Mutation Lys$^{758}$ → Ile of the Sarcoplasmic Reticulum Ca$^{2+}$-ATPase Enhances Dephosphorylation of $E_2P$ and Inhibits the $E_2$ to $E_1Ca_2$ Transition*

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The highly conserved lysine residue Lys$^{758}$ in the fifth stalk segment of the sarcoplasmic reticulum Ca$^{2+}$-ATPase was substituted with either isoleucine or arginine by site-directed mutagenesis. The substitution with arginine was without significant effects on Ca$^{2+}$-ATPase function, whereas multiple changes of functional characteristics were observed with the Lys$^{758}$ → Ile mutant. These included insensitivity of ATPase activity to the calcium ionophore A23187, an alkaline shift of the pH dependence of ATPase activity, reduced maximum molecular turnover rate and steady-state phosphorylation level, reduced apparent affinities for Ca$^{2+}$ and inorganic phosphate, as well as increased sensitivity to inhibition by vanadate. Analysis of the partial reaction steps of the enzyme cycle traced these changes to two steps. The rate of dephosphorylation of the ADP-insensitive phosphoenzyme intermediate ($E_P$) was increased, irrespective of variations of pH, K$^+$, Ca$^{2+}$, and dimethyl sulfoxide concentration. In addition, the rate of conversion of the dephosphoenzyme with low Ca$^{2+}$ affinity ($E_d$) to the Ca$^{2+}$-bound form activated for phosphorylation ($E_0Ca_d$) was reduced in the mutant, and the ATP-induced rate enhancement of this step required higher ATP concentrations in the mutant compared with the wild type.

The Ca$^{2+}$-ATPase of sarco(endo)plasmic reticulum is an energy transducing membrane-bound enzyme that pumps Ca$^{2+}$ at the expense of chemical energy being released by hydrolysis of ATP. As in other P-type ATPases, the proposed enzyme cycle (Fig. 1) comprises a series of consecutive conformational changes that couple vectorial ion transport processes with the formation and breakdown of an aspartyl-phosphorylated intermediate (1–4). The polypeptide chain of the Ca$^{2+}$-ATPase is folded to form two major cytoplasmic loops that are connected through four "stalk segments," S2-S5, to a membrane domain thought to comprise 10 transmembrane helices M1-M10 (2, 5, 6). The ATP-binding site and the catalytic center for phosphorylation and dephosphorylation are located in the cytoplasmic domain, whereas residues involved in the binding and occlusion of Ca$^{2+}$ are found in the membrane sector (2, 3, 7–11). Residues involved in the deoccluding transformation of $E_0PCa_d$ to $E_0P$ (Fig. 1) have been found as well cytoplasmic as membranous subdomains, attesting to the global nature of this conformational change (3, 12–15). Point mutations that affect the dephosphorylation of $E_0P$ have so far only been identified in the membrane embedded segments M4, M5, and M6 (3, 8, 16–18). The observation that mutations in the Ca$^{2+}$-binding membrane domain can block conformational changes in the catalytic site and the formation or decomposition of the acylphosphate illustrates the functional linkage between these spatially well separated domains and the occurrence of long-range intramolecular signaling during enzyme turnover, although the nature of this interaction remains obscure. The stalk segments S4 and S5 that physically link the largest cytoplasmic loop containing the phosphorylated aspartic acid residue (Asp$^{351}$) to transmembrane segments M4 and M5 are logical candidates for structural elements being involved in the intramolecular signaling between the membrane domain and the catalytic site, and indeed several of the residues shown to be crucial to the $E_0PCa_d$ to $E_0P$ transition are located in the S4 segment (3, 12, 14, 15). The recent finding (16) that replacement of the tyrosine Tyr$^{163}$ at the M5S5 boundary with glycine resulted in a mutant catalyzing ATP hydrolysis without net accumulation of Ca$^{2+}$ in the microsomal vesicles awards this subdomain a central role in energy coupling or in formation of a gate controlling the cytoplasmic entrance to the pathway for Ca$^{2+}$ translocation (16), but the role of the remainder of stalk segment S5 has not been elucidated in any detail.

