatible with cell viability, permitting analysis of their effects on the overall and partial reactions of the Na\textsuperscript{+},K\textsuperscript{+}-transport cycle. In Ile\textsuperscript{279} → Ala and Ile\textsuperscript{283} → Ala, the \( E_1 \) form accumulated, whereas in His\textsuperscript{285} → Lys and His\textsuperscript{285} → Glu, \( E_P \) accumulated. Phe\textsuperscript{286} → Ala displaced the conformational equilibria of dephosphoenzyme and phosphoryzme in parallel in favor of \( E_2 \) and \( E_3P \), respectively, and showed a unique enhancement of the \( E_3P \) → \( E_2P \) transition rate. These effects suggest that M3 undergoes significant rearrangements in relation to \( E_1E_2P \) and \( E_2P-E_3P \) conformational changes. Because the \( E_1E_2P \) and \( E_2P-E_3P \) conformational equilibria were differentially affected by some of the mutations, the phosphorylated conformations seem to differ significantly from the dephospho forms in the M3 region. Mutation of His\textsuperscript{285} furthermore increased the Na\textsuperscript{+}-activated ATPase activity in the absence of K\textsuperscript{+} ("Na\textsuperscript{+}-ATPase activity"). Ile\textsuperscript{279} → Ala, Ile\textsuperscript{283} → Ala, and His\textsuperscript{288} → Ala showed reduced Na\textsuperscript{+} affinity of the \( E_1 \) form. The rate of Na\textsuperscript{+}-activated phosphorylation from ATP was reduced in Ile\textsuperscript{279} → Ala and Ile\textsuperscript{283} → Ala, and these mutants showed evidence similar to Glu\textsuperscript{282} → Gln of destabilization of the Na\textsuperscript{+}-occluded state.

The Na\textsuperscript{+},K\textsuperscript{+}-ATPase\textsuperscript{1} present in the plasma membranes of all animal cells utilizes the energy derived from hydrolysis of ATP to drive transport of Na\textsuperscript{+} and K\textsuperscript{+} ions in opposite directions against their electrochemical gradients (1). This enzyme, which is a member of the P-type ATPase superfamily (2), is a heterodimer consisting of a large catalytic \( \alpha \)-subunit (\(-110\) kDa) and a glycosylated \( \beta \)-subunit (\(-55\) kDa). The catalytic subunit is common to all P-type ATPases, and, for the Ca\textsuperscript{2+}-ATPase of sarcoplasmic reticulum, the structure has been determined at high resolution (3, 4), showing that the protein consists of 10 membrane-spanning segments, M1–M10, and three large cytoplasmic domains: the nucleotide-binding (N) domain, the phosphorylation (P) domain, and the actuator (A) domain. The N- and P-domains are formed by the loop between M4 and M5 and the A-domain by two helices near the amino terminus, before M1, and the loop between M2 and M3. The overall structure of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase \( \alpha \)-subunit resembles that of the Ca\textsuperscript{2+}-ATPase (5, 6), allowing the Ca\textsuperscript{2+}-ATPase of sarcoplasmic reticulum to be used as a model for the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, as shown laid out in two dimensions in Fig. 1. From mutational studies it emerges that the residues of Na\textsuperscript{+},K\textsuperscript{+}-ATPase most likely to be involved in Na\textsuperscript{+} and K\textsuperscript{+} binding are located in the transmembrane segments M4, M5, and M6 (Fig. 1, red residues; Refs. 7–16). Several of these residues are conserved in the Ca\textsuperscript{2+}-ATPase and donate ligands to Ca\textsuperscript{2+} binding (17–20). The similarity between the ion binding pockets of Na\textsuperscript{+},K\textsuperscript{+}-ATPase and Ca\textsuperscript{2+}-ATPase (14, 19, 21) is further confirmed in a recent study in which homology modeling of Na\textsuperscript{+},K\textsuperscript{+}-ATPase, based on the Ca\textsuperscript{2+}-ATPase crystal structure, was carried out using valence searching to identify two Na\textsuperscript{+}/K\textsuperscript{+} sites at the positions equivalent to the Ca\textsuperscript{2+}-binding sites, and a third contiguous Na\textsuperscript{+} site (22). However, virtually nothing is known about the determinants of the specific cation binding properties and the pathways of ion migration to and from the binding pocket.

Another important issue concerns the mechanism of energy transduction: how ATP hydrolysis is linked to ion transport? This must involve long range interaction between the cytoplasmic domains and the cation coordinating residues located some 40 Å away in the transmembrane segments. During the course of the enzyme cycle (Scheme 1), the aspartic acid residue Asp\textsuperscript{371} in the \( E_1P \) → \( E_2P \) transition, whereas the \( E_2P \) and \( E_3P \) conformations undergo a conformational transition (\( E_3P \) → \( E_2P \) and \( E_2 \) → \( E_1 \)), resulting in release of occluded Na\textsuperscript{+} and K\textsuperscript{+}, respectively (23–25). As indicated in Fig. 1 (blue residues), mutagenesis studies have pinpointed residues in the cytoplasmic part as well as the transmembrane part of the protein that are important for these conformational changes, encompassing Glu\textsuperscript{233} in domain A (26), Gly\textsuperscript{263} and Arg\textsuperscript{264} in the flexible loop connecting domain A with transmembrane segment M3 (27), Gly\textsuperscript{330} and Leu\textsuperscript{332} near the unwound part of M4 (28), and Glu\textsuperscript{282} in the NH\textsubscript{2}-terminal part of M3 (29).

Interestingly, mutations of Glu\textsuperscript{282} in M3 affected, in addition to the major conformational changes of the enzyme, also the functional properties of the cation binding sites, in the cytoplasmically facing \( E_1 \) form as well as in the extracellularly facing \( E_3P \) form (29). Certain mutations of Glu\textsuperscript{282} reduced the affinity of the ATPase for ATP (19).

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‡ The abbreviations used are: Na\textsuperscript{+},K\textsuperscript{+}-ATPase, the Na\textsuperscript{+} and K\textsuperscript{+}-transporting adenosine triphosphatase (EC 3.6.1.37); \( E_1 \) and \( E_2 \) conformational states of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase; \( E_3P \) and \( E_2P \), phosphorylated conformational states; \( K_{\text{cat}} \), ligand concentration giving half-maximal activation or inhibition; M1–M10, putative transmembrane segments numbered from the NH\textsubscript{2}-terminal end of the peptide chain.

\textsuperscript{2} All numbering of Na\textsuperscript{+},K\textsuperscript{+}-ATPase residues in this article refers to the sequence of the rat \( \alpha_1 \) isoform.
apparent affinity for Na\(^+\) in the E\(_1\) form, whereas others allowed Na\(^+\) to substitute for K\(^+\) at the extracellularly facing sites of the E\(_P\) form, leading to an unusually high Na\(^+\)-induced ATPase activity in the absence of K\(^+\) (29). The latter effect has also been observed for mutation of certain residues located in or close to the cation-binding pocket (residues shown as **diamonds** in Fig. 1; Refs. 9, 10, 21, and 30–32). Hence, Glu\(^{282}\) mutants are included in several of the Na\(^+\), K\(^+\)-ATPase mutant classes depicted in Fig. 1, indicating a central role of Glu\(^{282}\) in the coordination of events occurring in the various regions of the protein in connection with ion translocation (29).

