Importance of Leu$^{99}$ in Transmembrane Segment M1 of the Na$^+,K^+$-ATPase in the Binding and Occlusion of K$^{+*}$

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Twenty-six point mutations were introduced into the N-terminal and middle parts of transmembrane segment M1 of the Na$^+,K^+$-ATPase and its cytosolic extension. None of the alterations to charged and polar residues in the N-terminal part of M1 and its cytosolic extension had any major effect on the cation binding properties, thus rejecting the hypothesis that these residues are involved in cation selectivity. By contrast, specific residues in the middle part of M1, particularly Leu$^{99}$, were found critical to K$^+$ interaction of the enzyme. Hence, mutation L99A reduced the affinity for K$^+$ activation of E$_2$P dephosphorylation 17-fold, and L99F reduced the equilibrium level of the K$^+$-occluded intermediate [K$_2$]E$_2$ and increased the rate of K$^+$ deocclusion 39-fold, i.e. more than seen for mutation E329Q of the cation-binding glutamate in M4. L99Q affected K$^+$ interaction in yet another way, the equilibrium level of [K$_2$]E$_2$ being slightly increased despite an increased rate of K$^+$ deocclusion, suggesting that the K$^+$ ions leave and enter the occlusion pocket more frequently than in the wild type. L99Q furthermore affected the ability to discriminate between Na$^+$ and K$^+$ on the extracellular side. Our findings can be explained by a structural model in which Leu$^{99}$ and Glu$^{329}$ interact and cooperate in K$^+$ binding and gating of the K$^+$ sites. The disturbance of K$^+$ interaction in mutants with alteration to Leu$^{91}$, Phe$^{95}$, Ser$^{96}$, or Leu$^{99}$ could be a consequence of the roles of these residues in positioning the M1 helix optimally for the interaction between Leu$^{99}$ and Glu$^{329}$. Phe$^{95}$ may serve to stabilize the pivot for movement of M1 through interaction with Ile$^{287}$ in M3.

The Na$^+,K^+$-ATPase$^2$ (EC 3.6.3.9), located in the plasma membrane, is an ATP-driven ion pump that transports three Na$^+$ out of the cell in exchange for two K$^+$ being transported into the cell for each ATP hydrolyzed, thereby creating the gradients for Na$^+$ and K$^+$ essential to cellular function (1, 2). It is a member of the superfamily of P-type ATPases, which in addition includes H$^+,K^+$-ATPases, H$^+$-ATPases, Ca$^{2+}$-ATPases, as well as heavy metal ion-transporting ATPases. The distinguishing biochemical feature of P-type ATPases is the formation during their reaction cycle of a covalent acylphosphate enzyme intermediate, resulting from the transfer of the γ-phosphoryl group of ATP to a conserved aspartyl residue within the protein. Scheme 1 depicts the reaction cycle of the Na$^+,K^+$-ATPase that describes how ion movement is linked with ATP hydrolysis through conformational changes in the enzyme (1, 2).

The translocation of Na$^+$ across the membrane during the $E_1P \rightarrow E_2P$ transition and the translocation of K$^+$ during the $E_2 \rightarrow E_1$ transition involve so-called “occluded” states (indicated in Scheme 1 by brackets), where the intramembranously bound ions are inaccessible to the medium on either side of the membrane, their dissociation being prevented by “gates” (1, 3–5).

The main polypeptide chain of the Na$^+,K^+$-ATPase is composed of two major parts as follows: a transmembrane part consisting of 10 transmembrane α-helices (M1–M10) (6, 7) and a large cytoplasmatic part consisting of three distinct domains designated “actuator,” “nucleotide-binding,” and “phosphorylation” domain in the terminology of the homologous Ca$^{2+}$-ATPase (8). Site-directed mutagenesis in combination with biochemical assays and homology modeling have implicated several residues in transmembrane segments M4 (Glu$^{329}$),$^3$ M5 (Thr$^{809}$, Ser$^{877}$, Asn$^{878}$, and Glu$^{781}$), M6 (Asp$^{806}$, Thr$^{809}$, and Asp$^{810}$), M8 (Glu$^{925}$), and M9 (Glu$^{956}$) in cation binding (9–19). Of these residues, Glu$^{329}$ and Ser$^{877}$ have been pinpointed as gating residues (12, 18, 20, 21).

No high resolution structure is yet available for the Na$^+,K^+$-ATPase, but the structure of the closely related Ca$^{2+}$-ATPase has been determined at atomic resolution for several conformational states (8, 21–25). Structural comparison of these atomic models has revealed major rearrangements both in the cytoplasmatic and the membranous parts of the molecule. In connection with the formation of the Ca$^{2+}$-occluded intermediate, the helical structure of M1 bends (at Asp$^{199}$), and the M1–M2 hairpin is shifted in the direction perpendicular to the membrane toward the cytoplasmatic side. Glu$^{809}$ in transmembrane segment M4 (the residue homologous to Glu$^{329}$ in the Na$^+,K^+$-ATPase) seems to work as a cytoplasmatic gate of the Ca$^{2+}$ binding pocket by changing its side chain conformation (18, 26). It has been proposed that the structural changes in M1 close the cytoplasmatic entry pathway, thereby resulting in occlusion of the bound Ca$^{2+}$ ions (21, 24). This proposal is in agreement with functional studies demonstrating that mutation of Asp$^{59}$,

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$^2$The abbreviations used are: Na$^+,K^+$-ATPase, Na$^+$-, and K$^+$-transporting adenosine triphosphatase; M1–M10, transmembrane segments numbered from the N terminus; MES, 4-morpholineethanesulfonic acid.

$^3$All numbering of Na$^+,K^+$-ATPase residues in this article refers to the sequence of the rat α1-isoform.
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located at the bending point, and mutation of Leu\textsuperscript{65}, suggested to lock the conformation of Glu\textsuperscript{399}, as well as mutations of other hydrophobic residues in M1 interfere with Ca\textsuperscript{2+} occlusion (27). Based on the crystal structure of the Ca\textsuperscript{2+}-ATPase in the Ca\textsubscript{2+}E\textsubscript{1} form, it was also proposed that Asp\textsuperscript{395} and three other negatively charged residues in the N-terminal part of M1 and its cytoplasmic extension provide a cytoplasmic entry port to the Ca\textsuperscript{2+} sites (28). The amino acid sequence homology between the Na\textsuperscript{+},K\textsuperscript{+}-ATPase and the Ca\textsuperscript{2+}-ATPase is rather low in the M1 region (Fig. 1), and it is therefore unclear whether M1 has similar functions in the two ATPases. The presence of two positively charged (Lys\textsuperscript{86} and Arg\textsuperscript{89}) and one negatively charged (Glu\textsuperscript{83}) side chains in the N-terminal part of M1 of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase and its cytoplasmic extension versus four carboxylate groups in the same region of the Ca\textsuperscript{2+}-ATPase (see Fig. 1) is reminiscent of voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels, where the ion selectivity filter is formed by two negatively charged side chains and one positive side chain in the Na\textsuperscript{+} channel and by four negatively charged side chains in the Ca\textsuperscript{2+} channel (29, 30). Lys\textsuperscript{86} is fully conserved throughout the Na\textsuperscript{+},K\textsuperscript{+}-ATPase family, as is the positive charge at position 89 (arginine or lysine); hence it might be hypothesized that M1 and its cytosolic extension contribute to a cation selectivity filter. In a recent study of the two glycines, Gly\textsuperscript{393} and Gly\textsuperscript{394}, in the M1 segment of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase (31), we found that Gly\textsuperscript{394} is critical for proper Na\textsuperscript{+} and K\textsuperscript{+} interaction, particularly in the \( E_{1P} \) form. So far, this is the only study characterizing the importance of M1 in the Na\textsuperscript{+},K\textsuperscript{+}-ATPase for cation interaction.

