Mutations Phe785Leu and Thr618Met in Na\(^+\),K\(^+\)-ATPase, Associated with Familial Rapid-onset Dystonia Parkinsonism, Interfere with Na\(^+\) Interaction by Distinct Mechanisms*

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The Na\(^+\),K\(^+\)-ATPase plays key roles in brain function. Recently, missense mutations in the Na\(^+\),K\(^+\)-ATPase were found associated with familial rapid-onset dystonia parkinsonism (FRDP). Here, we have characterized the functional consequences of FRDP mutations Phe785Leu and Thr618Met. Both mutations lead to functionally altered, but active, Na\(^+\),K\(^+\)-pumps, that display reduced apparent affinity for cytoplasmic Na\(^+\), but the underlying mechanism differs between the mutants. In Phe785Leu, the interaction of the E1 form with Na\(^+\) is defective, and the E1-E2 equilibrium is not displaced. In Thr618Met, the Na\(^+\) affinity is reduced because of displacement of the conformational equilibrium in favor of the K\(^+\)-occluded E2\((K_2)\) form. In both mutants, K\(^+\) interaction at the external activating sites of the E1P phosphoenzyme is normal. The change of cellular Na\(^+\) homeostasis is likely a major factor contributing to the development of FRDP in patients carrying the Phe785Leu or Thr618Met mutation. Phe785Leu moreover interferes with Na\(^+\) interaction on the extracellular side and reduces the affinity for ouabain significantly. Analysis of two additional Phe785 mutants, Phe785Leu/Thr618Phe and Phe785Tyr, demonstrated that the aromatic function of the side chain, as well as its exact position, is critical for Na\(^+\) and ouabain binding. The effects of substituting Phe785 could be explained by structural modeling, demonstrating that Phe785 participates in a hydrophobic network between three transmembrane segments. Thr618 is located in the cytoplasmic part of the molecule near the catalytic site, and the structural modeling indicates that the Thr618Met mutation interferes with the bonding pattern in the catalytic site in the E1 form, thereby destabilizing E1 relative to E2\((K_2)\).

The Na\(^+\),K\(^+\)-ATPase catalyzes ATP-driven exchange of intracellular Na\(^+\) for extracellular K\(^+\) across the plasma membranes of animal cells with a stoichiometry of 3Na\(^+\)/2K\(^+\) (1, 2). Vital cellular functions, such as the control of cell volume and the resting membrane potential, as well as electrical signaling via action potentials in excitable tissue, depend on the Na\(^+\) and K\(^+\) concentration gradients established by the Na\(^+\),K\(^+\)-ATPase. Furthermore, the Na\(^+\) gradient provides the driving force for transport of other ions (e.g. Ca\(^2+\), H\(^+\), I\(^-\), and phosphate), nutrients, and neurotransmitters (e.g. glutamate, serotonin, and dopamine). Two different neurological disorders are now known to be associated with mutations in the Na\(^+\),K\(^+\)-ATPase: the migraine subtype familial hemiplegic migraine type 2 (FHM) (3, 4) and the movement disorder familial rapid-onset dystonia parkinsonism (FRDP) (5). FRDP is characterized by an abrupt onset of dystonia and is usually accompanied by signs of parkinsonism.

The Na\(^+\),K\(^+\)-ATPase belongs to the family of P-type ATPases, which are characterized by the transient formation of an aspartyl-phosphorylated intermediate during the functional cycle. The reaction sequence in Reaction 1,

\[
E_1 + 3Na_{cyt}^+ \rightarrow E_1N_3 \rightarrow E_1P(N_3) \rightarrow E_2P + 3Na_{ext}^+ \rightarrow E_2P + 2K_{ext}^+ \rightarrow E_2PK_2 \rightarrow E_2(K_2) \rightarrow E_1 + 2K_{cyt}^+
\]

Reaction 1

describes how the phosphorylation and dephosphorylation of the Na\(^+\),K\(^+\)-ATPase protein and transitions between the major conformational states \(E_1\) and \(E_2\) lead to ATP hydrolysis coupled with ion translocation across the membrane (1, 2). During the transport process, the ions become occluded (indicated by parentheses), i.e. bound in the protein in such a way that they do not have access to the cytoplasmic or extracellular medium.

The Na\(^+\),K\(^+\)-ATPase is a hetero-oligomer consisting of a catalytic α-subunit and a glycosylated β-subunit. The α-subunit is encoded by four different human genes corresponding to α1, α2, α3, and α4 isoforms (6, 7). The isoforms share a high amino acid sequence homology, but are differentially expressed in various cell types/tissues. The α-subunit is made up of 10 transmembrane helices, M1–M10 (8, 9), and a cytoplasmic head piece. No high resolution structure of the Na\(^+\),K\(^+\)-ATPase is yet available, but the overall structure of the α-subunit is thought to be similar to that of the closely related sarco(endo)plasmic reticulum Ca\(^2+\)-ATPase, for which the structure has been determined at atomic resolution in several conformational states (10–12). Residues with oxygen-containing side chains in the transmembrane segments M4, M5, M6, M8, and M9 have been suggested to make up the cytoplasmically facing Na\(^+\) binding sites in the \(E_1\) conformation of the Na\(^+\),K\(^+\)-ATPase, on the basis of biochemical and mutagenesis experiments in conjunction with homology modeling based on the Ca\(^2+\)-ATPase structure (13–20). Some of the Na\(^+\) binding residues seem to contribute as well to the binding of K\(^+\) from the extracellular side in the \(E_2\) conformation (17, 19). Because most of the residues with oxygen-containing side chains thought to bind the cations are conserved between the Na\(^+\),K\(^+\)-ATPase and the Ca\(^2+\)-ATPase, it is likely that other (hydrophobic?) residues contribute to determine the specific cation binding properties by adjusting the distances between the ligand groups. A
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remarkable example is provided by the cuff of aromatic residues that hold the pore open at its proper diameter for K⁺ selection in K⁺ channels (21).