The results obtained with Glu\(^{282}\) mutants raised the question whether other M3 residues are of crucial functional importance. Glu\(^{282}\) could be unique among M3 residues, because of its ability to form hydrogen bonds to residues in the loop connecting transmembrane segments M6 and M7 (29), but nothing is known about the functional and structural roles of other M3 residues. Because M3 is directly attached through the peptide backbone to domain A (see Fig. 1) and through noncovalent bonds at the NH\(_2\)-terminal end to domain P (3), the whole M3 helix could be instrumental in the conformational rearrangements mediating communication between the cytoplasmic domains and the transmembrane sector. The recently determined crystal structure of Ca\(^{2+}\)-ATPase in a thapsigargin-stabilized E\(_2\) state further shows that a water accessible channel has opened up between M1 and M3, forming a putative entry pathway for Ca\(^{2+}\) (4). The atomic model for the E\(_2\) state indicates that M3 residues might be involved in control of cation migration to and from the binding sites, although it is not clear to what extent the binding of thapsigargin perturbs the structure. Comparison of the two Ca\(^{2+}\)-ATPase crystal structures (3, 4) indicates that, during the transition from E\(_1\)Ca\(^{2+}\) to E\(_2\), M3 is shifted downward (away from the cytoplasm) by 5 Å together with M4, whereas M1 moves upwards, and in addition M3 inclines and becomes strongly curved. It is, however, not yet clear whether this movement really represents a conformational change occurring in the native enzyme during the functional cycle. In apparent contradiction with the large movement predicted from the Ca\(^{2+}\)-ATPase crystal structures, it has recently been concluded that M3 of the Na\(^+\), K\(^+\)-ATPase is static relative to M1 and the cytoplasmic surface during the major transport-associated conformational transitions, because metal-catalyzed cleavage thought to occur near His\(^{285}\) in M3 was unaffected by these transitions (33, 34).

The present study elucidates the functional importance of residues in the NH\(_2\)-terminal part of M3 near the cytoplasmic surface of the membrane in the vicinity of Glu\(^{282}\). This part of M3 is located about midway between the catalytic site and the ion binding pocket. We have selected Ile\(^{279}\), Ile\(^{283}\), Glu\(^{284}\), His\(^{285}\), Phe\(^{286}\), and His\(^{288}\) for this study. The two hydrophobic residues Ile\(^{279}\) and Ile\(^{283}\) are located one α-helical turn away from each other in M3 and are equivalent to the residues Leu\(^{449}\) and Leu\(^{535}\) of the Ca\(^{2+}\)-ATPase, which form an integral part of the hydrophobic wall of the channel seen in the E\(_2\) crystal structure. His\(^{285}\) and His\(^{288}\) are contained within the sequence HFIIH pinpointed to be near the metal-binding site in M3 mediating metal-catalyzed cleavage (33, 34). The positions equivalent to His\(^{285}\) and His\(^{288}\) in the Na\(^+\), K\(^+\)-ATPase are occupied by the negatively charged glutamic acid residues Glu\(^{545}\) and Glu\(^{548}\) in the Ca\(^{2+}\)-ATPase, which could imply a cation-specific function. Phe\(^{286}\) of the Na\(^+\), K\(^+\)-ATPase is conserved in the Ca\(^{2+}\)-ATPase, where it interacts with the specific inhibitor thapsigargin that prevents Ca\(^{2+}\) binding.

Our results indicate that the NH\(_2\)-terminal part of M3 plays an important role in control of the E\(_1\), E\(_2\), and E\(_P\)-E\(_P\)-P conformational equilibria. For the first time we report on a Na\(^+\), K\(^+\)-ATPase mutant (Phe\(^{286}\) → Ala) displaying an enhanced rate of the E\(_1\)-P-E\(_P\)-P conformational transition. Furthermore, Ile\(^{279}\) and Ile\(^{283}\) are crucial for the Na\(^+\) binding properties of the E\(_1\) form. The results are summarized by the symbol code in Fig. 1 and the classification according to conformational shift in Table I.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Basic Functional Characterization—Oligonucleotide-directed mutagenesis** (35) of the cDNA encoding the ouabain-resistant rat α\(_i\)-isoform of Na\(^+\), K\(^+\)-ATPase, expression of mutants and wild type in COS-1 cells, using 5 μM 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 (7, 36).

ATPase activity was measured on sodium deoxycholate- or smamethion-treated leaky plasma membranes at 37 °C as described previously (7, 36). To eliminate the contribution of the endogenous COS-1 cell enzyme, 10 μM 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 mM) that inhibits all Na\(^+\), K\(^+\)-ATPase activity from that measured at 10 μM ouabain.

Studies of the Na\(^+\) dependence of steady-state phosphorylation from [γ\(^{32}\)P]ATP, the time course of ADP-dependent dephosphorylation, and the determination of the active site concentration by phosphorylation in the presence of 150 mM KCl and oligomycin (25 μM) to inhibit dephosphorylation were carried out at 0 °C as previously described (28).

**Rapid Kinetic Phosphorylation Experiments—** Rapid kinetic phosphorylation experiments at 25 °C were performed with either a Bio-Logic quench-flow module QFM-5 as described previously (27), or a Bio-Logic quench-flow module SFM-400/Q (Bio-Logic Science Instruments, Claix, France), that works by the same principle as the QFM-5 module. The phosphorylation rate of enzyme present in the Na\(^+\)-saturated form stabilized by oligomycin was determined in single-mixing experiments according to the previously described “Protocol 1” (27, 29).

**Data Analysis and Statistics—** Data normalization, averaging, and nonlinear regression analysis was carried out as previously (27). 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 dependence values of the ATPase activity and the Na\(^+\) dependence of phosphorylation were analyzed by applying the Hill equation. The ouabain dependence of the ATPase activity was analyzed by applying a function with the ouabain-inhibited enzyme represented by the sum of two Hill functions, one corresponding to the exogenous rat enzyme and one corresponding to the endogenous COS cell Na\(^+\), K\(^+\)-ATPase. The time course of K\(^+\) deocclusion was analyzed by use of the biexponential time function described previously (14, 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 EiNa\(_2\) form was fitted by a monoeponential function, and the dephosphorylation time courses following chase with ATP or ADP by a biexponential function.

**RESULTS**

**Expression, Ouabain Sensitivity, and Catalytic Turnover Rate—** The mammalian COS cell system was used to express wild type and mutants of the rat α\(_i\)-isoform of Na\(^+\), K\(^+\)-ATPase under ouabain-selective pressure, taking advantage of the difference between the ouabain sensitivities of the exogenous rat enzyme (K\(_{0.5}\) for ouabain inhibition > 100 μM; cf. Table I) and the endogenous COS cell Na\(^+\), K\(^+\)-ATPase (K\(_{0.5}\) 0.3–0.8 μM). Transfection was carried out with Ile\(^{279}\) → Ala, Ile\(^{283}\) → Ala, Glu\(^{286}\) → Ala, His\(^{285}\) → Ala, His\(^{288}\) → Glu, His\(^{288}\) → Lys, Phe\(^{286}\) → Ala, and His\(^{288}\) → Ala, and all these mutations with the exception of His\(^{285}\) → Ala were compatible with cell viability in the presence of ouabain. Hence, replacement of His\(^{285}\)
with either a glutamate or a lysine resulted in cell growth, suggesting that the sign of the charge is irrelevant, whereas an alanine at this position led to cell death, indicating that the expression level or the transport activity was reduced to very low levels in His285 Ala.

Titration of the ouabain concentration dependence of Na+/H+,K+/H+–ATPase activity revealed that the $K_{0.5}$ value for ouabain inhibition displayed by the mutants Glu284 Ala and His288 Ala was very similar to that of the wild type, whereas the $K_{0.5}$ value was increased ~4-fold in Ile279 Ala, His285 Glu, and His285 Lys, and ~6-fold in Ile283 Ala and Phe286 Ala relative to the wild type (Table I). To avoid any contribution from the endogenous enzyme, all measurements of ATPase activity and phosphoenzyme to be described below were carried out in the presence of 10 μM ouabain.

To compare the catalytic turnover rates, the ATPase activity was measured in the presence of 3 mM ATP and optimal concentrations of Na+ and K+ and related to the active site concentration determined by phosphorylation in the presence of oligomycin to maximally stabilize the phosphoenzyme. As seen from Table I, the catalytic turnover rate was considerably reduced in the mutants Ile279 Ala, Ile283 Ala, and Phe286 Ala relative to wild type, and only slightly reduced in His285 Glu, whereas in Glu284 Ala, His285 Lys, and His288 Ala the catalytic turnover rate was wild type-like.

To investigate the functional properties of the mutants in detail, a panel of assays for the overall and partial reactions was applied and the results are presented below.