In this study of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, we have introduced 26 point mutations in M1 and its cytoplasmic extension and analyzed the consequences for the overall and partial reactions. Two possible alignments of the sequences of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase and the Ca\textsuperscript{2+}-ATPase in this region are shown in Fig. 1 together with the amino acid replacements analyzed in the present and the previous study (31). The residues selected for mutagenesis were initially examined by alanine scanning except in the case of the tryptophan (Trp\textsuperscript{100}), which was replaced by leucine to avoid a too drastic reduction in size. Because one aim of this study was to examine the roles of the charged and polar residues in the N-terminal part with a view to the possibility that they might contribute to cation selectivity, Lys\textsuperscript{86} and Arg\textsuperscript{89} were replaced not only by alanine but also by glutamate, which according to the alignment in Fig. 1A is the corresponding residue in the Ca\textsuperscript{2+}-ATPase. Moreover, the glutamine Glu\textsuperscript{329} (highly conserved according to the alignment by Sweadner and Donnet (7) shown in Fig. 1B) was replaced by leucine and arginine in addition to alanine, the rationale behind the choice of arginine being that its bulkiness and positive charge would interfere with cation migration to the binding site, if the glutamine were located along the cation pathway. Leu\textsuperscript{91} was likewise replaced not only by alanine but also by proline in order to test the importance of the helical structure of the N-terminal part of M1, which probably is distorted by insertion of the “helix breaker” proline. Because alanine replacement of Phe\textsuperscript{95} led to a nonfunctional enzyme, the importance of the phenylalanine was studied further by substitution with tyrosine, arginine, and leucine, whose side chains differ in polarity, charge, and aromaticity but have bulkiness similar to that of the phenylalanine. According to the alignment shown in Fig. 1B, the leucine found important for Ca\textsuperscript{2+} occlusion in the Ca\textsuperscript{2+}-ATPase (Leu\textsuperscript{99}) is conserved in the Na\textsuperscript{+},K\textsuperscript{+}-ATPase (Leu\textsuperscript{99}), and because the first series of mutations in this study showed that the alanine replacement of Leu\textsuperscript{99} affects K\textsuperscript{+} binding markedly, this interesting residue was further studied by replacement with isoleucine, phenylalanine, and glutamine. We demonstrate that the side chains of specific residues in the middle part of M1, particularly Leu\textsuperscript{99}, are critical for K\textsuperscript{+} interaction of the enzyme. Our findings are consistent with a gate-locking function of Leu\textsuperscript{99} in the K\textsuperscript{+}-occluded [K\textsubscript{2}]E\textsubscript{2} state, restricting the conformational flexibility of the side chain of the gating residue Glu\textsuperscript{329} in M4, and the data furthermore indicate a crucial role for Leu\textsuperscript{99} in K\textsuperscript{+} binding to the \( E_{1P} \) form.

**Experimental Procedures**

**Site-directed Mutagenesis and Expression**—Mutations were introduced directly into the full-length cDNA of the ouabain resistant rat \( \alpha_{1} \)-isoform of Na\textsuperscript{+},K\textsuperscript{+}-ATPase (32) contained in the expression vector pHMT2 (33) by use of the QuickChange site-directed mutagenesis kit (Stratagene). For expression, the resulting recombinant plasmids were transfected into mammalian COS-1 cells, and stable clones were selected with ouabain as described previously (5, 11, 32). The crude plasma membrane fraction containing expressed wild-type or mutant Na\textsuperscript{+},K\textsuperscript{+}-ATPase was isolated by differential centrifugation (32), and the protein concentration was determined by the dye binding method of Bradford (34). Prior to functional analysis, the plasma membranes were made leaky with alamethicin or deoxycholate.

The presence of the desired mutations in the cDNA was verified by sequencing the CsCl gradient purified cDNA before transfection into the COS-1 cells, and in some cases the DNA stably integrated into the genome of the isolated ouabain-resistant COS-1 cell lines was also sequenced. For the latter purpose, genomic DNA was isolated using the GenElute mammalian genomic DNA miniprep kit (Sigma). Information about the exon-intron boundaries allowed design of primers that selectively anneal to the exon-exon boundaries of the exogenous cDNA stably integrated into the genome and not to the endogenous COS-1 cell Na\textsuperscript{+},K\textsuperscript{+}-ATPase genomic DNA. Using these
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primers, the entire coding region of the exogenous mutant cDNA was amplified by PCR and sequenced.

Functional Analysis—Measurements of ATPase activity were performed as described previously by following the liberation of Pi (11, 32, 35). The catalytic turnover rate was calculated as the ratio between the Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity and the active site concentration (the maximum capacity for phosphorylation measured at 0 °C in the presence of 150 mM NaCl and oligomycin) (36). For steady-state and transient kinetic measurements of partial reactions, a manual mixing technique or a quench-flow module (QFM-5 or SFM-400/Q, Bio-Logic Science Instruments, Claix, France) was used, according to previously described principles and as detailed in the legends to the figures. The Na\textsuperscript{+} dependence of phosphoenzyme formation and the time course of ADP/ATP-induced dephosphorylation (36), the time course of K\textsuperscript{+} deocclusion (18, 37), the rapid kinetics of phosphorylation of \( E_i \) (37, 38), and the rapid kinetics of \( E_i \text{P} \) dephosphorylation (31, 38) were analyzed as described. In all phosphorylation experiments, the reaction was terminated by acid quenching, and the acid-precipitated enzyme was analyzed by SDS-PAGE under acidic conditions (36). The radioactivity associated with the isolated Na\textsuperscript{+},K\textsuperscript{+}-ATPase band on the gel was quantified using a Packard Cyclone\textsuperscript{TM} Storage Phosphor System.

Data Analysis—All data represented in the figures and tables are average values of at least two (usually more) independent determinations performed on different enzyme preparations. The data were analyzed by nonlinear regression using the SigmaPlot program (SPSS Inc.) (37). To analyze the ouabain concentration dependence of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, a function represented by the sum of two hyperbolic components, a high affinity component corresponding to endogenous COS-1 cell Na\textsuperscript{+},K\textsuperscript{+}-ATPase and a low affinity component corresponding to recombinant exogenous rat enzyme, was fitted to the data (39). The other ligand concentration dependences were analyzed by applying the Hill equation. A biexponential function was fitted to the time courses of ADP/ATP-induced dephosphorylation, whereas a monoexponential function was fitted to the time courses of \( E_i \) phosphorylation and K\textsuperscript{+}-induced dephosphorylation. The time course of K\textsuperscript{+} deocclusion was analyzed using the biphasic time function described previously (18, 37), in which the component corresponding to the rapid phase is at maximum from the beginning. The best fits are shown as lines in the figures, and the extracted parameters with the standard errors calculated by the SigmaPlot program are given in the tables or in the text. In the tables, the largest deviations from wild type are highlighted in boldface.

RESULTS

Expression, Ouabain Sensitivity, and Catalytic Turnover Rate—The 26 point mutations investigated in this study are shown in Fig. 1 together with the two previously investigated glycine mutations (31). The mutations were introduced into the cDNA encoding the ouabain-resistant rat \( \alpha_\text{9} \)-isoform of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase. For expression of recombinant wild-type and mutant enzyme in COS-1 cells, the ouabain selection methodology was used. This expression strategy takes advantage of the difference in ouabain sensitivity between the endogenous COS-1 cell Na\textsuperscript{+},K\textsuperscript{+}-ATPase (\( K_{0.5} \sim 0.3 \) μM) and the exogenous rat Na\textsuperscript{+},K\textsuperscript{+}-ATPase (\( K_{0.5} > 100 \) μM), permitting isolation of stable cell lines expressing exogenous rat Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the presence of 5 μM ouabain because of the preferential inhibition of the endogenous Na\textsuperscript{+},K\textsuperscript{+}-ATPase (5, 11). Of the 26 mutants constructed, all except F95A, F95R, and L99A were able to confer ouabain resistance to the COS-1 cells grown in the presence of 5 mM K\textsuperscript{+}, 155 mM Na\textsuperscript{+}, and 5 μM ouabain, indicating that the mutant enzymes were able to transport Na\textsuperscript{+} and K\textsuperscript{+} at a rate compatible with cell viability. The inability of F95A, F95R, and L99A to confer ouabain resistance indicated that the ouabain concentration in the growth medium to 10 mM and lowering the ouabain concentration to 1 μM, indicating that the affinity for extracellular K\textsuperscript{+} was reduced and the affinity for ouabain increased in this mutant. The functional characteristics of the mutants isolated from the viable cells were examined as described below (for overview, see tables).

To study the effects of the mutations on the ouabain sensitivity, the ouabain concentration dependence of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity was determined, and the \( K_{0.5} \) value for ouabain inhibition of the exogenous expressed rat enzyme is shown in Table 1. Relative to the wild-type enzyme, the apparent affinity for ouabain was increased 2–6-fold (\( K_{0.5} \) reduced) in L91P, F95L, L99A, and L99I, whereas a significant reduction of the ouabain affinity (\( K_{0.5} \) increased) was observed for F87Q and W100L (2–3-fold, cf. Table 1). The \( K_{0.5} \) value for ouabain inhibition displayed by the remaining mutants differed less than 2-fold from that of the wild type. To eliminate any contribution from endogenous Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity and phosphorylation described below, ouabain was included in the reaction medium (1 μM for L91P, F95L, L99A, and L99I, and 10 μM for the remaining mutants).

The catalytic turnover rate (molecular ATP hydrolysis activity) was calculated as the ratio between the specific Na\textsuperscript{+},K\textsuperscript{+}-ATPase...
ATPase activity, determined at 37 °C in the presence of 130 mM Na\(^+\), 20 mM K\(^+\), and 3 mM MgATP, and the active-site concentration determined as the maximum capacity for phosphorylation (Table 1). Mutant L91P displayed the most pronounced reduction of the catalytic turnover rate (to 21% of wild type). Furthermore, the catalytic turnover rate was reduced more than 2-fold in L99A (39%) and L99F (37%) (Table 1).