In the present study, we have analyzed the functional consequences of replacing the highly conserved lysine, Lys$^{758}$, in stalk segment S5 of the Ca$^{2+}$-ATPase with isoleucine or arginine. Mutation Lys$^{758}$ → Ile results in multiple changes of phenotypic characteristics, which can be traced to an increased rate of dephosphorylation of $E_0P$ and a decreased rate of the Ca$^{2+}$-binding transition $E_d$ to $E_0Ca_2$. These changes have not previously been observed with a point mutant of the Ca$^{2+}$-ATPase.

EXPERIMENTAL PROCEDURES

Mutation and Expression—The construction of mutant Ca$^{2+}$-ATPase CDNAs and the expression of wild type and mutants in COS-1 cells have previously been described (12, 16). The expression level was quantitated by a specific sandwich enzyme-linked immunosorbent assay (19) and by determination of the active site concentration by phosphorilation (see below), and was related to the total microsomal protein determined by the dye-binding method of Bradford (20).

ATPase Activity—The rate of ATP hydrolysis catalyzed by the microsomal membranes was determined at 37 °C by measurement of the amount of P liberated over a period of 20 min using the Baginski
method (21). The hydrolysis of ATP was terminated by dilution of 0.5 ml of the reaction mixture in 1.0 ml of ice-cold 0.5 M HCl, 4 mM ammonium heptamolybdate, 170 mM ascorbic acid. Subsequently, 1.5 ml of 150 mM sodium-m-arsenite, 70 mM sodium citrate, and 0.35 mM CH₃COOH were added, and the mixture kept at 37 °C for 10 min to complete the reaction (21–23). Measurements of absorbance at 850 nm were related to a standard of known Pi concentration. In some cases the rate of ATP hydrolysis was measured spectrophotometrically at 37 °C by a NADH-coupled assay (16) in the presence of 0.15 mM NADH, 1 mM phosphoenolpyruvate, 10 IU of lactate dehydrogenase/ml, 10 IU of pyruvate kinase/ml.

To calculate the activity referable to Ca²⁺-ATPase, the amount of P formed in the presence of 4 mM EGTA was subtracted from that formed in the presence of Ca²⁺. The molecular turnover rate was calculated as the ratio between the specific Ca²⁺-ATPase activity (nanomoles of ATP hydrolyzed per mg of microsomal protein per second) and the active-site concentration (nanomoles of enzyme per mg of microsomal protein) measured by quantitation of phosphorylation from ³²P, as described below.

In experiments examining the Ca²⁺ concentration dependence, various concentrations of CaCl₂ were added, and the free Ca²⁺ concentrations were calculated using the computer program MAXC and the stability constants therein (24). To study vanadate inhibition of ATPase activity, the indicated concentrations of monovanadate were obtained from monovanadate as described previously (25).

Relation between Ca²⁺-activated ATP Hydrolysis and ATP-driven Ca²⁺ Uptake—Combined measurements of ATPase activity and Ca²⁺ transport were performed at 37 °C in a medium containing 20 mM MOPS/Tris, pH 7.0, 100 mM KCl, 7 mM MgCl₂, 5 mM potassium oxalate, 0.5 mM EGTA, and 0.55 mM CaCl₂. Oxalate was included to act as Ca²⁺ trap inside the microsomal vesicles (12). The medium used for Ca²⁺ uptake measurements in addition contained 10⁵ Bq of ⁴⁵Ca²⁺/ml to trace Ca²⁺ accumulation in the microsomes. The reaction was initiated by the addition of ATP to a final concentration of 5 mM. ATP hydrolysis was monitored by the Baginski (21) method, and Ca²⁺ uptake in the microsomal vesicles was measured by Millipore filtration following quench with LaCl₃ as described previously (12).