**ATP Dependence of Na+/K+-ATPase Activity**—In the wild-type Na+/K+-ATPase, ATP, binding with high affinity, phosphorylates the $E_1$Na$_3$ form, whereas ATP binding with low affinity to $E_2$K$_2$ accelerates the rate-limiting transition between $E_2$K$_2$ and $E_1$Na$_3$ (boxed ATP in Scheme 1). The apparent affinity for ATP in the activation of Na+/K+-ATPase activity is therefore increased, relative to wild type, in mutants with a shift of the steady-state distribution of $E_2$K$_2$ and $E_1$Na$_3$ in
favor of $E_1\text{Na}_3$, and reduced in mutants where $E_3(K_2)$ prevails (cf. Refs. 7, 27–30, and 36). The ATP concentration dependence of the Na$^+$,K$^+$-ATPase activity could be fitted by the Hill equation giving a Hill coefficient close to 1, and the $K_{0.5}$ value for ATP extracted from the data is listed in Table I. Compared with the wild type, Ile$^{279}$ → Ala and Ile$^{283}$ → Ala displayed a conspicuous increase of the apparent affinity for ATP, corresponding to a decrease in $K_{0.5}$ of 12- and 8-fold, respectively, whereas in Phe$^{286}$ → Ala the apparent affinity for ATP was reduced 2.3-fold ($K_{0.5}$ increased). Table I moreover shows that, in His$^{285}$ → Glu and His$^{285}$ → Lys, the $K_{0.5}$ value for ATP was slightly reduced, 1.4- and 1.7-fold, respectively, whereas it was wild type-like in Glu$^{284}$ → Ala and His$^{288}$ → Ala.

### Scheme 1. Minimum reaction cycle of the Na$^+$,K$^+$-ATPase. Included ions are shown in parentheses. Modulatory ATP is boxed.

![Scheme 1](image)

### Table I

| Mutation | $K_{0.5}$ ouabain | Turnover rate | $K_{0.5}$ ATP | $K_{0.5}$(K) | $K_{0.5}$ vanadate | Shift of conformational equilibrium |
|----------|------------------|---------------|---------------|--------------|-------------------|-----------------------------------|
| Wild type | 136 ± 10 | 8474 ± 165 | 416 ± 29 | 664 ± 14 | 2.5 ± 0.07 | E$_1$ |
| Ile$^{279}$ → Ala | 589 ± 49 | 4096 ± 347 | 34 ± 2 | 503 ± 4 | 133 ± 30 | (n$_H$ = 0.96) |
| Ile$^{283}$ → Ala | 851 ± 203 | 3073 ± 153 | 55 ± 4 | 303 ± 21 | 15.6 ± 2.5 | E$_1$ |
| Glu$^{284}$ → Ala | 134 ± 16 | 9348 ± 386 | 429 ± 36 | 625 ± 30 | 2.9 ± 0.08 | Wild type |
| His$^{285}$ → Glu | 614 ± 74 | 6852 ± 569 | 304 ± 14 | 151 ± 10 | 4.2 ± 0.18 | E$_2$/E$_P$ |
| His$^{285}$ → Lys | 489 ± 64 | 8436 ± 791 | 244 ± 8 | 332 ± 12 | 3.7 ± 0.13 | E$_1$/E$_P$ |
| Phe$^{286}$ → Ala | 825 ± 176 | 3866 ± 182 | 942 ± 205 | 111 ± 19 | 0.8 ± 0.04 | E$_2$/E$_P$ |
| His$^{288}$ → Ala | 104 ± 10 | 9233 ± 327 | 378 ± 35 | 691 ± 18 | 3.0 ± 0.14 | Wild type |

$^a$ Determined by ouabain titration of Na$^+$,K$^+$-ATPase activity. Measurements of ATP hydrolysis were carried out at 37 °C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM MgCl$_2$, 30 mM histidine buffer (pH 7.4), 1 mM EGTA, and various concentrations of ouabain. A function giving a Hill coefficient close to 1, and the corresponding active site concentration determined on the same membrane preparation by phosphorylation at 0 °C with [γ-32P]ATP in the presence of the optimal Na$^+$ concentration together with oligomycin (20 μg/ml) as described previously (28). Average values ± S.E. of 5-11 independent turnover determinations are shown.

$^b$ The catalytic turnover rate calculated as the ratio between the maximal Na$^+$,K$^+$-ATPase activity (measured in the presence of 3 mM ATP and optimal concentrations of Na$^+$ and K$^+$, and 10 μM ouabain) and the corresponding active site concentration determined on the same membrane preparation by phosphorylation at 0 °C with [γ-32P]ATP in the presence of the optimal Na$^+$ concentration together with oligomycin (20 μg/ml) as described previously (28). Average values ± S.E. of 5-11 independent turnover determinations are shown.

$^c$ The dependence of ATPase activity in the presence of ATP and inhibition to 60–70% activity requires more than 100 μM K$^+$ at this Na$^+$ concentration (30). K$^+$ inhibition may be caused by competition with Na$^+$ at the cytoplasmically facing sites of the dephosphoenzyme (see further below).

Moreover, in Fig. 2 the K$^+$ dependence of ATPase activity in mutants His$^{285}$ → Glu, His$^{285}$ → Lys, and Phe$^{286}$ → Ala shows another characteristic feature, an unusual ordinate intercept well above the origin, corresponding to 40, 18, and 1%, respectively. This ability to hydrolyze ATP in the absence of K$^+$ and mere presence of Na$^+$ (“Na$^+$-ATPase activity”) is further described below.

### Vanadate Dependence of Na$^+$,K$^+$-ATPase Activity—As vanadate binds to $E_2(K_2)$ but not to $E_1$, vanadate inhibition of ATPase activity provides information about the relative amount of the $E_2$ form accumulated at steady state. Fig. 3 shows the results of vanadate titration experiments, and the $K_{0.5}$ for vanadate inhibition is listed in Table I. Relative to wild type, mutant Ile$^{279}$ → Ala exhibited a conspicuous reduction of the apparent affinity for vanadate corresponding to an increase

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**Notes:**
- $E_3(K_2)$
- $E_1$
- $E_1\text{Na}_3$
- $2K^+_c$ ATP
- $E_2P$
- $E_2PK_2$
- $E_3PK_2$ ATP
- $3Na^+_c$ ADP
- $E_3(2K_2)$ ATP

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**Table I:**

| Mutation | $K_{0.5}$ ouabain | Turnover rate | $K_{0.5}$ ATP | $K_{0.5}$(K) | $K_{0.5}$ vanadate | Shift of conformational equilibrium |
|----------|------------------|---------------|---------------|--------------|-------------------|-----------------------------------|
| Wild type | 136 ± 10 | 8474 ± 165 | 416 ± 29 | 664 ± 14 | 2.5 ± 0.07 | E$_1$ |
| Ile$^{279}$ → Ala | 589 ± 49 | 4096 ± 347 | 34 ± 2 | 503 ± 4 | 133 ± 30 | (n$_H$ = 0.96) |
| Ile$^{283}$ → Ala | 851 ± 203 | 3073 ± 153 | 55 ± 4 | 303 ± 21 | 15.6 ± 2.5 | E$_1$ |
| Glu$^{284}$ → Ala | 134 ± 16 | 9348 ± 386 | 429 ± 36 | 625 ± 30 | 2.9 ± 0.08 | Wild type |
| His$^{285}$ → Glu | 614 ± 74 | 6852 ± 569 | 304 ± 14 | 151 ± 10 | 4.2 ± 0.18 | E$_2$/E$_P$ |
| His$^{285}$ → Lys | 489 ± 64 | 8436 ± 791 | 244 ± 8 | 332 ± 12 | 3.7 ± 0.13 | E$_1$/E$_P$ |
| Phe$^{286}$ → Ala | 825 ± 176 | 3866 ± 182 | 942 ± 205 | 111 ± 19 | 0.8 ± 0.04 | E$_2$/E$_P$ |
| His$^{288}$ → Ala | 104 ± 10 | 9233 ± 327 | 378 ± 35 | 691 ± 18 | 3.0 ± 0.14 | Wild type |
of the $K_{0.5}$ value of as much as 53-fold. Furthermore, the $K_{0.5}$ value was increased 6.3-fold in mutant Ile283A → Ala. These effects may be accounted for by a change of the distribution of $E_2$($K_2$) and $E_1$Na$_3$ in favor of $E_1$Na$_3$, in line with what can be deduced from the above-described increased apparent affinity for ATP. By contrast, in Phe286A → Ala the apparent affinity for vanadate was increased 3-fold, suggesting accumulation of the
Role of M3 Mutations in Na\(^{+},K^{+}\)-ATPase