**Table 1**

Analysis of the overall ATPase reaction

| Mutant | Catalytic turnover rate | $K_{0.5}$ (Na\(^+\)) | $K_{0.5}$ (ATP) | $K_{0.5}$ (Na\(^+\),K\(^+\)) | $K_{0.5}$ (VO\(^{3-}\)) |
|--------|------------------------|------------------------|-----------------|--------------------------|------------------------|
| Wild type | 1.00                   | 1.00                   | 1.00            | 1.00                      | 1.00                   |
| E83A   | 0.77 ± 0.07            | 0.98 ± 0.02            | 0.77 ± 0.08     | 0.94 ± 0.04              | 1.19 ± 0.06            |
| K86E   | 1.51 ± 0.13            | 0.94 ± 0.05            | 0.97 ± 0.03     | 0.84 ± 0.03              | 1.37 ± 0.07            |
| K89E   | 1.24 ± 0.08            | 1.05 ± 0.03            | 0.97 ± 0.03     | 0.87 ± 0.02              | 0.97 ± 0.03            |
| L97A   | 1.40 ± 0.10            | 0.93 ± 0.02            | 0.64 ± 0.03     | 0.93 ± 0.04              | 1.54 ± 0.08            |
| R98L   | 3.23 ± 0.19            | 0.56 ± 0.02            | 0.62 ± 0.05     | 0.55 ± 0.05              | 1.36 ± 0.07            |
| L98A   | 1.25 ± 0.11            | 1.28 ± 0.05            | 0.95 ± 0.11     | 0.86 ± 0.03              | 1.08 ± 0.07            |
| R99E   | 0.89 ± 0.09            | 1.13 ± 0.01            | 0.72 ± 0.04     | 1.04 ± 0.04              | 1.23 ± 0.07            |
| Q90E   | 1.30 ± 0.10            | 0.96 ± 0.01            | 0.49 ± 0.02     | 0.95 ± 0.03              | 2.31 ± 0.11            |
| Q90L   | 0.96 ± 0.11            | 0.93 ± 0.03            | 0.84 ± 0.09     | 1.09 ± 0.04              | 1.08 ± 0.04            |
| Q90R   | 0.45 ± 0.13            | 0.21 ± 0.02            | 0.46 ± 0.03     | 0.21 ± 0.20              | 1.47 ± 0.14            |
| L91A   | 0.65 ± 0.04            | 0.87 ± 0.03            | 0.28 ± 0.05     | 2.57 ± 0.05              | 5.13 ± 0.39            |
| F95L   | 0.38 ± 0.02            | 0.86 ± 0.03            | 0.32 ± 0.02     | 1.85 ± 0.04              | 4.61 ± 0.31            |
| F95Y   | 0.76 ± 0.08            | 1.02 ± 0.05            | 0.12 ± 0.01     | 6.10 ± 0.27              | 27 ± 0.03              |

* Data are from the ouabain dependence of the Na\(^+\),K\(^+\) -ATPase activity at 37 °C. The medium contained 30 mM histidine buffer (pH 7.4), 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM MgCl\(_2\), 1 mM EGTA, and various concentrations of ouabain. A function with the ouabain-inhibited enzyme represented by the sum of two hyperbolic components (Fig. 4).

**Dependence and the Rate of Phosphorylation from Na\(^{2+}\).**

The time course of phosphorylation was determined in the presence of 130 mM Na\(^+\), 20 mM K\(^+\), and 3 mM MgATP, and the active-site concentration determined as the maximum capacity for phosphorylation from Na\(^{2+}\). The $K_{0.5}$ values determined for the endogenous enzyme were 0.84 ± 0.03 μM. The $K_{0.5}$ values of the exogenous mutant enzymes are shown relative to that of the exogenous wild type determined in the same series of experiments (136 ± 5 μM).

**The catalytic turnover rate was calculated as the ratio between the Na\(^+\),K\(^+\) -ATPase activity, determined at 37 °C in the presence of 30 mM histidine buffer (pH 7.4), 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM MgCl\(_2\), 1 mM EGTA, and 10 μM ouabain (1 μM for L91P, F95L, L99A, and L99F), and the active site concentration (maximum capacity for phosphorylation measured at 0 °C in the presence of 150 mM NaCl and oligomycin) (36). The turnover rates are shown relative to that of the wild type (474 ± 17 μM min\(^{-1}\)).**

**Data obtained as for Fig. 4.** $K_{0.5}$ values obtained by fitting a Hill function are shown relative to that of the wild type determined in the same series of experiments (419 ± 15 μM). Because mutants L99A and L99F were inhibited by high ATP concentrations, a biphasic function with an activating phase and an inhibition phase represented by the sum of two Hill components was used for fitting the data in these cases, and the $K_{0.5}$ value shown in the table corresponds to the activating phase.

**Data obtained as for Fig. 4.** $K_{0.5}$ values shown relative to that of the wild type determined in the same series of experiments (2.69 ± 0.08 μM).

**Data are from the ouabain dependence of the Na\(^+\),K\(^+\) -ATPase activity at 37 °C.** The medium contained 30 mM histidine buffer (pH 7.4), 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM MgCl\(_2\), 1 mM EGTA, 10 μM ouabain (1 μM for L91P, F95L, L99A, and L99F), and various concentrations of vanadate. The equation $V = V_{max}\left(1 - [VO_3^{3-}]/(K_{0.5}^{3-} + [VO_3^{3-}])\right)$ was fitted to the data. The $K_{0.5}$ values shown in Table 2 relative to that of the wild type (WT) determined in the same series of experiments (538 ± 14 μM).

**Phosphate** by leucine also led to a significant reduction of the apparent Na\(^+\) affinity (2-fold) relative to wild type (Fig. 2).

The time course of phosphorylation was determined in the presence of 2 μM [γ-32P]ATP and a saturating Na\(^+\) concentration of 100 mM. For this purpose, a quench-flow technique (37, 38) allowing measurements in the millisecond range was used.

**FIGURE 2.** Na\(^+\) dependence of phosphorylation from [γ-32P]ATP. Phosphorylation was performed at 0 °C for 15 s in 20 mM Tris (pH 7.5), 3 mM MgCl\(_2\), 2 μM [γ-32P]ATP, 10 μM ouabain (1 μM for L91P, F95L, L99A, and L99F), 20 μg/ml oligomycin, the indicated concentrations of NaCl, and various concentrations of Na\(^+\),K\(^+\) -ATPase, determined in the same series of experiments (538 ± 14 μM).
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## Table 2
Analysis of partial reactions

| K$_{0.5}$(Na$^+$) for phosphorylation$^a$ | Phosphorylation rate$^a$ | [K$_{0.5}$]E$_2^+$ | K$^+$/Rb$^+$ deocclusion rate$^b$ | EP/(EP + oligomycin)$^c$ | t$_E$P $\rightarrow$ E$_2$P$^*$ |
|--------------------------------------|------------------------|------------------|-------------------------------|-----------------------|-----------------------------|
| Wild type                           | 1.00                   | 0.81 ± 0.07      | 0.03 ± 0.04                   | 0.03 ± 0.04           | 0.03 ± 0.04                 |
| E83A                                | 1.59 ± 0.06            | 0.93 ± 0.07      | 0.35 ± 0.04                   | 0.36 ± 0.04           | 0.36 ± 0.04                 |
| K86E                                | 1.76 ± 0.05            | 0.91 ± 0.07      | 0.42 ± 0.04                   | 0.43 ± 0.04           | 0.43 ± 0.04                 |
| F87A                                | 1.48 ± 0.05            | 0.90 ± 0.07      | 0.38 ± 0.04                   | 0.39 ± 0.04           | 0.39 ± 0.04                 |
| F92A                                | 1.76 ± 0.05            | 0.91 ± 0.07      | 0.42 ± 0.04                   | 0.43 ± 0.04           | 0.43 ± 0.04                 |
| L99A                                | 1.04 ± 0.05            | 0.91 ± 0.07      | 0.42 ± 0.04                   | 0.43 ± 0.04           | 0.43 ± 0.04                 |
| L99Q                                | 1.21 ± 0.05            | 0.91 ± 0.07      | 0.42 ± 0.04                   | 0.43 ± 0.04           | 0.43 ± 0.04                 |

$^a$ Data were obtained as for Fig. 2. K$_{0.5}$ values are shown relative to that of the wild type determined in the same series of experiments (358 ± 14 μM).