In the present study of Na⁺,K⁺-ATPase, we have focused on the function of a phenylalanine, Phe⁷⁸⁵, near the extracellular end of transmembrane segment M5. Recently, this phenylalanine was found mutated to leucine in the α3-isoform of Na⁺,K⁺-ATPase in patients with familial rapid-onset dystonia parkinsonism, but the consequences of this mutation for the functional properties of the Na⁺,K⁺-ATPase were not reported (5). To understand the pathophysiological mechanism, it is essential to characterize functionally the disease-causing Na⁺,K⁺-ATPase mutants. This phenylalanine is fully conserved in all known Na⁺,K⁺-ATPases, encompassing different species and isoforms, as well as in the non-gastric H⁺,K⁺-ATPase, whereas it is conservatively replaced by tyrosine in the gastric H⁺,K⁺-ATPase and by isoleucine in the sarco(endo)plasmic reticulum Ca²⁺-ATPases. Here we have studied the functional consequences of introducing the Phe785Leu mutation in the rat Na⁺,K⁺-ATPase α1-isoform. Using a panel of functional assays, we have inquired about the effects of mutation Phe785Leu on the affinities for Na⁺ and K⁺ and the rates of the partial reaction steps in the Na⁺,K⁺-ATPase enzyme cycle. In addition to Phe785Leu, we have expressed and characterized mutant Phe785Tyr, as well as a mutant in which the two residues Phe785 and Leu⁷⁸⁶ are swapped. In this way, we have been able to examine the importance of the exact position of the aromatic side chain and its functionality. We find that the aromatic side chain of Phe⁷⁸⁵ is important for the Na⁺ binding properties, but not for K⁺ binding. Na⁺ binding to E₁ seems to be directly affected by mutation of Phe⁷⁸⁵ without change of the E₁-E₂ equilibrium. The findings with Phe785Leu led us to ask the question whether there are functional similarities between the mutants that cause the FRPD disorder, and we therefore characterized an additional FRPD mutant, Thr618Met (5).

Unlike the other residues found mutated in FRDP, Thr⁶¹⁸ is located in the cytoplasmic part of the molecule near the catalytic site and is universally conserved among Na⁺,K⁺-ATPases, H⁺,K⁺-ATPases, and Ca²⁺-ATPases. Like Phe785Leu, the Thr618Met mutation also affects the Na⁺ affinity, but by a different mechanism, which involves a displacement of the conformational equilibrium in favor of E₂(K₅).

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—Mutations Phe785Leu and Thr618Met were introduced directly into full-length cDNA encoding the rat kidney Na⁺,K⁺-ATPase (α1-isoform) using the QuikChange Site-Directed Mutagenesis kit (Stratagene), whereas mutations Phe785Tyr and Phe785Leu/Leu⁷⁸⁶Phe were introduced into appropriate restriction fragments (15). Mutants and wild type were expressed in COS-1 cells, using 5 μM ouabain in the growth medium to inhibit the endogenous COS cell enzyme and thereby select stable transfectants (22, 23). The rat α1-isoform was used, because this isoform has relatively low affinity for ouabain because of the presence of charged residues in the M1-M2 loop (24), allowing the use of ouabain to selectively eliminate the endogenous COS cell enzyme.

Functional Analysis—The isolation of the plasma membrane fraction, which was made leaky prior to functional analysis, and the measurement of ATPase activity by following the release of Pi, at 37 °C were carried out as previously (15, 23). For steady-state and transient kinetic measurements of phosphorylation from [γ-³²P]ATP we used a manual mixing technique at 0 °C (15, 25) and

A quench-flow technique for rapid kinetic studies at 25 °C (26–29). The phosphorylation rate of enzyme present in the EᵢNaᵢ form was determined in single mixing experiments according to the previously described “Protocol 1” (26, 27). To monitor the rate of EᵢP dephosphorylation, a double-mixing procedure was applied, phosphorylating the enzyme in the first step and following the dephosphorylation next (“Protocol 2b,” Refs. 27 and 29). To determine the active site concentration, phosphorylation was carried out at 0 °C in the presence of 150 mM NaCl and oligomycin (20 μg/ml) to inhibit dephosphorylation (15, 25, 27, 28). To eliminate the contribution from endogenous Na⁺,K⁺-ATPase in the functional assays, ouabain was included in the reaction media as detailed in the legends to figures.

Data Analysis—Each data point shown is the average value corresponding to at least two independent measurements. For each result, the number of independent experiments (n) is indicated in the figure legends. Data normalization, averaging, and nonlinear regression analysis were carried out as previously (26), and the results are reported ± S.E. Generally, the Hill equation was used for analysis of ligand concentration dependences. For determination of the amount of K⁺-occluded enzyme and the rate constant corresponding to deocclusion, the time dependence of phosphorylation was analyzed as a biphasic time course (17) in Equation 1.

\[
EP = (EP_{max} - E_{occluded}) + E_{occluded}(1 - e^{-kt})
\]

(Eq. 1)

\(E_{occluded}\) represents the part of the enzyme that phosphorylates slowly because of its initial presence as the occluded E₂(K₅) form.

The ouabain dependence of the Na⁺,K⁺-ATPase activity was analyzed by applying a function with the ouabain-inhibited enzyme represented by the sum of two hyperbolic components, one corresponding to the exogenous rat enzyme and the other corresponding to the endogenous COS cell Na⁺,K⁺-ATPase in Equation 2.

\[
V = V_{tot} - A_1[O]/(K_1 + [O]) - A_2[O]/(K_2 + [O])
\]

(Eq. 2)

\(V_{tot}\) is the total activity in the absence of ouabain. [O] is the concentration of ouabain. \(A_1\) and \(A_2\) are maximal activities of the exogenous rat enzyme and the endogenous COS cell Na⁺,K⁺-ATPase, respectively, i.e. \(A_1 + A_2 = V_{tot}\). \(K_1\) and \(K_2\) are the respective ouabain concentrations giving half-maximal inhibition. If the maximal molecular turnover rates of the exogenous and endogenous enzyme are denoted \(TO_1\) and \(TO_2\), respectively, and the respective numbers of exogenous and endogenous Na⁺,K⁺-ATPase molecules in the preparation are \(M_1\) and \(M_2\), then in Equations 3 and 4,

\[
A_1 = \left(\left[TO_1 \times M_1\right]/\left(\left[TO_1 \times M_1 + TO_2 \times M_2\right]\right)\right) \times V_{tot}
\]

(Eq. 3)

\[
A_2 = \left(\left[TO_2 \times M_2\right]/\left(\left[TO_1 \times M_1 + TO_2 \times M_2\right]\right)\right) \times V_{tot}
\]

(Eq. 4)

from which Equation 5 can be derived,

\[
M_1/(M_1 + M_2) = (A_1/TO_1)/(A_1/TO_1 + A_2/TO_2)
\]

(Eq. 5)