Phosphorylation from [γ-³²P]ATP—Phosphorylation with [γ-³²P]ATP was performed on microsomal membranes (usually corresponding to about 0.5 pmol of Ca²⁺-ATPase) according to the previously described principles (13, 16), at 0 °C for 1–30 s in 100 µl of the medium described in the figure legends. The reaction solutions contained in Eppendorf tubes immersed in ice water were stirred efficiently by a vertically orientated tiny magnet bar (8 mm). To examine the pre-steady-state kinetics of phosphorylation starting from enzyme in the E₁Ca₂ form or the E₂ form, the microsomes were preincubated for at least 10 min in media containing, in addition to other components described in the figure legends, 0.1 mM CaCl₂ or 1 mM EGTA, respectively. Acid quenching of the phosphorylated enzyme was performed at serial time intervals by addition of 1 ml of ice-cold 7% (w/v) trichloroacetic acid containing 1 mM P₃. The acid-precipitated microsomal protein was washed by centrifugation and subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0 (13), and the radioactivity associated with the separated Ca²⁺-ATPase was quantitated by electronic autoradiography of the dried gels using a Packard InstantImager. Phosphorylation levels calculated as the ratio between the concentration of phosphorylzyme and the active site concentration are presented in the figures, except when otherwise indicated.

Phosphorylation from ³²P—To determine the total phosphorylation capacity of the expressed Ca²⁺-ATPase ("active site concentration") phosphorylation was carried out on microsomal membranes at 25 °C for 10 min in a medium containing 100 mM MES/Tris, pH 6.0, 10 mM MgCl₂, 2 mM EGTA, 0.5 mM PMT, and 30% (v/v) dimethyl sulfoxide. Dimethyl sulfoxide was present to increase the affinity for Pi, and thus ensure that 0.5 mM P₃ was saturating (26, 27). Titration of apparent affinity for Pi was performed at varying concentrations of ³²P, in the same medium as described above with 20% dimethyl sulfoxide. To study the time course of dephosphorylation, a phosphorylated sample was cooled to 0 °C and subsequently diluted 20-fold in ice-cold medium without dimethyl sulfoxide or containing 5 mM non-radioactive Pi to terminate the phosphorylation and permit monitoring of dephosphorylation. As indicated, the microsomes were equilibrated with 1 µl of the calcium ionophore A23187 before phosphorylation to allow rapid equilibration across the membrane of Ca²⁺ present in the dilution medium. The amount of phosphorylated protein was quantitated following acid quenching as described above for phosphorylation with [γ-³²P]ATP.

Curve Fitting and Kinetic Simulation—Data were fitted by nonlinear regression using the Sigmaplot program (Jandel Scientific). The analysis of ligand concentration dependences was based on the Hill equation,

\[ [EL] = \frac{[E]_{total} \times [L]^n}{K_a + [L]^n} \]  
(Eq. 1)

Time courses of phosphorylation and dephosphorylation were fitted to first-order kinetic equations whenever possible. Kinetic simulations of the enzyme cycle were carried out on an IBM-compatible PC using a program (SimZyme) based on the 4th order Runge-Kutta approximation method.

RESULTS

Expression—When the cell culture is working optimally, expression levels of 200–400 pmol of Ca²⁺-ATPase/mg of total microsomal protein can be obtained for the exogenous wild-type enzyme, corresponding to several hundredfold the level of the endogenous COS-1 cell Ca²⁺-ATPase. Mutants Lys⁷⁵⁸→Ile and Lys⁷⁵⁸→Arg were both found to be expressed to a level similar to that of the wild type.