$^b$ Data were obtained as for supplemental Fig. S1. Rapid kinetic measurements of E$_2$ phosphorylation from [γ-32P]ATP carried out at 25 °C using the Bio-Logic quench-flow module SFM-400/QK and a previously described mixing protocol (37, 38). Enzyme preincubated in 40 mM Tris (pH 7.5, 100 mM NaCl, 3 mM MgCl$_2$, 1 mM EGTA, 10 μM ouabain (1 μM for L91P, F95L, L99A, and L99I), and 20 μM/L oleoglycerin, was mixed with an equal volume of the same buffer containing 4 μM [γ-32P]ATP, followed by acid quenching at the indicated time intervals. A monophasic function was fitted to the data. Phosphorylation rates are shown relative to that of the wild type determined in the same series of experiments (26 ± 1 μM) (1). Relative to the deocclusion rates of mutants L99A and L99F, which correspond to the conditions in Fig. S4C. The deocclusion rates are shown relative to that of the wild type determined in the same series of experiments (0.01 ± 0.01 s$^{-1}$ corresponding to Fig. S4A and 0.0005 ± 0.0005 s$^{-1}$ corresponding to Fig. S5C) (2).

$^c$ Ratio between phosphorylation levels without and with oligomycin (20 μM/L). Phosphorylation was carried out at 15 s at 0°C in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 3 mM MgCl$_2$, 1 mM EGTA, 10 μM ouabain (1 μM for L91P, F95L, L99A, and L99I), and 2 μM [γ-32P]ATP.

$^d$ Data were obtained as for Fig. 6B. t$_E$ values are shown relative to that of the wild type determined in the same series of experiments (5.6 ± 0.23 s).

$^e$ Data are from Refs. 5 and 18.

Relative to wild type, the rate constant of phosphorylation was reduced to 41% in L91P (Table 2 and supplemental Fig. S1). In the other mutants characterized, the phosphorylation rate deviated less than 2-fold from that of the wild type (Table 2), as exemplified by mutants L99Q and F95L in supplemental Fig. S1. The effect of the L91P mutation on the phosphorylation rate is likely a consequence of the defective Na$^+$ interaction, interfering with the Na$^+$-induced propagation of conformational changes to the catalytic site.

### ATP Dependence of Na$^+$,K$^+$-ATPase Activity

Fig. 3 shows the ATP concentration dependence of the Na$^+$,K$^+$-ATPase activity for selected mutants, and the K$_{0.5}$(ATP) values are indicated in Table 1 for all the mutants. Several exhibited an increased affinity for ATP relative to wild type (reduced K$_{0.5}$ value). The most substantial effect, corresponding to a 5–7-fold decrease in the K$_{0.5}$ for ATP, was observed for the enzymes carrying alanaline and phenylalanine substitutions of Leu$^{99}$. Moreover, the mutants Q90A, Q90R, L91A, L91P, F95L, F95Y, L98A, and L99I showed a significant increase in the apparent ATP affinity. Because ATP binds with low affinity to the K$^+$-occluded [K$_{0.5}$]E$_2$ form and accelerates the [K$_{0.5}$]E$_2$ $\rightarrow$ E$_1$ step (Scheme 1, reaction 6), whereas it binds with high affinity to E$_1$, the apparent affinity for ATP is expected to be increased in mutants where the mutation has caused an acceleration of [K$_{0.5}$]E$_2$ $\rightarrow$ E$_1$ (1, 2, 18, 42). Hence, the increased apparent affinity for ATP may be secondary to such an acceleration of [K$_{0.5}$]E$_2$ $\rightarrow$ E$_1$. Alternatively, the intrinsic affinity of the [K$_{0.5}$]E$_2$ form for ATP might be increased in these mutants. As seen in Fig. 3, the L99A and L99F mutants furthermore exhibited an unusual inhibition at high ATP concentrations, most pronounced for the L99F mutant. To our knowledge, such an inhibition has not been reported previously in the literature for any Na$^+$,K$^+$-
ATPase mutant. Because, as described below, the $K^+$ binding properties of the L99A and L99F mutants resemble those of the previously characterized E329Q mutant with alteration to the cation-binding glutamate in M4 (5, 10, 13, 17, 18), the ATP dependence of this mutant was reexamined in this study for comparison (Fig. 3, right panel). It is seen that E329Q displayed an increased apparent affinity for ATP, and furthermore the data reveal a tendency for inhibition at high ATP concentrations, which is reminiscent of the inhibition seen with L99A and L99F.

$K^+$ Dependence of Na$^+$,K$^+$-ATPase Activity—The Na$^+$,K$^+$-ATPase activity was measured at various $K^+$ concentrations in the presence of 3 mM ATP. Fig. 4 shows the results for some of the mutants, and Table 1 summarizes the extracted $K_{0.5}(K^+)$ values for all mutants. F87Q and W100L exhibited an ~2-fold increased apparent affinity for $K^+$ relative to wild type ($K_{0.5}$ reduced). By contrast, the apparent $K^+$ affinity for activation was reduced by 1.8–3-fold (K$_{0.5}$ increased) in L91P, F95L, F95Y, L98A, L99A, and L99I (Fig. 4). The other mutants were wild type-like or showed less significant reduction of the apparent $K^+$ affinity (Table 1). The apparent $K^+$ affinity of mutant L99Q was similar to that of the wild type, but for this mutant the ordinate intercept was well above zero (17%), indicating that L99Q hydrolyzes ATP in the mere presence of Na$^+$ without $K^+$. This so-called “Na$^+$-ATPase activity” was analyzed in further detail below (see Fig. 8A).

Because of the well known $K^+$-ouabain antagonism (43), the defects in $K^+$ binding observed here for several mutants might be expected to promote ouabain binding. It is therefore interesting to note the inverse correlation between the $K_{0.5}$ value of ouabain inhibition and that of $K^+$ activation of ATPase activity for several mutants in Table 1. In line with this trend, the decrease in $K_{0.5}(K^+)$ seen for mutants F87Q and W100L is matched by an increase in $K_{0.5}$ for ouabain inhibition (Table 1).

$K^+$ and Rb$^+$ Occlusion—To study the occlusion of $K^+$ and its congener Rb$^+$, and the rate of deocclusion (Scheme 1, reaction 6), the wild-type or mutant enzyme was pre-equilibrated with $K^+$ or Rb$^+$ in the absence of Na$^+$ and ATP. The time course of phosphorylation from ATP was determined following a 10-fold dilution of the pre-equilibrated enzyme into a medium containing Na$^+$ and [γ-32P]ATP (18). In this assay, phosphorylation of the $K^+$- or Rb$^+$-occluded form proceeds through the reaction sequence $[K_2/Rb_2]E_2 \rightarrow E_1 \rightarrow Na_2E_1 \rightarrow Na_3E_2P$, where the deocclusion of $K^+/Rb^+$ is rate-limiting. For the wild type, this results in a biphasic time course with a fast and a slow component; the fast component reflecting enzyme present in the non-occluded $E_1$ form, which reacts almost instantaneously with Na$^+$ and ATP, and the slow component reflecting the release of $K^+/Rb^+$ from $[K_2/Rb_2]E_2$. The fraction of the enzyme present as $[K_2/Rb_2]E_2$ at equilibrium, determined by extrapolating the slow phase back to the ordinate intercept and subtracting this value from 100%, depends on the $K^+$ or Rb$^+$ concentration and the equilibrium constant for the interconversion between $[K_2/Rb_2]E_2$ and $E_1$. The data obtained following equilibration with 1 mM $K^+$ are shown in Fig. 5A for selected mutants, and the extracted parameters are listed in Table 2 for these and most of the remaining mutants. In the wild type, the equilibrium level of the $K^+$-occluded $[K_2]E_2$ form amounted to 95% of the enzyme (Fig. 5A and Table 2). It was reduced to 50–60% in F95L and F95Y and to 70–80% in Q90A, L91A/L91P, and L98A. The rate constant reflecting the deocclusion of $K^+$ was increased 2–3-fold in the mutants with alteration to Leu$^{91}$, Phe$^{95}$, Ser$^{96}$, and Leu$^{98}$ relative to wild type. A most conspicuous effect was seen for mutant L99F, the equilibrium level of $[K_2]E_2$ being reduced to 25%. The other Leu$^{99}$ mutants showed variable effects, the $[K_2]E_2$ level being 44, 78, and 100% in L99A, L99I, and L99Q, respectively. All the Leu$^{99}$ mutants, including L99Q, exhibited an increased rate of $K^+$ deocclusion relative to wild type. For L99I and L99Q, the rate could be accurately determined under the conditions corresponding to Fig. 5A, revealing an increase of 2- and ~4-fold, respectively, relative to wild type. The fact that the equilibrium level of $[K_2]E_2$ was slightly increased in L99Q, despite an increased rate of $K^+$ deocclusion, indicates that also the rate of the reverse occlusion reaction was enhanced in this mutant. For the wild type as well as L99A and L99F, experiments were also conducted following pre-equilibration with 8 mM $K^+$ (Fig. 5B). Under these conditions the equilibrium levels of $[K_2]E_2$ were 97, 47, and 18%, respectively, i.e. very similar to the levels determined following pre-equilibration with 1 mM $K^+$. This is in accordance with the reaction scheme $E_1 + 2K^+ \leftrightarrow K_2E_1 \leftrightarrow [K_2]E_2$, assuming that the reaction $E_1 + 2K^+ \leftrightarrow K_2E_1$ is saturated already at 1 mM $K^+$ and that the reason for the reduced equilibrium level of $[K_2]E_2$ in L99A and L99F is a left shift of $K_2E_1 \leftrightarrow [K_2]E_2$. Hence, it seems that the $K^+$-occluded $[K_2]E_2$ form is destabilized in these mutants, whereas the affinity of the $E_1$ form for $K^+$ is normal. Because the level of $[K_2]E_2$ was too low to allow an accurate determination of the rate of $K^+$ deocclusion in L99A and L99F, deocclusion experiments were also carried out following pre-equilibration in the presence of 8 mM Rb$^+$ (a congener of $K^+$ known to stabilize the occluded state more than $K^+$ (42)) and at pH 6.5 (known to stabilize the occluded state more than pH 7.5 (42)).