All numbering of Na⁺,K⁺-ATPase residues in this article refers to the sequence of the rat α1-isoform. In the human α3-isoform, the residues equivalent to those studied here are Phe⁷⁸⁵ and Thr⁶¹⁸ (5).
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[Image: FIGURE 1. Na\(^+\) affinity and phosphorylation rate of wild type and Phe785Leu. A, Na\(^+\) dependence of phosphorylation from [\(\gamma\)-\(^32\)P]ATP. Phosphorylation was carried out at 0 °C for 15 s in 20 mM Tris (pH 7.5), 3 mM MgCl\(_2\), 2 \(\mu\)M [\(\gamma\)-\(^32\)P]ATP, 10 \(\mu\)M ouabain, 20 \(\mu\)g/ml oligomycin, and the indicated concentrations of NaCl with N-methyl-\(^6\)glucamine added to maintain the ionic strength. Each line shows the best fit of the Hill equation, and the extracted \(K_n\) values with standard errors, the number of independent experiments (n), and Hill numbers (n\(_H\)) are as follows: wild type, \(K_n = 0.44 \pm 0.02 \text{ mM} (n = 7), n_H = 1.5;\) Phe785Leu, \(K_n = 5.8 \pm 0.37 \text{ mM} (n = 4), n_H = 1.5, 8, \) and Hill numbers (n\(_H\)) are as follows: wild type, \(K_n = 0.44 \pm 0.02 \text{ mM} (n = 7), n_H = 1.5;\) Phe785Leu, \(K_n = 5.8 \pm 0.37 \text{ mM} (n = 4), n_H = 1.5.\) B, ATP dependence of the initial rate of phosphorylation from ATP at 25 °C. Phosphorylation was carried out as described for B in the presence of 0.5, 1, 2, or 5 \(\mu\)M [\(\gamma\)-\(^32\)P]ATP, and the initial rate, expressed relative to the enzyme concentration to obtain the molecular rate in ms\(^{-1}\), is shown as a function of the ATP concentration in double-reciprocal plots. Each number of independent experiments carried out with wild type and Phe785Leu is n = 2–4 for each ATP concentration. The maximal rate (V\(_{max}\)) of the phosphorylation reaction, corresponding to infinite ATP concentration, and the \(K_m\) value for ATP, determined from this plot, are indicated in the figure.

thus allowing calculation of the relative contribution of the Na\(^+\),K\(^+\)-ATPase molecules corresponding to the exogenous expressed enzyme to the total number of Na\(^+\),K\(^+\)-ATPase molecules in the preparation.

RESULTS

Mutant Phe785Leu Can Be Expressed and Is Functional, although with Reduced Turnover Rate—The wild-type rat 1-isoform of Na\(^+\),K\(^+\)-ATPase and the Phe785Leu mutant were expressed in mammalian COS cells in the presence of ouabain in the growth medium to inhibit the endogenous COS cell Na\(^+\),K\(^+\)-ATPase enzyme, thereby selecting for stable transfectants (22, 23). Like the wild type, the Phe785Leu mutant sustained cell growth, indicating that it is functional in transport of Na\(^+\) and K\(^+\), and the expression level of the mutant was similar to that of the wild type (data not shown). At substrate concentrations that are fully saturating for the wild-type enzyme (130 mM Na\(^+\), 20 mM K\(^+\), and 3 mM MgATP), the molecular turnover rate for Na\(^+\),K\(^+\)-ATPase activity, calculated as the ratio between Na\(^+\)-K\(^+\)-ATPase activity and the active site concentration, was 4.4-fold reduced in the mutant relative to wild type (905 ± 101 min\(^{-1}\) (n = 6) versus 8474 ± 165 min\(^{-1}\) (n = 11) for wild type).

The Mutation Phe785Leu Reduces the Affinity for Na\(^+\) and the V\(_{max}\) of Phosphorylation from ATP—The activation of phosphorylation from ATP requires binding of Na\(^+\) at cytoplasmically facing high affinity sites of the E\(_1\) form. Concomitantly with the phosphorylation, the Na\(^+\) ions become occluded. Fig. 1 presents results of phosphorylation experiments with Phe785Leu and the wild type, in which we examined their Na\(^+\)-binding properties in the absence of K\(^+\) (Fig. 1A) and the rate of phosphorylation of E\(_1\)Na\(_3\) from ATP leading to the Na\(^+\)-occluded E\(_1\)P(Na\(_3\)) form (Fig. 1, B and C). Relative to the wild-type Na\(^+\),K\(^+\)-ATPase, the Phe785Leu mutant exhibited a conspicuous 13-fold reduction of the affinity (increase of \(K_{0.5}\)) for Na\(^+\) activation of phosphorylation. Fig. 1B shows that the phosphorylation rate constant at 2 \(\mu\)M ATP was 2-fold reduced for Phe785Leu compared with wild type. To determine the maximal phosphorylation rate and the apparent affinity for ATP of the E\(_1\)Na\(_3\) form, the enzyme was further analyzed in phosphorylation experiments carried out in the presence of various ATP concentrations under conditions otherwise identical to those described for Fig. 1B. The results are shown in the form of double-reciprocal plots of the initial phosphorylation rate per ATPase molecule as a function of the concentration of ATP (Fig. 1C). It is seen that both plots are linear, allowing extraction of the V\(_{max}\) and the Michaelis constant (\(K_m\)). The V\(_{max}\) of phosphorylation was 4-fold reduced in Phe785Leu relative to wild type. Moreover, the apparent affinity for ATP was 2.6-fold increased (i.e. \(K_m\) reduced) in the mutant relative to the wild type. The latter effect can be accounted for by the reduced phosphorylation rate, which leads to an increased accumulation of enzyme with ATP bound (for equations describing this relation, see e.g. Ref. 30, Scheme 1). The reduced V\(_{max}\) of phosphorylation of the E\(_1\)Na\(_3\) form is likely related to the reduced affinity for Na\(^+\), because the binding and occlusion of Na\(^+\) at the cytoplasmically facing sites normally leads to enzyme conformational changes that are propagated to the catalytic site, thereby controlling the rate of phosphoryl transfer from ATP.