| Mutation | 200 mM Na\(^{+}\) | 400 mM Na\(^{+}\) | Phosphorylation | E\(_{d}\) E\(_{p}\) | E\(_{d}\) E\(_{p}\) | E\(_{d}\) E\(_{p}\) | E\(_{d}\) E\(_{p}\) |
|---------|-----------------|-----------------|---------------|---------------|---------------|---------------|---------------|
|         | E\(_{d}\), E\(_{p}\) | E\(_{d}\), E\(_{p}\) | E\(_{d}\), E\(_{p}\) | E\(_{d}\), E\(_{p}\) | E\(_{d}\), E\(_{p}\) | E\(_{d}\), E\(_{p}\) | E\(_{d}\), E\(_{p}\) |
| Wild type | 2.07 ± 0.02 | 2.07 ± 0.02 | 2.07 ± 0.02 | 2.07 ± 0.02 | 2.07 ± 0.02 | 2.07 ± 0.02 | 2.07 ± 0.02 |
| Ile\(_{279}\) → Ala | 2.10 ± 0.03 | 2.10 ± 0.03 | 2.10 ± 0.03 | 2.10 ± 0.03 | 2.10 ± 0.03 | 2.10 ± 0.03 | 2.10 ± 0.03 |
| Glu\(_{284}\) → Ala | 2.00 ± 0.01 | 2.00 ± 0.01 | 2.00 ± 0.01 | 2.00 ± 0.01 | 2.00 ± 0.01 | 2.00 ± 0.01 | 2.00 ± 0.01 |

From Fig. 8, rate constant corresponding to the slow phase indicated by the fitted line. Data for Glu\(_{329}\) → Ala and His\(_{288}\) → Ala were obtained in the same way.

Glu\(_{329}\) is believed to be directly involved in Na\(^{+}\) binding and to function as a gating residue right at the entrance of the ion binding pocket (14, 22) (cf. Fig. 12). As can be seen in Fig. 5, the Hill number was conspicuously increased in this mutant (to 2.9), relative to wild type, thus confirming the expectation based on the reaction cycle in Scheme 1 has demonstrated that the intrinsic affinity of one of the three Na\(^{+}\) sites was selectively reduced (37), or if both the binding (“on”) and the dissociation (“off”) rate for all the ions were enhanced (i.e., a defective gating mechanism) (38). In the present study, the consequence of a defective gating mechanism for the apparent cooperativity was further examined by including mutant Glu\(_{329}\) → Gln in the studies carried out in the absence of oligomycin (Fig. 5). Glu\(_{329}\) is believed to be directly involved in Na\(^{+}\) binding and to function as a gating residue right at the entrance of the ion binding pocket (14, 22) (cf. Fig. 12). As can be seen in Fig. 5, the Hill number was conspicuously increased in this mutant (to 2.9), relative to wild type, thus confirming the expectation based on computation and demonstrating the similarity of the functional change observed for Ile\(_{279}\) → Ala and Ile\(_{283}\) → Ala to that of Glu\(_{329}\) → Gln.

Oligomycin Effect on Phosphoenzyme Level—When the wild-type Na\(^{+},K^{+}\)-ATPase is phosphorylated from ATP at low temperature (0 °C) and 2 µM ATP, the steady-state level of phoshoenzyme built up at a saturating Na\(^{+}\) concentration of 150 mM corresponds to ~80% of the active site concentration. In the presence of oligomycin, the phosphoenzyme level increases, because oligomycin stabilizes the Na\(^{+}\)-occluded form, thereby reducing the dephosphorylation rate. It is seen in Table II that, for Ile\(_{279}\) → Ala and Ile\(_{283}\) → Ala, the steady-state phosphorylation level reached in the absence of oligomycin was remarkably low, only 39 and 16%, respectively, of that reached in the presence of oligomycin. In His\(_{329}\) → Glu, His\(_{328}\) → Lys, and Phe\(_{266}\) → Ala, the value was also reduced somewhat relative to wild type, corresponding to 73, 67, and 58%, respectively, whereas in Glu\(_{329}\) → Ala and His\(_{328}\) → Ala, the value was wild type-like, corresponding to 84 and 85%, respectively. The level of phosphoformdehyde built up at steady state depends on the rate of phosphorylation and the rate of dephosphorylation, these characteristics of the phosphoenzyme were further studied (see below).

Time Course of Phosphorylation at 2 µM [γ-32P]ATP—The quench flow technique described previously (27, 29) was used to study the kinetics of the reaction sequence consisting of ATP binding to the E\(_{d}\)N\(_{a}\) form with subsequent phosphorylation to form E\(_{p}\)P(Na\(_{a}\)) at a millisecond time scale at 25 °C, in the presence of 2 µM [γ-32P]ATP and 100 mM Na\(^{+}\) with oligomycin (Fig. 6 and Table II). As shown in Fig. 6, the appearance of phosphoenzyme following addition of ATP to the Na\(^{+}\)-saturated enzyme could be satisfactorily fitted by a monoeponential function, and the derived rate constant, k\(_{obs}\) is listed in Table II. For His\(_{329}\) → Glu, His\(_{328}\) → Lys, Phe\(_{266}\) → Ala, and His\(_{328}\) → Ala, k\(_{obs}\) was very similar to the value of 29 s\(^{-1}\) determined for the wild type, whereas significantly lower k\(_{obs}\) values of 21 and 17 s\(^{-1}\) were found for Ile\(_{279}\) → Ala and Ile\(_{283}\) → Ala, respectively, suggesting that either the phosphorylation reaction or the binding of ATP is defective in these mutants.

Maximum Phosphorylation Rate and ATP Dependence—To
Further analyze mutant Ile283 → Ala, exhibiting the largest reduction of phosphorylation rate, the ATP concentration dependence of this mutant was compared with that of the wild type under conditions otherwise identical to those described for Fig. 6. Fig. 7 shows the results in the form of double-reciprocal plots of the initial phosphorylation rate per ATPase molecule as a function of the concentration of ATP. The plots are seen to be linear, allowing extraction of the V_max and the Michaelis constant (K_m). The V_max was 2-fold reduced in Ile283 → Ala relative to wild type (60 versus 115 s⁻¹), whereas the K_m for ATP was similar to that of the wild type (5.1 versus 5.5 μM), indicating that the phosphorylation reaction rather than the binding of ATP is defective in this mutant. K⁺ Occlusion—The previously described assay (14, 27, 29, 30) was used to estimate the level of the K⁺-occluded intermediate, E₂(K₂), formed at equilibrium, and the rate of the K⁺ deocclusion reaction E₂(K₂) → E₁ + 2K⁺. The enzyme was equilibrated with K⁺ in the absence of Na⁺ and ATP. Oligomycin was then added to block dephosphorylation, and subsequently the phosphorylation reaction was initiated by diluting the enzyme 10-fold in a solution containing [γ-³²P]ATP and Na⁺, and the time course of phosphoenzyme formation was followed. The reaction sequence leading to phosphorylation of E₂(K₂) proceeds through the steps E₂(K₂) → E₁ → E₁Na₃ → E₁P(Na₃), where the release of occluded K⁺ is rate-limiting. Under these conditions the time course of phosphorylation normally consists of a rapid phase, reflecting phosphorylation of the enzyme pool present initially as the non-occluded E₁ form, and a slow phase corresponding to release of occluded K⁺ from the E₁ enzyme pool through the rate-limiting step in the reaction sequence. Hence, the data in Fig. 8 were analyzed as a biphasic time function in which the component corresponding to the rapid phase for simplicity was reduced to a constant, because the non-occluded E₁ enzyme pool binds Na⁺ and phosphorylates instantaneously (cf. Refs. 14, 27, 29, and 30). In Fig. 8, the line shows the best fit corresponding to the slow phase reflecting K⁺ deocclusion, and the extent of the slow phase corresponding to the amount of enzyme initially present as E₂(K₂) equals 100% minus the ordinate intercept. Table II shows for all mutants the parameters extracted by this fitting procedure.