**FIGURE 4. $K^+$ dependence of Na$^+$,K$^+$-ATPase activity in the presence of 3 mM ATP.** Measurement of Na$^+$,K$^+$-ATPase activity was performed at 37°C in a medium containing 30 mM histidine buffer (pH 7.4), 40 mM NaCl, 3 mM ATP, 3 mM MgCl$_2$, 1 mM EGTA, 10 μM ouabain (1 μM for L91P, F95L, L99A, and L99I), and the indicated concentrations of $K^+$. The equation $V = \frac{V_{\text{max}} - V_0}{K_{\text{0.5}} + [K^+] + V_0}$ was fitted to the data. The extracted $K_{0.5}$ values are shown in Table 1 relative to that of the wild type (WT) determined in the same series of experiments ($58 \pm 17$ μM).
Importance of Leu99 in M1 of the Na\(^{+},K^{+}\)-ATPase

**Na\(^{+},K^{+}\)-ATPase Activity at Low ATP Concentration**—To further study the [K\(_2\)]\(E_2\) → \(E_1\) step in the L99F mutant, the catalytic turnover rate was determined under conditions where [K\(_2\)]\(E_2\) → \(E_1\) is the rate-limiting step for the overall ATPase reaction. This was achieved by lowering the ATP concentration from the 3 mM used for Fig. 4 to 50 \(\mu\)M (supplemental Fig. S2), because as described above high ATP concentrations in the millimolar range accelerate [K\(_2\)]\(E_2\) → \(E_1\) by binding with low affinity to [K\(_2\)]\(E_2\). In the presence of 50 \(\mu\)M ATP, the mutant displayed a maximal catalytic turnover rate 1.5-fold higher than that of the wild type, unlike the situation at 3 mM ATP where the maximal catalytic turnover rate of the mutant constituted only 37% that of the wild type. The relatively high turnover rate observed for the mutant at a low ATP concentration, where [K\(_2\)]\(E_2\) → \(E_1\) is rate-limiting for the overall reaction, is consistent with the [K\(_2\)]\(E_2\) → \(E_1\) step being faster in the L99F mutant relative to wild type. Likewise the higher \(K_{0.5}(K^+)\) value found for the mutant relative to wild type under these conditions (supplemental Fig. S2) may be accounted for by the enhancement of the rate-limiting [K\(_2\)]\(E_2\) → \(E_1\) step (44). These effects of the L99F mutation are very similar to the observations with E329Q using the same assay of ATP hydrolysis at 50 \(\mu\)M ATP (18).

**Vanadate Dependence of Na\(^{+},K^{+}\)-ATPase Activity**—Vanadate, acting as an analog of the phosphoryl group in the transition state occurring during dephosphorylation, inhibits the Na\(^{+},K^{+}\)-ATPase by binding preferentially to the [K\(_2\)]\(E_2\) form (45). The apparent affinity for vanadate inhibition was determined from the vanadate concentration dependence of the Na\(^{+},K^{+}\)-ATPase activity (Table 1). Substitution of Leu\(^{99}\) with phenylalanine caused a most dramatic reduction of the apparent vanadate affinity, corresponding to a more than 80-fold increase of \(K_{0.5}(V^+)\) relative to wild type. The \(K_{0.5}\) for vanadate inhibition was somewhat increased in several other mutants as well, as much as 5-fold in F95L, F95Y, and L99A. The latter mutants together with L99F are the ones that showed the largest decrease in the level of [K\(_2\)]\(E_2\) determined in the occlusion assay (Fig. 5A and Table 2), and it is therefore likely that the reduced affinity for vanadate inhibition of the Na\(^{+},K^{+}\)-ATPase activity reflects a reduction of the level of the vanadate-reactive [K\(_2\)]\(E_2\) form present during the cycling of the enzyme. Hence, the 80-fold reduction of vanadate affinity observed for L99F may in part be a consequence of the destabilization of [K\(_2\)]\(E_2\) giving rise to the marked acceleration of the [K\(_2\)]\(E_2\) → \(E_1\) step in this mutant. Moreover, in L99F the transition state occurring during dephosphorylation of \(E_P\) may be destabilized in a similar way as [K\(_2\)]\(E_2\), and a reduced dephosphorylation rate (see below and Table 3), producing less [K\(_2\)]\(E_2\), may contribute to reduce the steady-state level of [K\(_2\)]\(E_2\) present during enzyme cycling.

**Oligomycin Effect on Phosphoenzyme Level**—The data in Fig. 2 were obtained in the presence of oligomycin, which is known to promote Na\(^{+}\) occlusion and stabilize the phosphoenzyme by inhibiting the \(E_P\) → \(E_P\) transition (2, 46). In the wild-type enzyme, the level of phosphoenzyme formed at 0°C in the presence of saturating amounts of Na\(^{+}\) (150 mM) with no added

| Time (s) | Phosphorylation (%): L91A, L91P, WT | Phosphorylation (%): L99A, L99F, L99Q, L99I, S99A | Phosphorylation (%): L99A, L99F, L99I |
|---------|--------------------------------------|-----------------------------------------------|----------------------------------|
| 0       | 10%                                  | 10%                                           | 10%                             |
| 10      | 20%                                  | 20%                                           | 20%                             |
| 20      | 30%                                  | 30%                                           | 30%                             |
| 30      | 40%                                  | 40%                                           | 40%                             |
| 40      | 50%                                  | 50%                                           | 50%                             |
| 50      | 60%                                  | 60%                                           | 60%                             |
| 60      | 70%                                  | 70%                                           | 70%                             |
| 70      | 80%                                  | 80%                                           | 80%                             |
| 80      | 90%                                  | 90%                                           | 90%                             |
| 90      | 100%                                 | 100%                                          | 100%                            |

Under these circumstances the level of [Rb\(_2\)]\(E_2\) amounted to as much as 99, 97, and 73% in the wild type, L99A, and L99F, respectively (Fig. 5C). This allowed an accurate determination of the rate of deocclusion, which was found 5- and 39-fold enhanced in L99A and L99F, respectively relative to wild type (Table 2). Hence, for these mutants the reduced equilibrium levels of [K\(_2\)/Rb\(_2\)]\(E_2\) could be explained by an increased rate of deocclusion. It should be noted that the conspicuous 39-fold enhancement of the rate of Rb\(^{+}\) deocclusion in L99F is even larger than the 26-fold enhancement observed for the E329Q mutant with alteration to the cation-binding glutamate in M4 (Table 2).