Ionophore Sensitivity and Ca²⁺ Dependence of ATPase Activity of Mutant Lys⁷⁵⁸→Ile—Steady-state rates of ATP hydrolysis were determined for the wild type and mutant Lys⁷⁵⁸→Ile at various Ca²⁺ concentrations at pH 7.0, in the presence and absence of the calcium ionophore A23187 (Fig. 2). By allowing passive efflux of Ca²⁺ accumulated inside the microsomal vesicles the calcium ionophore increases the ATPase activity of the wild type 2–3-fold, due to relief of the “back inhibition” of the E₁PCa₂ → E₂P transformation imposed by a high luminal Ca²⁺ concentration (cf. Fig. 1). By contrast, the calcium ionophore did not increase the ATPase activity of mutant Lys⁷⁵⁸→Ile, but gave rise to a slight inhibition. This observation was confirmed in measurements where the NADH-coupled spectrophotometric assay was used to study in detail the time course of ATP hydrolysis before and immediately following the addition of ionophore (inset of Fig. 2).

Moreover, there was a conspicuous 3-fold difference between the maximum molecular turnover numbers for ATP hydrolysis measured with the Lys⁷⁵⁸→Ile mutant and the wild type, and the apparent calcium affinity displayed by the mutant was approximately 3-fold lower than that of the wild type (Fig. 2). At Ca²⁺ concentrations above 10 µM, the ATPase activity of the wild type starts to decline, an effect which is most pronounced in the presence of the calcium ionophore and is, at least under these conditions, partly due to Ca²⁺ binding at the low affinity luminal inhibitory sites. In addition, some of the inhibition of the wild type at high Ca²⁺ concentration may result from the substitution of Ca²⁺ for catalytic Mg²⁺ in the phosphorylated complex, slowing the conversion of E₁PCa₂ to E₂P (28). In the mutant, the inhibitory effect of high Ca²⁺ concentration was less pronounced compared with the wild type (Fig. 2).

pH Dependence of Ca²⁺-ATPase Activity of Mutant Lys⁷⁵⁸→Ile—...
was added to a final concentration of 1 absorbance unit. At the absence of Ca2+

Lys758 predominantly from a decrease in the rate of activity not referable to Ca2+

whereas on the alkaline side the decline in activity stems considerably higher than that of the wild type.

It was studied in the pH range 6–9 (Fig. 3). The wild type displays a pH optimum in the physiological pH range around pH 7. The decline in ATPase activity observed for the wild type at the acidic side of the pH optimum is caused primarily by an inhibitory influence of protons on the E2 to E1Ca2+ conversion, whereas on the alkaline side the decline in activity stems predominantly from a decrease in the rate of E1P dephosphorylation (29–35).

For the Lys758→Ile mutant, the pH optimum was shifted toward a more alkaline value relative to the pH optimum of the wild type. In the alkaline pH range up to pH 9 there was almost no decrease in the ATPase activity of the mutant, and at pH 9 the turnover rate of the mutant was considerably higher than that of the wild type.

Ile—The pH dependence of the Ca2+-activated ATPase activity was studied in the pH range 6–9 (Fig. 3). The wild type displays a pH optimum in the physiological pH range around pH 7. The decline in ATPase activity observed for the wild type at the acidic side of the pH optimum is caused primarily by an inhibitory influence of protons on the E2 to E1Ca2+ conversion, whereas on the alkaline side the decline in activity stems predominantly from a decrease in the rate of E1P dephosphorylation (29–35).

For the Lys758→Ile mutant, the pH optimum was shifted toward a more alkaline value relative to the pH optimum of the wild type. In the alkaline pH range up to pH 9 there was almost no decrease in the ATPase activity of the mutant, and at pH 9 the turnover rate of the mutant was considerably higher than that of the wild type.

Ca2+ Uptake in Microsomal Vesicles by Mutant Lys758→Ile—The absence of an activating effect of the calcium ionophore on the wild type was abolished toward the extremes of the pH interval studied, possibly because the E1P Ca2+ to E1P transition sensitive to luminal Ca2+ becomes less rate-limiting under these conditions. The inhibitory effect of the calcium ionophore on the ATPase activity of the Lys758→Ile mutant was most pronounced at neutral and acidic pH values.

Ca2+ and pH Dependence of ATPase Activity of Mutant Lys758→Arg—In contrast to the situation with the Lys758→Ile mutant, the ionophore-mediated activation, the Ca2+ and pH dependencies of ATPase activity, and the maximum molecular turnover rate of the mutant with the more conservative amino acid substitution Lys758→Arg were similar to those of the wild type (Fig. 5), as was the coupling ratio between Ca2+ accumulation and ATP hydrolysis (results not shown).