For the wild-type Na⁺, K⁺-ATPase, 88% of the enzyme was present in the K⁺-occluded E₂(K₂) form following equilibration with 1 mM K⁺, and similar amounts of E₂(K₂) were formed in Ile283 → Ala, Glu284 → Ala, His285 → Glu, His285 → Lys, and His288 → Ala. In Phe286 → Ala, the amount of E₂(K₂) constituted as much as 100% under identical conditions, whereas the amount of E₂(K₂) formed in Ile279 → Ala was only 39%.

As further seen in Fig. 8 and Table II, the rate constant corresponding to the slow phase reflecting release of occluded K⁺ was significantly higher for Ile279 → Ala, Ile283 → Ala, His285 → Glu, and His285 → Lys as compared with the wild type, corresponding to an increase of 2.6-, 4.1-, 2-, and 1.7-fold, respectively. The corresponding rate constants corresponding to Glu284 → Ala and His288 → Ala were very similar to that of the wild type, whereas the rate constant of Phe286 → Ala was 2-fold reduced relative to wild type. It should be emphasized that, although the line corresponding to Ile279 → Ala gives the visual impression of being parallel to that corresponding to the wild type, the extracted rate constant was higher that of the wild type, because the extent of the slow phase was smaller for Ile279 → Ala than for the wild type.

Na⁺-ATPase Activity—As revealed in Fig. 2 by the unusually high ordinate intercept, some of the mutants were able to hydrolyze ATP at a significant rate even in the absence of K⁺. The Na⁺ dependence of this "Na⁺-ATPase activity" is shown in Fig. 9 for selected mutants. For His285 → Glu and His285 → Lys, the Na⁺-ATPase activity reached a maximum level 7-
The expressed wild-type Na⁺,K⁺-ATPase and mutants were pre-equilibrated for 1 h at room temperature in a medium containing 20 mM Tris (pH 7.5) and 1 mM K⁺. Oligomycin (150 µg/ml) was then added, and the solution cooled to 10°C and diluted 10-fold by addition of a phosphorylation solution producing final concentrations of 1 µM [γ-32P]ATP, 100 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 20 mM Tris (pH 7.5), and 10 µM ouabain. The phosphorylation was monitored by acid quenching at the indicated time intervals, and data are shown as percentage of the phosphorylation level obtained with fully deoccluded enzyme, for which the 1-h incubation had been carried out in the presence of 50 mM Na⁺ and absence of K⁺. Average values corresponding to at least four independent experiments were analyzed by fitting a biphasic time function as previously described (14, 27, 29, 30). The extracted rate constant corresponding to the slow phase (deocclusion rate) and the extent of the slow phase, corresponding to the amount of E₃(K)K initially present, are listed in Table II. Wild-type (filled circles), Ile²⁷⁹ → Ala (filled triangles), Ile²⁸³ → Ala (open squares), His²⁸⁵ → Glu (open triangles pointing downward), His²⁸⁵ → Lys (open diamonds), and Phe²⁸⁶ → Ala (open triangles pointing upward).

4.5-fold higher, respectively, than that observed for the wild type. The Na⁺-ATPase activity reached in Phe²⁸⁶ → Ala was 1.7-fold higher than that of the wild type, whereas the Na⁺-ATPase activity in the other mutants was wild type-like, as exemplified in Fig. 9 by Ile²⁸³ → Ala.

For the wild type, the Na⁺ dependence of Na⁺-ATPase activity shows a complex activation and inhibition pattern. The activation phase corresponding to a K₀.₅ below 1 mM is a result of Na⁺ binding at the cytoplasmically facing Na⁺-transport sites. As previously discussed (10), this phase is followed by an inhibition at Na⁺ concentrations between 1 and 5 mM and subsequently by further activation at higher Na⁺ concentrations (K₀.₅ ~ 100 mM), because of Na⁺ binding at extracellularly facing sites (39). At very high NaCl concentrations above 300 mM, some inhibition also occurs, which may be ascribed to an effect of Na⁺ and/or Cl⁻ on the E₁P → E₃P equilibrium (40, 41).

In Phe²⁸⁶ → Ala, the Na⁺-ATPase activity was slightly increased throughout the Na⁺ concentrations studied relative to the wild type. For Na⁺ concentrations below 300 mM, however, the course of the curve resembles that of the wild type, whereas above 300 mM Na⁺ a further activation phase not seen for the wild type is noticed (Fig. 9). In contrast, the curves representing the Na⁺-ATPase activity of mutants His²⁸⁵ → Glu and His²⁸⁵ → Lys differ from that of the wild type throughout the Na⁺ concentration range studied. Thus, below 100 mM Na⁺ the intermediate inhibition phase is absent, and the two activation phases observed for the wild type below 100 mM Na⁺ merge. Furthermore, the inhibition at high NaCl concentrations starts already ~80 and 200 mM Na⁺, respectively (Fig. 9), and is very efficient at 1 mM NaCl.

ADP Sensitivity of the Phosphoenzyme—The phosphoenzyme is usually considered to consist of two major pools designated E₃P and E₃P. The E₃P intermediate is K⁺-insensitive but ADP-sensitive, i.e. able to donate the phosphoryl group back to ADP forming ATP. The E₃P intermediate can be distinguished from E₁P, because it is ADP-insensitive and K⁺-sensitive, K⁺ at the extracellularly facing sites inducing rapid hydrolysis of E₃P. By studying the ADP sensitivity of the phosphoenzyme at 0°C, the extent of each phosphoenzyme pool present at steady state was estimated. Fig. 10 presents results of experiments with the wild-type Na⁺,K⁺-ATPase and the mutants Ile²⁷⁹ → Ala, Ile²⁸³ → Ala, His²⁸⁵ → Glu, His²⁸⁵ → Lys, and Phe²⁸⁶ → Ala, in which the time course of dephosphorylation was monitored in the presence of 2.5 mM ADP together with 1 mM ATP, following phosphorylation by [γ-32P]ATP. The phosphorylation was carried out in the presence of 20 mM Na⁺ and absence of K⁺ to promote accumulation of the E₃P intermediate. The data points were fitted by a biexponential function, allowing estimation of the extents of the rapid and slow decay components, reflecting the initial amounts of the ADP-sensitive E₃P and the ADP-insensitive E₃P, respectively. In Fig. 10, the dotted lines show extrapolation of the slow decay component back to the ordinate intercept to indicate the amount of E₁P. Similar experiments were also conducted with Glu²⁸⁴ → Ala and His²⁸⁸ → Ala, and Table II lists the result of this analysis for all mutants. For the wild-type Na⁺,K⁺-ATPase, 21% of the phosphoenzyme was ADP-sensitive E₃P. For Ile²⁷⁹ → Ala, Ile²⁸³ → Ala, Glu²⁸⁴ → Ala, and His²⁸⁸ → Ala, the E₃P level was very similar to that of the wild type. For His²⁸⁵ → Glu and His²⁸⁵ → Lys, the E₃P level was significantly higher, 84% and 52%, respectively, corresponding to a 4- and 2.5-fold increase, relative to wild type. Hence, in the latter two mutants, the distribution of the phosphoenzyme intermediate between E₁P and E₃P was shifted considerably in favor of E₃P, relative to the wild type. The Phe²⁸⁶ → Ala mutant, on the other hand, displayed a reduced E₃P level of only 10% (Table II), indicating that the distribution between E₁P and E₃P is changed in favor of E₁P. The rate constant corresponding to the slow phase, reflecting the dephosphorylation of E₃P, was increased significantly in His²⁸⁵ → Glu and
The E2P form (to ensure rapid dephosphorylation of E1P). The data points are average values corresponding to four to seven independent experiments, calculated following normalization to the 100% value obtained by quenching after 10 s of phosphorylation without dephosphorylation. The lines show the best fits to the complete sets of normalized data of a biexponential time function. Note the logarithmic ordinate scale. The dotted lines show the extrapolation of the slow decay component corresponding to E1P back to ordinate intercept. The rate constant corresponding to the slow decay component (E1P hydrolysis) is as follows (s⁻¹): wild type, 0.030; Ile279→Ala, 0.047; Ile283→Ala, 0.055; His285→Glu, 0.186; His285→Lys, 0.170; Phe286→Ala, 0.069. The extents of the rapid and slow decay components corresponding to the initial values of E1P and E2P, respectively, are listed in Table II.