**FIGURE 5.** Time course of deocclusion following pre-equilibration with 1 mM KCl (A), 8 mM KCl (B), or 8 mM RbCl (C). A, enzyme was preincubated for 1 h at room temperature in the presence of 20 mM Tris (pH 7.5) and 1 mM KCl, followed by addition of oligomycin (150 \(\mu\)g/ml). One minute later, the sample was cooled to 10°C and diluted 10-fold in a phosphorylation solution producing final concentrations of 20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM \(\gamma\)-ATP, 1 mM MgCl\(_2\), 1 mM EGTA, and 10 \(\mu\)M ouabain (1 \(\mu\)M for L91P, F95L, L99A, and L99I). The reaction was terminated by acid quenching at the times indicated on the abscissa. For determination of the initial amount of the K\(^{+}\)-occluded enzyme and the rate constant corresponding to deocclusion, the time dependence of phosphorylation was analyzed as a biphasic time course EP = \(EP_{\text{max}} - E_{\text{occluded}}\) + \(E_{\text{occluded}} \times (1 - e^{-kt})\), where \(E_{\text{occluded}}\) represents the part of the enzyme that phosphorylates slowly because of its initial presence as the occluded [K\(_2\)]\(E_2\) form (18). The extracted equilibrium levels of [K\(_2\)]\(E_2\) and the rate constants of K\(^{+}\) deocclusion, expressed relative to that of the wild type (WT) determined in the same series of experiments (0.016 ± 0.001 s\(^{-1}\)), are listed in Table 2. B, experiments were conducted and analyzed as described for A, except that the preincubation medium contained 8 mM KCl in place of 1 mM KCl. The extracted equilibrium levels of [K\(_2\)]\(E_2\) are given in the text. C, experiments were conducted and analyzed as described for A, except that the preincubation medium contained 8 mM RbCl in place of 1 mM KCl and 20 mM MES/Tris (pH 6.5) in place of 20 mM Tris (pH 7.5). Likewise the phosphorylation solution contained 20 mM MES/Tris (pH 6.5) in place of 20 mM Tris (pH 7.5). Phosphorylation was carried out at 0°C. The rate constants for deocclusion of [Rb\(_2\)]\(E_2\) expressed relative to that of the wild type determined in the same series of experiments (0.0005 ± 0.0002 s\(^{-1}\)), are given in Table 2.
TABLE 3
Analysis of E\textsubscript{2}P dephosphorylation

| E\textsubscript{2}P at 20 mM Na\textsuperscript{+ +} | K\textsubscript{E2P} \rightarrow [K\textsubscript{3}]E\textsubscript{2}P\textsuperscript{c} | V\textsubscript{max} |
|--------------------------------|---------------------------------|------------|
| %                          |                                |            |
| Wild type                  | 75 ± 5                         | 1.0        |
| L91P                       | 80 ± 7                         | 0.6        |
| F95L                       | 71 ± 6                         | 1.3        |
| L98A                       | 67 ± 6                         | 1.8        |
| L99A                       | 88 ± 7                         | 17.0       |
| L99I                       | 89 ± 8                         | 3.0        |
| L99F                       | 82 ± 10                        | 0.6        |
| L99Q                       | 62                             | 1.0        |
| E329Q                      | 83\textsuperscript{d}         | 9.6\textsuperscript{e} |

\textsuperscript{a} Phosphorylation was carried out at 0 °C for 15 s in the presence of 20 mM Tris (pH 7.5), 20 mM NaCl, 130 mM choline chloride, 3 mM MgCl\textsubscript{2}, 1 mM EGTA, 2 \mu M \textsuperscript{32P}\textsubscript{ATP}, and 10 \mu M ouabain (1 \mu M for L91P, F95L, L99A, and L99I). Dephosphorylation was followed by addition of a solution producing final concentrations of 2.5 mM ADP and 1 mM ATP. A biexponential function was fitted to the data, and the extent of the slow component, corresponding to the fraction of phosphoenzyme which is ADP-insensitive, is indicated as E\textsubscript{2}P. Because mutant L99Q exhibited rapid dephosphorylation even in the absence of ADP (Na\textsuperscript{+}−ATPase activity), the ADP-insensitive fraction was in this case estimated simply by relating the amount of phosphoenzyme remaining after 2 s of dephosphorylation in the presence of ADP to that measured in the absence of ADP. The result of a similar calculation performed for the wild type was 81%.

\textsuperscript{b} Data were obtained as shown for Fig. 7. K\textsubscript{0.5} and V\textsubscript{max} values are shown relative to that of the wild type determined in the same series of experiments (2.46 ± 0.77 mm and 213 ± 19 s\textsuperscript{-1} respectively).

\textsuperscript{c} Data are from Ref. 18.

\textsuperscript{d} Data are from Ref. 31.

interconversion. For L91P, this type of measurement could not be conducted, because most of the phosphoenzyme was accumulated as E\textsubscript{2}P during phosphorylation with [\gamma\textsuperscript{32P}]\textsubscript{ATP} in the presence of a high NaCl concentration, as demonstrated by the insensitivity to ADP in Fig. 6A.

**K\textsuperscript{+} Interaction with E\textsubscript{2}P−K\textsuperscript{+}** stimulates ATP hydrolysis by binding to the E\textsubscript{2}P intermediate at the extracellularly facing transport sites, where it activates the dephosphorylation of E\textsubscript{2}P (Scheme 1). The reduced apparent K\textsuperscript{+} affinities revealed by the K\textsuperscript{+} dependence of the Na\textsuperscript{+}, K\textsuperscript{+}−ATPase activity of mutants L91P, F95L, F95Y, L98A, L99A, and L99I (Table 1) may be caused by interference with the interaction of K\textsuperscript{+} with E\textsubscript{2}P, or it may be secondary to changes in the rate constants of the reactions leading to and from E\textsubscript{2}P. To directly examine the binding of K\textsuperscript{+} at the external transport sites of E\textsubscript{2}P, we measured the K\textsuperscript{+} dependence of E\textsubscript{2}P dephosphorylation (Scheme 1, reactions 4 and 5). First, it was demonstrated that E\textsubscript{2}P accumulates in the mutants as well as the wild type when phosphorylation with ATP is carried out in the presence of a relatively low Na\textsuperscript{+} concentration of 20 mM and in the absence of K\textsuperscript{+} (Table 3, 1st column). For measurement of E\textsubscript{2}P dephosphorylation, the phosphoenzyme was formed under similar ionic conditions and by use of the quench−flow module for rapid mixing (31, 38). The dephosphorylation was then followed in the presence of varying K\textsuperscript{+} concentrations (0, 1, 5, 10, 20, or 65 mM), as shown represented for 1 mM K\textsuperscript{+} in Fig. 7A. Fig. 7B shows the rate constant of E\textsubscript{2}P dephosphorylation determined in this way as a function of the K\textsuperscript{+} concentration, allowing extraction of the K\textsubscript{0.5} values for K\textsuperscript{+} activation of E\textsubscript{2}P dephosphorylation as well as the V\textsubscript{max} values for dephosphorylation (Table 3). The K\textsubscript{0.5} value of 2.5 mM determined for the wild type in this way is in good agreement with the value of 1.9−4.5 mM reported in the literature for K\textsuperscript{+} binding at the extracellularly facing transport sites in sided systems (48, 49). L91P and L99F showed a significantly reduced V\textsubscript{max} of E\textsubscript{2}P dephosphorylation (to ~40% that of the wild type) and a slight increase of the apparent K\textsuperscript{+} affinity (K\textsubscript{0.5} reduced). In contrast, L98A and L99I showed 1.8− and
3.0-fold increase of $K_{0.5}$ for $K^+$ activation of dephosphorylation, respectively, the $V_{\text{max}}$ values being similar to that of the wild type or only slightly reduced. A most substantial reduction in the apparent $K^+$ affinity, corresponding to 17-fold increase of the $K_{0.5}$ value, was found for L99A (Fig. 7B), indicating that the function of the extracellularly facing transport sites of $E_P$ is severely perturbed in this mutant. The apparent $K^+$ affinity of L99Q was wild type-like. For comparison, the corresponding analysis of mutant E392Q with alteration to the cation-binding residue in M4 revealed a 10-fold reduction in the apparent affinity for $K^+$ activation (increase of the $K_{0.5}$ value), see Table 3.

$Na^+$-ATPase Activity—The ability of mutant L99Q to catalyze ATP hydrolysis in the absence of $K^+$, seen from the non-zero ordinate intercept in Fig. 4, prompted a more detailed investigation of this $Na^+$-ATPase activity. As evident from Fig. 8A, the maximum level of $Na^+$-ATPase activity reached in L99Q in the mere presence of $Na^+$ without $K^+$ was 3-fold higher than in the wild type (1672 versus 504 min$^{-1}$). The maximal $Na^+$-ATPase activity constituted ~6% of the maximal $Na^+,K^+$-ATPase activity in the wild type, but it amounted to ~20% in the mutant. Such a high $Na^+$-ATPase activity may reflect an unusual ability of $Na^+$ to replace $K^+$ in activation of $E_P$ dephosphorylation. As seen in Fig. 8B, examination of the dephosphorylation of $E_P$ in the presence of 200 mM $Na^+$ without $K^+$ showed that the rate constant of $Na^+$-activated $E_P$ dephosphorylation was 3-fold enhanced in L99Q relative to that of the wild type, whereas the other $Leu^{99}$ mutants showed a dephosphorylation rate somewhat lower than that of the wild type under these conditions. The increased rate of $Na^+$-activated dephosphorylation in L99Q explains the ability of L99Q to hydrolyze ATP in the mere presence of $Na^+$ and may also account for the low steady-state phosphorylation level observed for this mutant in the absence of oligomycin (cf. Table 2, ratio of phosphorylation levels without and with oligomycin 43%).