The E\(_1\)-E\(_2\) Equilibrium and K\(^+\) Occlusion Are Unaffected by the Mutation Phe785Leu—Because effects on the equilibrium between the major conformational states of the dephosphoenzyme, E\(_1\), and E\(_2\) would indirectly influence the apparent Na\(^+\) affinity, assays were carried out to characterize the conformational equilibrium. First, we studied the ATP dependence of the Na\(^+\),K\(^+\)-ATPase activity. Because ATP binding normally shifts the equilibrium between E\(_1\) and E\(_2\), in favor of E\(_2\), the apparent affinity for ATP in the activation of Na\(^+\),K\(^+\)-ATPase activity should increase, if the E\(_1\)-E\(_2\) equilibrium were displaced toward E\(_2\) by the mutation (cf. Ref. 26), and decrease if the equilibrium were displaced in favor of E\(_1\). As seen in Fig. 2A, the Phe785Leu mutant displayed wild type-like apparent affinity for ATP in this assay. Fig. 2B shows the vanadate-inhibition profile of the Na\(^+\),K\(^+\)-ATPase activity. Because vanadate is known to inhibit the Na\(^+\),K\(^+\)-ATPase activity by reacting with the E\(_2\) form, the sensitivity to inhibition by vanadate can be used as an index of the amount of E\(_2\) present. Thus, a shift in the equilibrium between E\(_1\) and E\(_2\) toward E\(_2\) should increase the apparent vanadate affinity, and a shift in the opposite direction in favor of E\(_1\) should decrease the apparent vanadate affinity. As seen in Fig. 2B, the apparent affinity for vanadate exhibited by the mutant was wild type-like. The results in Fig. 2A and B indicate that the E\(_1\)-E\(_2\) equilibrium is unaffected by the Phe785Leu mutation. On this basis, the results in Fig. 1 permit the conclusion that the interaction with Na\(^+\) at one or more of the cytoplasmically facing sites of the E\(_1\) state is defective in the Phe785Leu mutant.

Fig. 2C shows the results of experiments in which we determined the amount of the K\(^+\)-occluded E\(_2\)(K\(_3\)) form accumulated at equilibrium in the absence of Na\(^+\) and the rate of K\(^+\) deocclusion. The enzyme was equilibrated with 1 mM K\(^+\) in the absence of Na\(^+\), and the phosphorylation was followed upon dilution of the enzyme in a solution containing 0.37 mM Na\(^+\), 0.1 mM K\(^+\), 10 \(\mu\)M ouabain, and the indicated concentrations of NaCl with N-methyl-\(^6\)glucamine added to maintain the ionic strength. Each line shows the best fit of the Hill equation, and the extracted \(K_n\) values with standard errors, the number of independent experiments (n), and Hill numbers (n\(_H\)) are as follows: wild type, \(K_n = 0.44 \pm 0.02 \text{ mM} (n = 7), n_H = 1.5;\) Phe785Leu, \(K_n = 5.8 \pm 0.37 \text{ mM} (n = 4), n_H = 1.5, 8, \) and Hill numbers (n\(_H\)) are as follows: wild type, \(K_n = 0.44 \pm 0.02 \text{ mM} (n = 7), n_H = 1.5;\) Phe785Leu, \(K_n = 5.8 \pm 0.37 \text{ mM} (n = 4), n_H = 1.5.\)
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**FIGURE 2. Characterization of the E₁,E₂ and K⁺ occlusion and Na⁺ binding in wild type and Phe785Leu.**

A. ATP-dependence of Na⁺,K⁺-ATPase activity. The rate of ATP hydrolysis was determined at 37 °C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM MgCl₂, 1 mM EGTA, 10 μM ouabain, 30 mM histidine buffer (pH 7.4), and the indicated concentrations of Na⁺. Each line shows the best fit of the Hill equation. The K₅₀ values with standard errors, the number of independent experiments (n), and Hill numbers (n_H) are as follows: wild type, K₅₀ = 0.46 ± 0.08 mM (n = 4), n_H = 1.0; Phe785Leu, K₅₀ = 0.462 ± 0.075 mM (n = 5), n_H = 1.0. B. Vanadate sensitivity of Na⁺,K⁺-ATPase activity. The rate of ATP hydrolysis was determined at 37 °C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM MgCl₂, 1 mM EGTA, 10 μM ouabain, and 30 mM histidine buffer (pH 7.4), and the indicated concentrations of vanadate. Each line shows the best fit of the Hill equation. The K₅₀ values for vanadate inhibition with standard errors, the number of independent experiments (n), and Hill numbers (n_H) are as follows: wild type, K₅₀ = 2.34 ± 0.09 mM (n = 6), n_H = 0.96; Phe785Leu, K₅₀ = 2.19 ± 0.26 μM (n = 4), n_H = 1.0. C. K⁺ occlusion at equilibrium and rate of deocclusion. Following equilibration of the enzyme for 1 h at room temperature in the presence of 20 mM Tris (pH 7.5) and 1 mM KCl, oligomycin (150 μg/ml) was added, and the solution was cooled to 10°C and diluted 10-fold by addition of a phosphorylation solution of the same temperature, producing final concentrations of 1 μM [γ-32P]ATP, 100 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10 μM ouabain, and 20 mM Tris (pH 7.5). The time course of phosphorylation was followed by acid quenching at the indicated time intervals, and the data show the level of phosphorylation as percentage of the phosphorylation level of fully deoccluded enzyme, obtained by performing the 1-h incubation in the presence of 50 mM NaCl without K⁺. Each line shows the best fit of the time function described under "Experimental Procedures," in which the ordinate intercept reflects the non-occluded enzyme pool ready to bind Na⁺ and phosphorylate immediately, whereas the exponential part (shown by the line) reflects the phosphorylation of E₂(K₂) through the steps E₂(K₂) → E₁ → E₁Na₃ → E₁P(Na₃), where the release of occluded K⁺ is rate-limiting (for a more detailed description of this method, see Ref. 17). The fraction of enzyme initially present as E₂(K₂) equals 100% minus the ordinate intercept. It is seen that the E₂(K₂) fraction is close to identical for the wild type and the mutant (93.6% versus 91.8%), indicating that the E₂(K₂) → E₁ + K⁺ equilibrium is unaffected by the mutation, in line with the results presented above in Fig. 2, A and B. The rate of K⁺ deocclusion from E₂(K₂) was also very similar for wild type and mutant (wild type, 0.018 s⁻¹; mutant, Phe785Leu 0.014 s⁻¹).