Hi285→Lys, corresponding to 6.2- and 5.7-fold, respectively, and in Phe286→Ala it was increased 2.3-fold, relative to wild type (see legend to Fig. 10). These results suggest that the high Na⁺-ATPase activity observed for these mutants (Fig. 9) is the result of an increased ability of Na⁺ to activate dephosphorylation of E2P by binding in place of K⁺ at the extracellularly facing sites.

Dephosphorylation Kinetics at 0 °C of E1P Phosphoenzyme—The E1P→E2P interconversion was further examined for the wild type and the mutants that, according to the results in Fig. 10, deviated significantly from wild type with respect to ADP sensitivity: His285→Glu, His285→Lys, and Phe286→Ala. Phosphorylation was carried out in the presence of a high NaCl concentration of 600 mM to promote accumulation of the E1P form, even in the wild type (40, 41). That the major fraction of the phosphoenzyme present under these conditions indeed is the E1P intermediate was confirmed by demonstrating that the phosphoenzyme formed was fully ADP-sensitive, decaying within 2–4 s upon addition of ADP (data not shown). Dephosphorylation in the forward direction was then monitored following addition of a solution producing final concentrations of Na⁺ of either 200 mM (Fig. 11A) or 600 mM (Fig. 11B), 1 mM unlabeled ATP, and 20 mM K⁺ (to ensure rapid dephosphorylation of E2P so that E1P→E2P is rate-limiting for the dephosphorylation of E1P; cf. Scheme 1). As previously found for the wild type and other mutants (27, 28, 41), the phosphoenzyme decay is not monoeponential under these conditions (possibly because of heterogeneity of the lipid phase at 0 °C; Ref. 41), but a comparison of rate differences can be performed on the basis of the half-lives (T0.5) as shown in Table II. Under both dephosphorylation conditions, the T0.5 value was increased in Ile279→Ala and His285→Glu and His285→Lys relative to wild type. At 600 mM Na⁺ the increase amounted to at least 3-fold in His285→Glu and 1.5-fold in His285→Lys, indicating that the E1P→E2P conversion rate is reduced in these mutants, in line with the enhanced ADP sensitivity described above. By contrast, in Phe286→Ala, the T0.5 for dephosphorylation was conspicuously shortened relative to wild type under both sets of dephosphorylation conditions, indicating that the E1P→E2P conversion is accelerated in this mutant. In the presence of 200 mM Na⁺, the dephosphorylation of Phe286→Ala was actually too fast to be measured (Fig. 11A). At 600 mM Na⁺ (Fig. 11B), accurate measurements could be performed, and it is seen in Table II that the T0.5 value was 7.2-fold lower in Phe286→Ala compared with wild type.

**DISCUSSION**

E1P→E2P Conformational Changes—Relative to wild type, the mutants Ile279→Ala and Ile283→Ala displayed increased apparent affinity for ATP and decreased apparent affinity for vanadate (Table I), consistent with a change of the steady-state distribution of the phospho forms E2P(K2) and E1PNa3 in favor of the latter. These effects were more pronounced for Ile279→Ala than for Ile283→Ala. Accordingly, measurements of K⁺ deocclusion showed that the rate constant characterizing the K⁺-deoccluding E2P(K2)→E1P conversion was increased in Ile283→Ala and Ile283→Ala, and for Ile279→Ala, we found that the relative amount of E2P(K2) formed at equilib-
rium in the absence of Na\(^{+}\) and ATP was 39% versus 88% for wild type (Table II), indicating that the equilibrium between \(E_2(K_2)\) and \(E_1\) was displaced significantly in favor of \(E_1\). By contrast, the distribution of the phosphoenzyme conformations, \(E_1P\) and \(E_2P\), was wild type-like in Ile\(^{279}\) \(\rightarrow\) Ala and Ile\(^{283}\) \(\rightarrow\) Ala (Table II).

In His\(^{285}\) \(\rightarrow\) Glu and His\(^{285}\) \(\rightarrow\) Lys, on the other hand, the phosphoenzyme showed a significant change in the distribution of \(E_1P\) and \(E_2P\) in favor of \(E_1P\) (Table II), whereas the distribution of the dephospho forms \(E_2(K_2)\) and \(E_1K_2\) was changed little, relative to wild type, as judged from the small changes in the apparent affinities for ATP and vanadate (Table I) and in the amount of \(K^-\)-occluded enzyme and rate of \(K^-\) deocclusion (Table II). Dephosphorylation experiments initiated from \(E_1P\) showed that the \(E_2P\) \(\rightarrow\) \(E_1P\) conversion was inhibited in His\(^{285}\) \(\rightarrow\) Glu and His\(^{285}\) \(\rightarrow\) Lys, and more so in the former (Table II), thus explaining the change of the steady-state distribution of the phosphoenzyme intermediates in favor of \(E_1P\), indicated by the increased ADP sensitivity, as well as the reduction of the catalytic turnover rate observed for His\(^{285}\) \(\rightarrow\) Glu (Table I). It is also noteworthy that, although the charge-reversing glutamate substitution (corresponding to that found in the wild-type Ca\(^{2+}\)-ATPase) exerted a more pronounced effect on the \(E_1P\) \(\rightarrow\) \(E_2P\) conversion than the lysine substitution, the His\(^{285}\) \(\rightarrow\) Ala substitution, removing charge and polarity, led to complete loss of cell viability.

Mutations His\(^{285}\) \(\rightarrow\) Glu and His\(^{285}\) \(\rightarrow\) Lys furthermore gave rise to an increased Na\(^{+}\)-activated ATPase activity in the absence of K\(^{+}\) ("Na\(^{+}\)-ATPase activity"), similar to that induced by certain other mutations in the transmembrane region (Fig. 1, diamonds), including mutations of Glu\(^{282}\) in M3 (29). The Na\(^{+}\)-ATPase activity of His\(^{285}\) \(\rightarrow\) Glu and His\(^{285}\) \(\rightarrow\) Lys was inhibited at relatively low salt concentrations, to an extent that correlated with the reduction of the \(E_1P\) \(\rightarrow\) \(E_2P\) transition rate (compare Figs. 9 and 11). The Na\(^{+}\)-ATPase activity is the result of an enhanced rate of \(E_2P\) dephosphorylation (Fig. 10, slow phase), and the sensitivity to inhibition by salt can be explained by the \(E_1P\) \(\rightarrow\) \(E_2P\) transition being rate-limiting for the Na\(^{+}\)-ATPase cycle in these mutants, as this transition is known to be inhibited by salt (40, 41).