Inhibition by High ATP Concentration—To examine further the mechanism underlying the unusual inhibition of the $Na^+,K^+$-ATPase activity of mutant L99F at high ATP concentrations seen in Fig. 3, we studied the effect of increasing the ATP concentration from 1 to 3 mM on the $E_P$ dephosphoryl-
tion measured in the presence of 20 mM K+. As seen in Fig. 8C, this increase of the ATP concentration did not affect the dephosphorylation rate significantly in the wild type, whereas in the L99F mutant the dephosphorylation rate was reduced ~2-fold, thus explaining the inhibition of Na+,K+-ATPase activity seen for this mutant in Fig. 3.

### DISCUSSION

The sequence homology between the Na+,K+-ATPase and the Ca2+-ATPase is rather low in the M1 region (Fig. 1), and it might be speculated that the sequence differences, encompassing polar and charged residues in addition to some of the hydrophobic residues, contribute to determine the specific ion selectivity. However, none of the mutations altering the charged and polar residues Glu85, Lys86, Arg89, and Glu90 in the N-terminal part of M1 and its cytosolic extension had any major effect on the interaction of the enzyme with Na+. The most serious disturbances of K+ interaction were observed for mutants with alteration to Leu99. The cells were unable to survive.

The most serious disturbances of K+ interaction were observed for mutants with alteration to Leu99. The cells expressing L99A could only survive when the external K+ concentration was increased from 5 to 10 mM, indicating that the affinity for extracellular K+ was reduced in this mutant. This is supported by the finding of an increased $K_{0.5}^+$ for K+ activation of ATPase activity (Table 1). Direct evidence for a reduced K+ affinity of E2P was obtained in rapid kinetic experiments of dephosphorylation, in which a 17-fold increase of $K_{0.5}^+$, relative to wild type, was measured for L99A (Fig. 7 and Table 3). Hence, the K+ affinity was even lower than seen for the previously characterized E329Q mutant with alteration to the cation-binding glutamate in M4 (Table 3). Evidence was also obtained that the L99A mutation induces a considerable destabilization of the occluded [K2/Rb2]E2 form, the deocclusion rate being 5-fold enhanced, relative to wild type, and the equilibrium concentration of [K2]E2 being less than half that of the wild type (Fig. 5 and Table 2). When instead of alanine the larger isoleucine was used to replace Leu99, the effects were less dramatic, with a 3-fold decrease in the affinity of E2P for K+ and a 2-fold increase in the rate of K+ deocclusion. By contrast, substitution with the very bulky phenylalanine led to a slight increase of the affinity of E2P for K+ relative to wild type (Table 3), but at the same time the phenylalanine induced a remarkable 39-fold increase of the rate of Rb+ deocclusion from [Rb2]E2 with a corresponding >80-fold reduction in the sensitivity to inhibition by the transition state analog vanadate that binds to [K2]E2 (Tables 1 and 2), indicating that in L99F the occluded [K2/Rb2]E2 form and possibly the transition state occurring during dephosphorylation (K2E2P → [K2]E2) are even more destabilized than in L99A or E329Q, although the ability of E2P to bind K+ with high affinity is retained in L99F. The marked reduction of the $V_{max}$ of dephosphorylation (see Table 3) displayed by L99F makes it likely that the transition state of this reaction is destabilized, which may reflect difficulties in the formation of [K2]E2. The inhibition of K2E2P → [K2]E2 at a high ATP concentration (Fig. 8C) may also be related to an abnormal transition state in this reaction.

It should be stressed that in the wild type K+ interacts differently with the phosphorylated E2P form and the E2 dephosphoenzyme. In the latter form, K+ is occluded with no access to either side of the membrane, and deocclusion occurs toward the cytoplasmic side in connection with the transition [K2]E2 → K2E1 → E1 + 2K+ (Scheme 1, reaction 6). In E2P, the ion binding pocket has access to the extracellular side, and the K+ ions bound to E2P are therefore not occluded. K+ occlusion occurs either through the physiological route when K2E2P dephosphorylates forming [K2]E2, or through the “direct route” (2) where [K2]E2 is formed from E1 through E1 + 2K+ → K2E1 ↔ [K2]E2 (Scheme 1, reaction 6). It appears from our data that the various mutations of Leu99 affect K2E2P and [K2]E2 differently, the smaller alanine being most disturbing in the case of K2E2, whereas the large phenylalanine disturbs mostly the occluded [K2]E2 form. The L99Q mutation affected K+ interaction in yet another way. Judging from the normal vanadate and ATP dependences of ATPase activity (Table 1) and the 100% accumulation of [K2]E2 at equilibrium (i.e. slightly more than in the wild type, see Table 2), the [K2]E2 form is not destabilized in L99Q, but the K+ deocclusion rate was nevertheless enhanced 4-fold, relative to wild type, indicating that also the reverse reaction, the formation of [K2]E2 through the direct route, was accelerated. This is the feature expected for a mutation interfering with the gating mechanism of the ion binding pocket in such a way that the cytoplasmic gate opens and closes more frequently than in the wild type. L99Q is unique in the sense that it apparently interferes with gating without destabilizing [K2]E2 or reducing the affinity of E2P for K+ (Tables 2 and 3).

Three of the Leu99 mutations, L99A, L99I, and L99Q, furthermore increased the rate of the E2P → E1P transition (Fig. 6B). Because E1P is associated with Na+ release on the extracellular side, the acceleration of this step may be explained by an increased rate of opening of the extracellular gate. Interestingly, L99Q had the additional effect of allowing Na+ to stimulate the dephosphorylation of E2P in place of K+ (increased Na+-ATPase activity, see Fig. 8, A and B). The enhanced rate of both of the steps E2P → E1P → E2P involved in the dephosphorylation may be responsible for the reduced phosphorylation level observed with L99Q in the absence of oligomycin (Table 2). The increased rate of Na+-induced dephosphorylation of E2P touches upon the mechanism by which the Na+,K+-ATPase normally is able to distinguish between Na+ and K+ on the extracellular side. Apparently, a hydrophobic residue at the position of Leu99 prevents Na+, but not K+, from being an efficient activator of E2P dephosphorylation, whereas the glutamine promotes Na+ activation of E2P dephosphorylation (a possibility is that the side chain oxygen of the glutamine is directly involved in the binding of the Na+ ion).
**Importance of Leu**<sup>99</sup> in M1 of the Na<sup>+</sup>,K<sup>+</sup>-ATPase**

In a previous study, Leu<sup>65</sup> of the Ca<sup>2+</sup>-ATPase, which is homologous to Leu<sup>99</sup> of the Na<sup>+</sup>,K<sup>+</sup>-ATPase according to the alignment in Fig. 1B, was replaced by alanine, resulting in a change in Ca<sup>2+</sup> interaction as well as acceleration of the E<sub>P</sub> → E<sub>P</sub> transition and a block of the dephosphorylation of E<sub>P</sub> (27). The latter two effects bear a striking resemblance to some of our present findings with the Na<sup>+</sup>,K<sup>+</sup>-ATPase Leu<sup>99</sup> mutants. The block of E<sub>P</sub> dephosphorylation observed in the Ca<sup>2+</sup>-ATPase mutant L65A is analogous to the markedly reduced affinity of the extracellularly facing sites of E<sub>P</sub> for K<sup>+</sup> in the Na<sup>+</sup>,K<sup>+</sup>-ATPase mutant L99A and/or the reduced V<sub>max</sub> of dephosphorylation observed for L99F. The mechanism underlying the block of E<sub>P</sub> dephosphorylation in the Ca<sup>2+</sup>-ATPase mutant L65A was not defined previously, but on the basis of the present results it now seems reasonable to suggest that it reflects a disruption of the interaction of the E<sub>P</sub> form of the Ca<sup>2+</sup>-ATPase with the protons to be counter-transported, in the same way as the Na<sup>+</sup>,K<sup>+</sup>-ATPase interaction with K<sup>+</sup> is interfered with by mutation of Leu<sup>99</sup>. The parallel observations in this work and our previous study of the Ca<sup>2+</sup>-ATPase support the alignment proposed by Sweadner and Donnet (7) shown in Fig. 1B, where the leucine (Leu<sup>99</sup>/Leu<sup>65</sup>) is conserved, and suggest that despite a general low sequence homology between these ATPases in the M1 region, this segment plays similar important roles in cation interaction in both proteins.