**FIGURE 3. K⁺ dependence of Na⁺,K⁺-ATPase activity and dephosphorylation of E₂P in wild type and Phe785Leu.** A. K⁺ dependence of Na⁺,K⁺-ATPase activity. Measurements were performed at 37 °C in the presence of 40 mM NaCl, 3 mM ATP, 3 mM MgCl₂, 30 mM histidine buffer (pH 7.4), 1 mM EGTA, 10 μM ouabain, and the indicated concentrations of KCl. The number of independent experiments is n = 3 for wild type and n = 4 for Phe785Leu. B. K⁺ dependence of E₂P dephosphorylation. Phosphorylation was performed at 25 °C for 5 s in 20 mM NaCl, 130 mM ChoCl, 20 mM Tris (pH 7.5), 3 mM MgCl₂, 1 mM EGTA, 10 μM ouabain, and 2 μM [γ-32P]ATP. Dephosphorylation was studied upon addition of 1 mM unlabeled ATP together with 1 mM or 20 mM KCl and different concentrations of ChoCl to maintain ionic strength. Each line shows the best fit of a mono-exponential decay function, and the extracted rate constants with the number of independent experiments (n) are: wild type (closed symbols), 87 s⁻¹ (n = 7) and 217 s⁻¹ (n = 4); Phe785Leu (open symbols), 105 s⁻¹ (n = 4) and 217 s⁻¹ (n = 4), at 1 mM and 20 mM KCl, respectively. A control experiment with 600 mM NaCl added instead of KCl is indicated by an open square for Phe785Leu (n = 9) and closed triangles for wild type (n = 6).

[γ-32P]ATP and Na⁺. The data were analyzed by fitting a biphasic time function (see "Experimental Procedures"), in which the ordinate intercept reflects the non-occluded enzyme pool ready to bind Na⁺ and phosphorylate immediately, whereas the exponential part (shown by the line) reflects the phosphorylation of E₂(K₂) through the steps E₂(K₂) → E₁ → E₁Na₃ → E₁P(Na₃), where the release of occluded K⁺ is rate-limiting (for a more detailed description of this method, see Ref. 17). The fraction of enzyme initially present as E₂(K₂) at equilibrium is for wild type 93.6 ± 1.8% and for Phe785Leu 91.8 ± 1.4%. The rate of K⁺ deocclusion is for wild type 0.018 ± 0.002 s⁻¹ and for Phe785Leu 0.014 ± 0.001 s⁻¹. The number of independent experiments is n = 4 for both wild type and Phe785Leu.

The Affinity for K⁺ at the Extracellularly Facing Sites of E₂P Is Unaffected by the Mutation Phe785Leu—Further studies were carried out to obtain information about the interaction with K⁺ in the various conformational states. Fig. 3A shows the K⁺ concentration dependence of Na⁺,K⁺-ATPase activity. K⁺ present at submillimolar concentration activates ATP hydrolysis both in the wild type and in the Phe785Leu mutant. This activation is caused by stimulation of dephosphorylation by K⁺ binding at the external sites on E₂P. In addition to the K⁺ activation phase, the Phe785Leu mutant showed inhibition by K⁺ at concentrations above 2 mM. Such an inhibition phase can also be detected for the wild-type enzyme, if the Na⁺ concentration is lowered (31), but is not seen for the wild type under the prevailing conditions with 40 mM Na⁺ present, unless the K⁺ concentration is further increased above 30 mM. The inhibition is caused by K⁺ binding in competition with Na⁺ at the cytoplasmically facing E₁ sites, turning the enzyme into the K⁺-occluded E₂(K₂) form by the so-called "direct route" E₁ + K⁺ → E₂(K₂) (2). However, because the above described results (Fig. 2C) showed that in the absence of Na⁺ the E₂(K₂) ⇄ E₁ equilibrium of the mutant is wild type-like, it can be concluded that the enhanced K⁺ inhibitory effect is caused by the reduced Na⁺ affinity of the mutant rather than an increase of K⁺ affinity. Hence, the 13-fold reduction of Na⁺ affinity weakens the ability of Na⁺ to compete with K⁺ and drive the enzyme into the E₁ form in the presence of K⁺. The inhibitory effect of high K⁺ concentrations on the Na⁺,K⁺-ATPase activity combined with the reduction of the maximal rate of phosphorylation seems to account for
the observed decrease of the catalytic turnover rate of the mutant relative to wild type.

Because of the presence of the inhibition phase, an accurate value for the affinity for K\(^+\) at the activating extracellularly facing sites of E\(_2P\) cannot be obtained from the data in Fig. 3A. Fig. 3B shows results of experiments in which the binding of K\(^+\) at these sites was examined directly by studying the E\(_2P\) dephosphorylation induced by K\(^+\). Normally, the extracellular sites on E\(_2P\) possess low affinity for Na\(^+\) and high affinity for K\(^+\), and the binding of K\(^+\) leads to rapid dephosphorylation of the enzyme. Phosphoenzyme was formed from [\(\gamma^{32}\)P]ATP in the absence of K\(^+\) and in the presence of a low Na\(^+\) concentration of 20 mM at 25 °C, to ensure accumulation of the E\(_2P\) form in the wild-type enzyme as well as in the mutant (see further below). Subsequently, the dephosphorylation was followed upon addition of 1 mM or 20 mM K\(^+\), or 600 mM Na\(^+\) without K\(^+\) as control (Fig. 3B). For both the wild type and the Phe785Leu mutant, rapid dephosphorylation required K\(^+\), and the rate of the K\(^+\)-induced dephosphorylation of the mutant was wild type-like both in the presence of a non-saturating K\(^+\) concentration of 1 mM and at the saturating K\(^+\) concentration of 20 mM. Therefore, it can be concluded that mutation Phe785Leu affects neither the affinity for K\(^+\) at the activating extracellularly facing sites nor the maximal rate of K\(^+\)-induced dephosphorylation of E\(_2P\). Because it was shown above that there is no significant effect of the mutation on the E\(_1P\) = E\(_2P\) equilibrium of the dephosphoenzyme in the absence of Na\(^+\) (Fig. 2C), it appears that the interaction with K\(^+\) is normal in the mutant, both in the phosphorylated and the dephosphorylated state.