Phe\(^{286}\) \(\rightarrow\) Ala showed displacement of the conformational equilibria of dephosphoenzyme and phosphoenzyme in favor of \(E_1E_2(K_2)\) and \(E_2P\), respectively. Hence, Phe\(^{286}\) \(\rightarrow\) Ala exhibited reduced ATP affinity and increased vanadate affinity (Table I). Furthermore, 100% of the enzyme was in the \(K^-\)-occluded form, in the absence of other ligands, and the K\(^-\)-deocclusion rate was significantly reduced, relative to wild type (Table II). The latter effect may account for the reduced catalytic turnover rate of Phe\(^{286}\) \(\rightarrow\) Ala (Table I), as the deocclusion of K\(^+\) is rate-limiting for the overall reaction. With respect to the phosphoenzyme intermediates, a slightly higher level of ADP-insensitive \(E_2P\), relative to wild type, was noticed, and the rate constant corresponding to the \(E_2P\) \(\rightarrow\) \(E_1P\) conversion was enhanced, as much as 7-fold, relative to wild type, at 600 mM Na\(^{+}\) (Table II). Such a significant enhancement of the \(E_1P\) \(\rightarrow\) \(E_2P\) transition rate has to our knowledge not been previously reported for any Na\(^{+}\)-K\(^+\)-ATPase mutant. These changes in the conformational equilibria, favoring the accumulation of K\(^+\)-binding enzyme forms, also explain the increased apparent K\(^+\) affinity seen for Phe\(^{286}\) \(\rightarrow\) Ala in K\(^+\) titration of Na\(^{+}\)-K\(^+\)-ATPase activity (Table I).

The mutational effects on the conformational changes observed in the present study are summarized in Table I, using the descriptive terms \(E_1', E_2', E_1P'\) and \(E_2P'\) to indicate the forms accumulated to a higher extent in the mutant compared with wild type. This classification highlights the fact that mutations of Ile\(^{279}\) and Ile\(^{283}\) preferentially affects the conformational change of the dephosphoenzyme, whereas mutations of His\(^{285}\) preferentially affects the conformational change of the phosphoenzyme, with small effects on the dephosphoenzyme (as indicated by parentheses), and mutation of Phe\(^{286}\) affects both conformational equilibria in parallel. It is, furthermore, noteworthy that we previously reported that replacement of the M3 residue Glu\(^{282}\) with lysine displaces the \(E_1E_2\) and \(E_2P-E_1P\) equilibria in parallel in favor of \(E_1\) and \(E_2P\), respectively, i.e. in the opposite direction compared with the Phe\(^{286}\) \(\rightarrow\) Ala mutant described here, and that replacement of Glu\(^{282}\) with alanine displaces the \(E_1-E_2\) equilibrium in favor of \(E_2\) with little effect on the conformational equilibrium of phosphoenzyme (29).

The identification of several M3 residues that are important for the conformational equilibrium between the \(E_1\) and \(E_2\) forms of the dephosphoenzyme suggests that a significant movement of M3 occurs in relation to the transition between these forms. This is at odds with the conclusion based on metal ion-catalyzed cleavage, that M3 is static relative to M1 and the cytoplasmic surface (33, 34), but concurs with the large movement of M3 predicted from the two crystal structures of the Ca\(^{2+}\)-ATPase (4).

Interestingly, it is possible to explain the displacement of the conformational equilibria in favor of \(E_1E_2\) induced by the Phe\(^{286}\) \(\rightarrow\) Ala mutation by reference to the Ca\(^{2+}\)-ATPase crystal structures. In the Ca\(^{2+}\)-ATPase \(E_1E_2\) crystal structure, the side chain of the phenylalanine (Phe\(^{286}\)) equivalent to Phe\(^{286}\) of Na\(^{+}\)-K\(^+\)-ATPase is interposed between the hydrophobic side chains of M5 residues Ile\(^{761}\) (Ile\(^{771}\) in Na\(^{+}\)-K\(^+\)-ATPase) and Ile\(^{675}\) (Leu\(^{775}\) in Na\(^{+}\)-K\(^+\)-ATPase) in a cogwheel-like arrangement (Fig. 12, left panel), whereas in the \(E_2\) structure the distance to Ile\(^{761}\) is too large for any interaction to occur (Fig. 12, middle panel). The downward, sliding movement of M3, breaking van der Waals interactions involving Phe\(^{286}\) and to some extent being subject to steric hindrance by the bulkiness of Phe\(^{286}\) and Ile\(^{675}\), would conceivably be aided by substitution of the large aromatic side chain of the phenylalanine with the smaller alanine.

The lethal effect of the His\(^{285}\) \(\rightarrow\) Ala mutation, as well as the preferential effects of the His\(^{285}\) \(\rightarrow\) Glu and His\(^{285}\) \(\rightarrow\) Lys mutations on the conformational equilibrium of the phosphoenzyme, are less straightforward to explain on the basis of the structural knowledge we have from the Ca\(^{2+}\)-ATPase crystal structures, but it should be noted that neither of the Ca\(^{2+}\)-ATPase crystal structures represents phosphorylated enzyme. Because several M3 mutations affected the \(E_1E_2\) and \(E_2P-E_1P\) equilibria differentially, the structural rearrangements occurring in the Na\(^{+}\)-K\(^+\)-ATPase phosphoenzyme likely differ somewhat from those of the dephosphoenzyme in the M3 region. The side chains of His\(^{285}\) and Glu\(^{282}\) are sufficiently close to be able to interact, and ion pairing of these residues could be more important for the conformational change of the phosphoenzyme as compared with the dephosphoenzyme, thus explaining the preferential effect of the His\(^{285}\) mutations on the phosphoenzyme. Interestingly, either residue is replaced with one of opposite charge in the Ca\(^{2+}\)-ATPase (Glu\(^{282}\) and Lys\(^{283}\), respectively), and ion pairing would therefore also be possible in this enzyme as clearly seen in the \(E_2\) crystal structure (Fig. 12, right panel). The need for ion pairing between His\(^{285}\) and Glu\(^{282}\) to facilitate the conformational change of the Na\(^{+}\)-K\(^+\)-ATPase phosphoenzyme would explain the finding that the \(E_1P\) form accumulates both in His\(^{285}\) \(\rightarrow\) Glu and in Glu\(^{282}\) \(\rightarrow\) Lys (29). It also explains that the mutational effect is less pronounced in His\(^{285}\) \(\rightarrow\) Lys, the positively charged lysine being able to substitute at least partially for that of the histidine.
May at least in part be accounted for by the accumulation of E activating phosphoryl transfer from ATP, whereas the E1 form were probed by Na is furthermore seen that the M3 residues Leu249 and Leu253 together with M4 residues Ile315 and Leu319 form a hydrophobic wall of this pathway, rather close to the M1 binding may actually be more pronounced than revealed by the intraM1 conformations of the Na,K-ATPase in E1 and E2 conformations in the region corresponding to that studied here for the Na,K-ATPase. The correspondence between the indicated Ca2+-ATPase residues and equivalent Na,K-ATPase residues are as follows (Ca2+-ATPase residue, color used for the side chain, Na,K-ATPase residue): (Leu253, pink, Ile279), (Lys255, white, Glu282), (Leu253, pink, Ile279), (Glu255, yellow, His268), (Phe256, yellow, Phe266), (Glu258, yellow, His268), (Glu282, cyan, Val235), (Ile761, blue, Ile775), (Ile761, blue, Ile775). The left panel highlights the "cogwheel-like" arrangement of the Phe256 side chain in between the hydrophobic side chains of M3 residues Ile249 and Ile253 in the E2 structure. The middle panel shows that downward movement and inclination of M3 has occurred on transition to E2. The arrow indicates a possible entrance pathway for Ca2+, which has opened as a consequence of the sliding movement of M3 and bending of M1 (4). The right panel shows the view right into this entrance pathway with the Ca2+ binding residue Glu309 at the bottom. Note the proximity of the side chains of Lys252 (only shown on the right panel) and Glu255, allowing for formation of a salt bridge. It is furthermore seen that the M3 residues Leu249 and Leu253 together with M4 residues Ile311 and Leu319 form a hydrophobic wall of this pathway, and that Glu282 is located right at the mouth of the entrance.

Furthermore, there would be no need for ion pairing to neutralize Glu282 in the Glu282 → Ala mutant, explaining the lack of effect of this mutation on the conformational change of the phosphoenzyme (29).