In an attempt to understand the mutational effects on K<sup>+</sup> binding observed in this study, we have modeled the M1–M4 region of the Na<sup>+</sup>,K<sup>+</sup>-ATPase by replacing residues in the Ca<sup>2+</sup>-ATPase E<sub>2</sub>MgF<sub>4</sub> structure with the corresponding Na<sup>+</sup>,K<sup>+</sup>-ATPase residues (Fig. 9). The E<sub>2</sub>MgF<sub>4</sub> structure is thought to represent the E<sub>P</sub> product state (50), and although the Ca<sup>2+</sup>-ATPase binds protons instead of the K<sup>+</sup> ions bound by the Na<sup>+</sup>,K<sup>+</sup>-ATPase, the model shown in Fig. 9 may be considered a first approximation to the structure of the Na<sup>+</sup>,K<sup>+</sup>-ATPase with occluded K<sup>+</sup> ions. Importantly, the leucine corresponding to Leu<sup>99</sup> in the Na<sup>+</sup>,K<sup>+</sup>-ATPase makes van der Waals contact with the glutamate in M4 (Glu<sup>329</sup> in Na<sup>+</sup>,K<sup>+</sup>-ATPase), which binds K<sup>+</sup> and acts as a gate (18). This interaction may help to position the glutamate side chain such that the K<sup>+</sup> ions become occluded. In fact, the distance between these residues is even shorter in the E<sub>2</sub>MgF<sub>4</sub> form than it is in the E<sub>P</sub> form of the Ca<sup>2+</sup>-ATPase, where the interaction between Glu<sup>329</sup> and Leu<sup>65</sup> has been suggested to be of importance for Ca<sup>2+</sup> occlusion (21, 26). Our data lead to the hypothesis that Leu<sup>99</sup> and Glu<sup>329</sup> interact and cooperate in K<sup>+</sup> binding and gating of the K<sup>+</sup> sites of the Na<sup>+</sup>,K<sup>+</sup>-ATPase. Based on the model in Fig. 9, the conspicuous destabilization of the K<sup>+</sup>- or Rb<sup>+</sup>-occluded state caused by substitution of the leucine with a bulky phenylalanine may be explained by a steric clash with the glutamate as illustrated in Fig. 10, A and B (compare the positions taken by Glu<sup>329</sup> to avoid clash with Phe<sup>99</sup> with that taken by Glu<sup>329</sup> in Fig. 9). Because of the smaller size of the alanine, the L99A mutation would not lead to such a steric clash and hence be less destabilizing compared with the phenylalanine substitution. The interaction with the glutamate would, however, be weakened in the L99A mutant, thereby changing the position of the glutamate to some extent, which would be in accordance with our findings. The interference of the glutamine substitution with the gating process, allowing the K<sup>+</sup> ions to leave and enter the occlusion pocket more frequently, may be explained in various ways as illustrated in Fig. 10, C–F. Hence, hydrogen bond formation between a glutamate oxygen atom and the glutamine amide group may help release the K<sup>+</sup> ion from the glutamate (Fig. 10C). Electrostatic repulsion between the oxygens of the glutamate-glutamine pair may cause less strong fixation of the glutamate side chain (Fig. 10, D and E), and the same increase in flexibility of the glutamate will also result if a hydrogen bond is formed between the glutamine amide and a backbone carbonyl oxygen (Fig. 10F). Furthermore, a possibility is that the glutamine oxygen transiently binds the K<sup>+</sup> ion on its way to and from the occlusion pocket and thereby increases the rates of occlusion and deocclusion.

The finding that the K<sup>+</sup> affinity of E<sub>P</sub> is disturbed more by the alanine substitution of Leu<sup>99</sup> than by the phenylalanine substitution (Table 3), whereas the opposite is true for the stability of [K<sub>2</sub>]E<sub>2</sub> (Table 2), clearly attests that K<sub>2</sub>E<sub>2</sub>P and [K<sub>2</sub>]E<sub>2</sub> represent two different enzyme conformations. Because the cation binding pocket is open toward the extracellular side in K<sub>E</sub>P, there may be more space available around Leu<sup>99</sup> in K<sub>E</sub>P than in [K<sub>2</sub>]E<sub>2</sub>. This may prevent a steric clash between the phenylalanine and the glutamate in K<sub>E</sub>P, thus explaining the close normal K<sup>+</sup> affinity of F<sub>E</sub>P in L99F. The very low K<sup>+</sup> affinity of E<sub>P</sub> displayed by L99A, together with the less dramatic effect of L99I and the close to normal affinities of L99F and L99Q, indicates that irrespective of its polarity, a side chain of a certain size is required at the position of Leu<sup>99</sup> for proper binding of K<sup>+</sup> to E<sub>P</sub>. Again it may serve to lock the conformation of the glutamate side chain in the optimal position for interacting with the...
equilibrium away from the Na$^+$-binding $E_1$ form, as these mutations rather seem to stabilize $E_1$ relative to $E_2$, judging from the reduced level of $[K_2]E_2$ (Table 2) and the increased ATP affinity and reduced vanadate affinity (Table 1). Hence, it may be concluded that L91P and F95L interfere directly with the interaction of $E_1$ with Na$^+$ on the cytoplasmic side. In addition, the interaction of $E_3$P with Na$^+$ on the extracellular side may be severely disturbed in L91P, because the $E_3$P phosphoform predominated even in the presence of a high Na$^+$ concentration of 600 mM (Fig. 6A), indicating insensitivity of the $E_3$P-$E_3$P equilibrium to extracellular Na$^+$. This is reminiscent of our previous finding with the alanine mutation of Gly$^{94}$ in M1 (31). Moreover, L91P and F95L as well as L91A, F95Y, S96A, and L98A disturbed K$^+$ interaction significantly. Some of these mutations reduced the equilibrium concentration of $[K_2]E_2$ and increased the rate of K$^+$ deocclusion (Table 2), and L91P in addition reduced the $V_{\text{max}}$ of K$^+$-induced dephosphorylation (Table 3). S96A, like L99Q, seems to interfere with the gating of the cation binding pocket on both sides of the membrane, as indicated by the increased rate of K$^+$ deocclusion without concomitant reduction of the equilibrium concentration of $[K_2]E_2$ and the increased rate of the Na$^+$ releasing $E_1$P $\rightarrow$ $E_2$P transition.

The observed effects of mutation of Leu$^{91}$, Phe$^{95}$, Ser$^{96}$, and Leu$^{98}$ could be consequences of the roles of these residues in positioning the M1 helix optimally for the interaction between Leu$^{99}$ and Glu$^{329}$. The functional importance of Leu$^{91}$ demonstrated by the effects of the L91P and L91A substitutions may in part be understood by noting that in the model in Fig. 9 the hydrophobic side chain of Leu$^{91}$ points into the lipid phase, thereby likely anchoring the N-terminal part of M1 to the membrane. Such a role cannot be fulfilled by proline or alanine. The residue present at the corresponding position in the Ca$^{2+}$-ATPase is a phenylalanine, which like the leucine is able to interact efficiently with the lipid. Anchoring the N-terminal part of M1 may introduce constraints on the positioning of M1 when it moves, which may explain that the L91P and L91A mutations disturb K$^+$ occlusion in the $E_2$ form. The finding that the L91P mutant, but not L91A, was defective in Na$^+$ interaction as well furthermore suggests that the destabilization of the helix structure that likely occurs upon insertion of the proline is of particular importance in the $E_1$ form.

The side chain hydroxyl group of Ser$^{96}$ is located within hydrogen bonding distance to the backbone carbonyl oxygen of Gly$^{93}$ and within van der Waals interaction distance of Leu$^{99}$, and mutation of Ser$^{96}$ may therefore alter the angle between the two helical segments of M1. The model in Fig. 9 furthermore shows that the large hydrophobic side chains of Phe$^{95}$ in M1 and Ile$^{287}$ of M3 are close enough to interact through hydrophobic/van der Waals contacts. The importance of this interaction is evident from the finding that the alanine and arginine substitutions of Phe$^{95}$ were incompatible with cell growth. Apparently, only a large and hydrophobic side chain is tolerated at this position. Even the subtle changes to leucine and tyrosine had significant effects on the stability of the K$^+$-occluded [K$_2$]E$_2$ form (Table 2). Thus, it appears that the interaction between Phe$^{95}$ and Ile$^{287}$ is an essential element in the optimization of the interaction with the occluded K$^+$ ions. This may...
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be a consequence of the previously suggested role of the Ile\textsuperscript{287}–Gly\textsuperscript{94} pair as a pivot for movements of M1 (31). Hence, the bulky and hydrophobic side chain of Phe\textsuperscript{95} may serve to stabilize the pivot through the interaction with Ile\textsuperscript{287}, thereby keeping M1 in the correct position for interaction with the cation-binding M4 segment.

In conclusion, this study implicates in particular the middle region of M1 of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the mechanisms controlling the binding and occlusion of K\textsuperscript{+} in \(E_2/E_3P\) and the release of Na\textsuperscript{+} in connection with the \(E_1P \rightarrow E_2P\) transition, whereas this region seems of little importance for Na\textsuperscript{+} binding and occlusion in \(E_2P\). The results support a model in which Leu\textsuperscript{99} functions as a gate-locking residue that restricts the mobility of Gly\textsuperscript{94} pair as a pivot for movements of M1 (31). Hence, the bulky and hydrophobic side chain of Phe\textsuperscript{95} may serve to stabilize the pivot through the interaction with Ile\textsuperscript{287}, thereby keeping M1 in the correct position for interaction with the cation-binding M4 segment.

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