**Mutation Phe785Leu Interferes with the Na\(^+\) Interaction**

That Normally Shifts the E\(_1P\)-E\(_2P\) Equilibrium in Favor of E\(_2P\)—The two phosphoenzyme intermediates, E\(_1P\) and E\(_2P\), are distinguished by their difference in interaction with the cations transported and in the reactivity with ADP. The E\(_1P\) intermediate, which has Na\(^+\) bound in an occluded state, is ADP-sensitive, i.e. able to react with ADP and donate the phosphoryl group back to ADP, forming ATP. By contrast, E\(_2P\) is ADP-insensitive, but dephosphorylates by hydrolysis of the aspartyl phosphoryl bond. At 0 °C, the relative amounts of E\(_1P\) and E\(_2P\) can be determined by studying the time course of dephosphorylation upon addition of ADP to phosphoenzyme formed from [\(\gamma^{32}\)P]ATP (25, 26, 28), because the phosphoenzyme exhibits two decay phases, a rapid phase corresponding to E\(_1P\), reacting backward with ADP, and a slow phase corresponding to hydrolysis of E\(_2P\). In the presence of a relatively low Na\(^+\) concentration of 20 mM, the initial E\(_1P\)/E\(_2P\) concentration ratio, estimated by extrapolating the slow phosphoenzyme decay phase back to intercept, constituted 15/85 in Phe785Leu and 23/77 in the wild type (data not shown). Hence, there was a slight increase of the relative amount of E\(_1P\) in mutant Phe785Leu. In wild-type Na\(^+\)-K\(^+\)-ATPase, Na\(^+\) is released at the extracellularly facing side of the membrane in connection with the conformational transition from E\(_1P\) to E\(_2P\), and a high extracellular Na\(^+\) concentration displaces the E\(_1P\)-E\(_2P\) conformational equilibrium in favor of E\(_2P\). To study the effect of Na\(^+\) binding at the extracellularly facing sites of the phosphoenzyme, the E\(_1P\)/E\(_2P\) ratio was examined in the presence of a high Na\(^+\) concentration of 600 mM, which in the wild-type shifts the E\(_1P\)-E\(_2P\) equilibrium in favor of E\(_2P\). It is seen in Fig. 4A that all the wild-type phosphoenzyme dephosphorylated rapidly upon addition of ADP, reflecting the accumulation of the E\(_2P\) phosphoenzyme intermediate, whereas dephosphorylation of the phosphoenzyme formed by the mutant was rather slow, indicating that E\(_2P\) accumulated during phosphorylation of the mutant, despite the high Na\(^+\) concentration present. The dephosphorylation was also followed upon chase with a solution containing 20 mM K\(^+\) (Fig. 4B). The presence of K\(^+\) ensures rapid dephosphorylation of E\(_2P\), thereby making E\(_2P\) → E\(_1P\) rate-limiting for the phosphoenzyme decay. In wild-type enzyme, the phosphoenzyme decay was slow, because the accumulated E\(_2P\) phosphoenzyme had to be converted into E\(_1P\), before K\(^+\) could induce rapid dephosphorylation. By contrast, the decay of the Phe785Leu phosphoenzyme contained a large rapid component, indicating that K\(^+\)-sensitive E\(_1P\) accumulated during phosphorylation. Hence, from the results presented in Fig. 4, A and B it may be concluded that mutation Phe785Leu prevents 600 mM Na\(^+\) from shifting the E\(_1P\)-E\(_2P\) equilibrium in favor of E\(_1P\). It therefore appears that the mutation disturbs not only the interaction with Na\(^+\) at the cytoplasmically facing high affinity sites, but also the interaction with extracellular Na\(^+\), binding at a low affinity site. Either the affinity for extracellular Na\(^+\) is reduced relative to wild type, or bound Na\(^+\) is unable to exert its normal effect.

**Phosphate Is Involved in Ouabain Binding**—Fig. 5 shows the ouabain concentration dependence of Na\(^+\)-K\(^+\)-ATPase activity for the Phe785Leu mutant and the wild type. The biphasic nature of the curves reveals the presence of a minor quantity of the endogenous COS cell Na\(^+\)-K\(^+\)-ATPase in addition to the expressed exogenous mutant or wild-type rat enzyme (in all experiments described above the endogenous enzyme was silenced with 10 μM ouabain). The lines show the best fit of a function with ouabain-inhibited enzyme represented by the sum of two hyperbolic components: a high affinity component corresponding to the endogenous COS cell Na\(^+\)-K\(^+\)-ATPase and a low affinity component corresponding to the expressed exogenous rat enzyme (either wild type or mutant), see “Experimental Procedures” Equation 2. Taking into account the difference in turnover rates of wild type and mutant, it may be calculated from the relative contributions of the two components that in the wild-type enzyme preparation the fraction of Na\(^+\)-K\(^+\)-ATPase molecules contributed by the exogenous enzyme is 92% (8% being endogenous COS cell Na\(^+\)-K\(^+\)-ATPase), and that the

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**FIGURE 4.** Distribution of phosphoenzyme between E\(_1P\) and E\(_2P\) intermediates at 600 mM Na\(^+\), Phosphoenzyme was formed at 0 °C for 15 s in 600 mM NaCl, 20 mM Tris (pH 7.5), 3 mM MgCl\(_2\), 1 mM EGTA, 10 μM ouabain, and 2 μM [\(\gamma^{32}\)P]ATP. A, dephosphorylation was followed upon chase with 1 mM non-radioactive ATP and 2.5 mM ADP (maintaining the NaCl concentration at 600 mM). The number of independent experiments is for wild type, n = 5 and for Phe785Leu, n = 4. B, dephosphorylation was followed upon chase with 1 mM non-radioactive ATP and 20 mM KCl (maintaining the NaCl concentration at 600 mM). The number of independent experiments is for wild type, n = 4 and for Phe785Leu, n = 3.
corresponding number for the mutant enzyme preparation is 93% (7% endogenous COS cell Na⁺, K⁺-ATPase), see “Experimental Procedures.” The extracted $K_{d}$ values for ouabain inhibition (see legend to Fig. 5) reveal that the affinity for ouabain is 60-fold reduced in the Phe785Leu mutant relative to wild type. Similar experiments were carried out with another cardiac glycoside, ouabagenin, which lacks the sugar moiety of ouabain (32), and in this case the affinity was also reduced very significantly by the mutation (15-fold, data not shown), but less than seen for ouabain, suggesting that the interaction being disturbed by the mutation involves the sugar moiety as well as the remaining part of the ouabain molecule.