It is also interesting that those M3 mutants showing a change of conformational equilibrium furthermore exhibited a reduced apparent affinity for ouibain (4–6-fold; see Table I), although there seems to be no direct relation between the ouibain affinity and the direction of the change of conformational equilibrium. A reduction of ouibain affinity of the same magnitude was also seen for the Glu282 mutants (29). Ouibain binds somewhere in the extracellular part of the protein, and the loop between M1 and M2 is thought to be involved (42). A possible explanation of the reduced ouibain affinity seen for the M3 mutants is that the M3–M4 loop, which is located rather close to the M1–M2 loop in the Ca2+-ATPase E2 crystal, contributes to make up the ouibain binding site, and that the mutations change the position of this loop by affecting the inclination or the strong curvature of M3.

Na+ Binding Properties—The Na+ binding properties of the E1 form were probed by Na+ titration of phosphorylation. Na+ binds to E1 from the cytoplasmic side of the membrane, thereby activating phosphoryl transfer from ATP, whereas the E2 form is unable to phosphorylate from ATP. For the mutants Ile279 → Ala, Ile279 → Ala, His288 → Ala, and His288 → Ala, we found a 2–4.5-fold reduced apparent affinity for Na+ in the presence of oligomycin (Fig. 4 and Table I). For Phe286 → Ala, this effect may at least in part be accounted for by the accumulation of E2. For the other three mutants, however, the E1 → E2 equilibrium was either unaffected by the mutation (His288 → Ala) or poised in favor of the Na+ binding E1 form (Ile279 → Ala and Ile283 → Ala). Therefore, the reduced apparent affinity for Na+ indicates that the intrinsic Na+ affinity of E1 is reduced in these mutants. For Ile279 → Ala and Ile283 → Ala, the effect on Na+ binding may actually be more pronounced than revealed by the →4-fold reduction of apparent affinity, because it is masked by the displacement of the E1 → E2 equilibrium in favor of E1. The inhibition of ATPase activity by K+ at relatively low concentration seen for Ile279 → Ala and Ile283 → Ala (Fig. 2) is probably the consequence of an enhanced ability of K+ to compete with Na+ at the cytoplasmically facing sites because of the reduced Na+ affinity. Further evidence of defective Na+ binding in these mutants was obtained by comparing the Na+-stimulated phosphorylation observed in the presence and absence of oligomycin. By stabilizing the Na+-occluded form, oligomycin allows the maximum amount of phosphorylated enzyme to accumulate (43, 44). For the wild type, the amount of phosphoenzyme accumulated in the absence of oligomycin constitutes ~80% that measured in the presence of oligomycin, whereas for Ile279 → Ala and Ile283 → Ala, the corresponding values were only 39 and 16%, respectively (Table II). The latter is the lowest value reported so far for any Na+,K+-ATPase mutant. By conducting rapid kinetic measurements, we obtained evidence that the phosphorylation rate is reduced in Ile279 → Ala and Ile283 → Ala, even in the presence of oligomycin (Fig. 6), and it seems likely that a reduced phosphorylation rate rather than an increased dephosphorylation rate is responsible for the low phosphorylation level seen in the absence of oligomycin, as the hydrolysis of the phosphoenzyme occurred at a close to normal rate in these mutants (Fig. 10). The more detailed investigation of the ATP dependence of the phosphorylation rate carried out for Ile283 → Ala demonstrated that the Vmax of phosphorylation is only half that of the wild type, whereas the ATP affinity is normal (Fig. 7). The reduced Vmax of phosphorylation can furthermore account for the decrease of the catalytic turnover rate observed at high ATP concentration for this mutant (Table I). It is interesting to note the similarity to previous findings following glutamine substitution of Glu239 in the ion binding pocket. In the Glu239 → Gln mutant, the phosphorylation level in the absence of oligomycin was approximately one third that detected in its presence, also as a result of a reduced rate of phosphorylation (14, 21), and the defective phosphorylation seemed to arise from a reduced probability of finding the pocket with bound Na+ in the closed ("occluded")
configuration required for triggering phosphorylation. The same may be relevant for Ile\(^{279} \rightarrow \) Ala and Ile\(^{283} \rightarrow \) Ala, even though these residues are located at some distance from the ion binding pocket, whereas Glu\(^{299}\) is thought to be part of the pocket and function as a gate right at the entrance of the pocket (14, 21, 22) (cf. Fig. 12). Mutations Ile\(^{279} \rightarrow \) Ala and Ile\(^{283} \rightarrow \) Ala may directly or indirectly perturb the gateway to the ion binding pocket, or the pocket itself, such that the bound Na\(^+\) ions tend to dissociate more rapidly than in the wild type. In line with this hypothesis, we also observed an increased apparent cooperativity in Na\(^+\) activation of phosphorylation in the absence of oligomycin for Ile\(^{279} \rightarrow \) Ala and Ile\(^{283} \rightarrow \) Ala, but not for Phe\(^{286} \rightarrow \) Ala (Fig. 5). As demonstrated here for the first time, a similar increase of apparent cooperativity is seen for Glu\(^{299} \rightarrow \) Gln (Fig. 5), thus supporting the notion that such an effect may arise from an enhanced dissociation ("off") rate for the activating ions, as previously demonstrated theoretically by computation (37, 38).

The finding that alanine substitution of Ile\(^{279}\) or Ile\(^{283}\) affects the ion binding properties of the Na\(^+\),K\(^+\)-ATPase is strikingly similar to the recent report of an enhanced rate of Ca\(^{2+}\) dissociation toward the cytoplasmic side in the equivalent Ca\(^{2+}\)-ATPase mutants Leu\(^{249} \rightarrow \) Ala and Leu\(^{253} \rightarrow \) Ala (45) and suggests that the Na\(^+\),K\(^+\)-ATPase and the Ca\(^{2+}\)-ATPase are structurally and functionally similar in this region. In the Ca\(^{2+}\)-ATPase, Leu\(^{249}\) and Leu\(^{253}\) are close to two other hydrophobic residues, Ile\(^{312}\) and Leu\(^{419}\) (in Na\(^+\),K\(^+\)-ATPase: Val\(^{395}\) and Leu\(^{388}\), respectively; see Fig. 12), and the weakening of the hydrophobic/van der Waals interactions caused by the alanine substitution could conceivably lead to structural changes affecting the ion binding pocket or the entrance pathway/gating mechanism of this pocket.

Additionally, mutation His\(^{286}\) \rightarrow \) Ala reduced the Na\(^+\) affinity of the E\(_{1}\) form, although, as judged from the normal phosphorylation rate, there was no effect on the occlusion of Na\(^+\), such as proposed for Ile\(^{279} \rightarrow \) Ala and Ile\(^{283} \rightarrow \) Ala. This result may be accounted for by a reduced on-rate for Na\(^+\) in His\(^{286}\) \rightarrow \) Ala. In the thapsigargin-stabilized E\(_{2}\) crystal structure of the Ca\(^{2+}\)-ATPase, a water-filled channel has opened up between M1 and M3 (Fig. 12, middle panel, the arrow indicating the suggested entrance, and right panel), and this has been suggested as a putative Ca\(^{2+}\) entrance pathway (4). It is interesting to note that the Ca\(^{2+}\)-ATPase residue Glu\(^{286}\), equivalent to His\(^{286}\) of the Na\(^+\),K\(^+\)-ATPase, is located right at the mouth of the channel between M1 and M3.

**Conclusion**—The present results demonstrate the importance of the M3 residues Ile\(^{279}\), Ile\(^{283}\), His\(^{286}\), and Phe\(^{286}\) for the E\(_{1}\)-E\(_{2}\) and E\(_{2}\)-P-E\(_{2}\)-P conformational changes of the Na\(^+\),K\(^+\)-ATPase, which thereby seem to involve significant rearrangements of M3. Because the E\(_{1}\)-E\(_{2}\) and E\(_{2}\)-P-E\(_{2}\)-P conformational equilibria were differentially affected by some of the mutations, the phosphorylated conformations seem to differ significantly from the dephospho forms in the M3 region. Furthermore, Ile\(^{279}\), Ile\(^{283}\), and His\(^{286}\) are important for the Na\(^+\) binding properties of the enzyme in the E\(_{1}\) form, and the Ile\(^{279}\) and Ile\(^{283}\) mutants showed evidence of destabilization of the Na\(^+\)-occluded state.

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