Swapping of Phe785 and Leu786 Exerts Similar Effects as the Mutation Phe785Leu, whereas the Mutant Phe785Tyr Is Wild Type-like—To examine whether it was the removal of the aromatic side chain that disturbed Na⁺ and ouabain binding, and whether its exact position is important, we constructed two additional mutants in which Phe785 is swapped with the adjacent Leu786 residue or is substituted with tyrosine. The functional properties of these mutants were examined as described above for Phe785Leu. Swapping of Phe785 and Leu786 resulted in reduction of the turnover rate to 1367 ± 99 min⁻¹ (n = 8), i.e. a level similar to that observed for single mutation Phe785Leu, whereas in mutant Phe785Tyr the turnover rate was 10487 ± 285 min⁻¹ (n = 7), i.e. slightly increased relative to wild type. As can be seen in Fig. 6A, swapping of Phe785 and Leu786 led to severe reduction of the apparent Na⁺ affinity despite the presence of the aromatic side chain adjacent to its original position, in fact the double mutant showed even lower Na⁺ affinity (19-fold reduced relative to wild type) than observed for the single Phe785Leu mutation. The $K_{d}$ concentration dependence of the ATPase activity was also rather similar to that observed for Phe785Leu (Fig. 6B). Moreover, direct measurements similar to those presented in Fig. 3B indicated normal K⁺ affinity at the extracellularly facing sites of E₂P for the double mutant (data not shown). The Phe785Tyr substitution had only a small 2-fold effect on Na⁺ affinity, and no effect on K⁺ binding (Fig. 6, A and B). Analogous to the finding for mutant Phe785Leu, swapping of Phe785 and Leu786 led to a severe (9-fold) reduction of the $V_{\text{max}}$ of phosphorylation with reduced $K_{n}$ for ATP. In the Phe785Tyr mutant, the $V_{\text{max}}$ of phosphorylation was only 1.5-fold reduced relative to the wild-type enzyme (Fig. 6C). Also the affinities for ouabain and ouabagenin determined in inhibition studies were close to normal in the Phe785Tyr mutant (1.6- and 1.3-fold reduced, respectively, relative to wild type), whereas they were 127- and 54-fold reduced, respectively, following swapping of Phe785 and Leu786 (data not shown).

The Mutation Thr618Met, as Well, Reduces Na⁺ Affinity, but Only in the Presence of K⁺—Because the findings with Phe785Leu raised the question whether the reduced Na⁺ affinity represents a general pathophysiological mechanism in FRDP, we went on to characterize an additional FRDP mutant, Thr618Met (5). The expression level of this mutant was similar to wild type (data not shown), and the catalytic turnover number determined at saturating substrate concentrations was 2290 ± 89 min⁻¹ (n = 12), i.e. 3.7-fold reduced relative to wild type. The ouabain affinity determined as for Fig. 5 was 4-fold reduced in Thr618Met (data not shown). As seen in Fig. 7A, the apparent Na⁺ affinity of Thr618Met was only slightly reduced (1.3-fold) relative to wild type, when determined in the absence of K⁺ in phosphorylation experiments similar to those presented in Figs. 1A and 6A. However, the Na⁺ dependence of the Na⁺, K⁺-ATPase activity determined under the more physiological conditions in the presence of K⁺ indicated a significant 3-fold reduction in apparent Na⁺ affinity relative to wild type (Fig. 7B). In dephosphorylation experiments carried out as in Fig. 4A, Thr618Met behaved wild type-like, the phosphoenzyme being fully ADP sensitive at 600 mM Na⁺ (data not shown). Thus, the mutation does not interfere with Na⁺ interaction at the external sites, but affects Na⁺ interaction at the cytoplasmic side in the presence of K⁺.

The rising phase of the K⁺ dependence shown in Fig. 7C suggests high affinity for K⁺ binding at the activating extracellularly facing sites of E₂P, but as was the case for Phe785Leu, the presence of the inhibition phase precludes an accurate evaluation of the affinity for extracellular K⁺ on the
FRDP Mutations Phe785Leu and Thr618Met in Na\(^{+},K^{+}\)-ATPase

Further evidence for stabilization of \(E_{2}(K_{2})\) in Thr618Met was obtained in measurements of the ATP and vanadate dependences of the Na\(^{+},K^{+}\)-ATPase activity similar to those presented in Fig. 2, A and B. As seen in Fig. 8, B and C, the Thr618Met mutation reduced the apparent affinity for ATP and increased the apparent affinity for vanadate in accordance with a displacement of the conformational equilibrium in favor of \(E_{2}/E_{1}(K_{3})\) forms.

**DISCUSSION**

Here we report the novel observation that the Na\(^{+},K^{+}\)-ATPase mutations Phe785Leu and Thr618Met, associated with the neurological disorder FRDP, lead to functionally altered, but active, Na\(^{+},K^{+}\)-ATPase which was less pronounced than that displayed by the wild type. The reduced catalytic turnover rate of Thr618Met.

basis of the ATP activity measurement. Dephosphorylation studies similar to those presented for Phe785Leu in Fig. 3B showed a wild type-like rate of K\(^{+}\)-induced dephosphorylation of \(E_{2}P\) for Thr618Met (96 ± 10 s\(^{-1}\) (n = 3) versus 87 ± 2 s\(^{-1}\) (n = 3) for wild type at 1 mM K\(^{+}\)).

The K\(^{+}\) dependence of the Na\(^{+},K^{+}\)-ATPase activity (Fig. 7C) showed an inhibition phase which was less pronounced than that displayed by Phe785Leu. Because the Na\(^{+}\) affinity of Thr618Met was close to normal in the absence of K\(^{+}\), the K\(^{+}\) inhibition phase appears to be the result of stabilization of the \(E_{2}(K_{2})\) form in this case (see below).

**Mutation Thr618Met Stabilizes \(E_{2}(K_{2})\)**—Fig. 8A shows the results of experiments with Thr618Met similar to those presented in Fig. 2C for Phe785Leu, in which we determined the amount of the K\(^{+}\)-occupied \(E_{2}(K_{2})\) form accumulated at equilibrium in the absence of Na\(^{+}\) and the rate of K\(^{+}\) deocclusion. Following equilibration with either 100 \(\mu\)M or 1 mM K\(^{+}\), the rate of K\(^{+}\) deocclusion was 2–3-fold reduced in Thr618Met relative to wild type. Furthermore, at the non-saturating K\(^{+}\) concentration of 100 \(\mu\)M, the relative amount of the \(E_{2}(K_{2})\) form (100% minus the ordinate intercept) was significantly increased in the mutant relative to wild type (83% versus 64%), indicating that the \(E_{2}(K_{2})\) form was stabilized by the mutation. Assuming a simple hyperbolic binding isotherm, the observed change would correspond to a decrease of the dissociation constant for K\(^{+}\) from 56 \(\mu\)M to 20 \(\mu\)M and, hence, a ~3-fold increase of the apparent K\(^{+}\) affinity on the cytoplasmic side. The stabilization of \(E_{2}(K_{2})\) seems to account for the reduced apparent affinity for Na\(^{+}\) and the reduced catalytic turnover rate of Thr618Met.

Further evidence for stabilization of \(E_{2}(K_{2})\) in Thr618Met was obtained in measurements of the ATP and vanadate dependences of the Na\(^{+},K^{+}\)-ATPase activity similar to those presented in Fig. 2, A and B. As seen in Fig. 8, B and C, the Thr618Met mutation reduced the apparent affinity for ATP and increased the apparent affinity for vanadate in accordance with a displacement of the conformational equilibrium in favor of \(E_{2}/E_{1}(K_{3})\) forms.
by displacement of the $E_2-E_1$ equilibrium in favor of $E_2$. Therefore, the interaction of the $E_1$ form with $Na^+$ is defective in the Phe785Leu mutant.

For Thr618Met, we observed only a slight (1.3-fold) reduction of the apparent affinity for $Na^+$ in the absence of $K^+$ (Fig. 7A), but in the presence of $K^+$ a significant 3-fold reduction of the apparent Na$^+$ affinity was observed (Fig. 7B), and there was an increased apparent affinity for $K^+$ being occluded from the cytoplasmic side by the so-called “direct route,” corresponding to a displacement of the equilibrium $E_0 + K^+ \rightleftharpoons E_x(K)$ in favor of $E_x(K)$ (Fig. 8). In this respect, Thr618Met differs from Phe785Leu, for which the $E_1 + K^+ \rightleftharpoons E_x(K)$ equilibrium was found wild type-like. Hence, the reduction of the apparent Na$^+$ affinity observed with Thr618Met in the presence of $K^+$ can be accounted for by an enhanced ability of $K^+$ to compete with $Na^+$ for binding, and there seems to be little perturbation of the Na$^+$ binding sites in this case.

The dissociation of the effects on Na$^+$ and K$^+$ binding observed for the Phe785Leu mutant is rather unique among Na$^+$, K$^+$-ATPase mutants. Another prominent example is the Tyr773Leu mutant, which also showed severely reduced Na$^+$ affinity and wild type-like K$^+$ affinity (33). Recently, Tyr773 was proposed to be part of the so-called “third Na$^+$ binding site” thought to be located between M5, M6, and M9 and to be strictly Na$^+$ specific (19, 20), and it is possible that the Phe785Leu mutation in some way affects Na$^+$ binding at this site without modifying the two other cation sites supposed to interact with both Na$^+$ and K$^+$.

The reduction of the apparent affinity for Na$^+$ observed for Phe785Leu as well as Thr618Met may be of importance for understanding the pathophysiology of FRDP. Recently, FRDP was shown to be associated with mutations of the α3-isof orm of the Na$^+$, K$^+$-ATPase α-subunit, the Phe785Leu and Thr618Met mutations being two of six mutations found in patients with the disease (5). The pathophysiological mechanisms underlying the development of FRDP and the other neurological disorder associated with genetic alteration of Na$^+$, K$^+$-ATPase, FHM, are unknown. None of the FHM mutations analyzed functionally affect Na$^+$ affinity (34), and because the α2-isof orm, found mutated in FHM, is expressed in glial cells (35), it has been hypothesized that the Na$^+$, K$^+$-ATPase mutations causing hemiplegic migraine lead to impaired clearance of brain K$^+$, producing a wide cortical depolarization (3, 4). The α3-isof orm of Na$^+$, K$^+$-ATPase, found mutated in FRDP, is expressed in neurons, but not in glial cells (35), and a convincing hypothesis concerning the mechanism underlying FRDP has been missing (36). Overall, the α3-isof orm exhibits a high level of sequence homology with the other isoforms (90% amino acid identity with α1) with full conservation of the residues found mutated in FRDP patients. It is, thus, likely that perturbations of enzyme function similar to those reported here for the Phe785Leu and Thr618Met mutations of the α1-isof orm occur in the FRDP patients carrying the equivalent mutation of the α3-isof orm. It is noteworthy that the Phe785Leu and Thr618Met mutants are well expressed and active. The reduced Na$^+$ affinity without reduction of K$^+$ affinity may, thus, be a major factor contributing to the development of FRDP in patients carrying mutation Phe785Leu or Thr618Met, and could in fact represent a general pathophysiological mechanism in FRDP. Significantly in this connection, a third FRDP mutant, Glu282Lys, was previously characterized functionally in mutagenesis studies carried out before the relation between FRDP and mutation of Na$^+$, K$^+$-ATPase was established, and it was found that this mutation reduces the apparent Na$^+$ affinity of the $E_1$ form 3-fold (27). Hence, for all the three FRDP mutants characterized so far the low Na$^+$ affinity could possibly lead to an increase of the intracellular Na$^+$ concentration. In the neuronal cells, changes in intracellular Na$^+$ may lead to secondary changes in Ca$^{2+}$ via the Na$^+$/Ca$^{2+}$ exchange system, with consequences for the signaling cascade triggered by changes in Ca$^{2+}$, and subsequent excessive liberation of excitatory amino acids. Another possibility is that the Na$^+$-coupled neuronal uptake of dopamine is affected, because the Na$^+$ gradient is diminished due to the rise of intracellular Na$^+$. In contrast to Parkinsons disease, there is no widespread neurodegeneration or loss of dopamine production in patients with FRDP (3, 36, 37), but the disturbance of the Na$^+$-gradient, providing
Hence, it appears that Phe785 is a crucial determinant of the intrinsic extent than in the wild type, an increase of the apparent affinity for Na\(^+\) for Na\(^+\) engagement in a hydrophobic network with residues of M3 and M4, which (11), it seems likely that also in\(^+\)K\(^+\)-ATPase, Phe785 was placed in a recent model for the ouabain binding site (41), Phe785 was placed relative to threonine, one might even imagine a steric clash between the methionine and the arginine in the mutant. Thus, the presence of the methionine likely interferes with the interaction between the aspartate and the arginine, leading to destabilization of the E\(_1\) form. Because the Ca\(^2+\)-ATPase crystal structures show that the arginine has moved away from the aspartate in the E\(_1\) conformation (11, 44, 45), the Thr618Met mutation is less likely to cause problems in this conformation, thus explaining the shift of the conformational equilibrium in favor of E\(_2\)(K\(_2\)) observed for the Thr618Met mutant.

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