To elucidate the basis for the differences between the characteristics of the overall ATPase activities of the wild type and mutant Lys758→Ile, the partial reaction steps of the enzyme cycle were analyzed under various experimental conditions, as described below.

Titration of Phosphorylation from ATP and PPi—The steady-state level of phosphoenzyme formed from ATP was examined at 0 °C, where the ratio between the rates of phosphorylation and dephosphorylation favors accumulation of phosphoenzyme in the wild type even at rather low ATP concentrations. The phosphorylation from ATP is activated by Ca2+ binding at the high-affinity cytoplasmically facing sites (Fig. 1). As seen in Fig. 6A, the Lys758→Ile mutant required higher levels of Ca2+.
for activation of phosphorylation than the wild type, the $K_{0.5}$($Ca^{2+}$) values being 1.2 and 0.4 mM, respectively, in accordance with the $Ca^{2+}$ titrations of ATPase activity presented in Fig. 2. In Fig. 6A, the results for wild type and mutant were normalized separately taking the maximal values as 100%, and were fitted to the Hill equation with $K_{0.5}$ values of 0.4 mM and 1.2 mM for the wild type and Lys758→Ile mutant, respectively. B, phosphorylation was carried out as described for A, in the presence of variable concentrations of $[^32P]ATP$ and 100 mM CaCl$_2$ without EGTA. The measured phosphorylation levels are shown as percentage of the total phosphorylation capacity of the preparation determined with $^{32}$P$_2$ in the presence of 30% (v/v) dimethyl sulfoxide (active site concentration, see “Experimental Procedures”). The data were fitted to the Hill equation with $K_{0.5}$ values of 0.17 and 0.34 mM for the wild type and Lys758→Ile mutant, respectively. C, phosphorylation from 32Pi was carried out at 25 °C for 10 min in the presence of 100 mM MES/Tris, pH 6.0, 10 mM MgCl$_2$, 2 mM EGTA, 20% (v/v) dimethyl sulfoxide, and variable concentrations of $^{32}$P$_2$. The measured phosphorylation levels are shown as percentage of the active site concentration of the preparation (see above). The data were fitted to the Hill equation with $K_{0.5}$ values of 20 mM and 90 mM for the wild type and Lys758→Ile mutant, respectively.

levels calculated by relating the concentration of phosphoenzyme to the active site concentration. Contrary to the wild type, which was 100% phosphorylated at 10 mM ATP, the Lys758→
Ile mutant was only 60% phosphorylated at this ATP concentration, even though the data obtained with the mutant could be fitted to a hyperbolic dependence on the ATP concentration with an $V_{0.5}$ value of only 0.34 μM.

The $E_2$ form of the Ca$^{2+}$-ATPase can be phosphorylated backwards from $P_i$ (Fig. 1). The apparent affinity for $P_i$ was determined under optimal conditions for accumulation of $P_i$ and/or an increased rate of dephosphorylation. The reduced apparent affinity for $P_i$ found with the Lys 758 Ile mutant is the result of either a reduced rate of phosphorylation of the wild-type phosphoenzyme.

When $K^+$ was omitted from the dilution medium (Fig. 7B), the half-life of the wild-type phosphoenzyme increased at least 3-fold, in accordance with the literature (37), while there was no detectable change in the dephosphorylation rate of the mutant. Thus, there was a marked difference between the dephosphorylation rates of the Lys$^{758} \rightarrow$ Ile mutant and wild type under these conditions.

As seen in Fig. 7C, addition of 5 mM Ca$^{2+}$ in the presence of calcium ionophore at pH 7.0 reduced the rate of dephosphorylation of the wild type severalfold, due to the back inhibition by Ca$^{2+}$-binding at the low affinity luminal sites described above. In contrast to the conspicuous effect of Ca$^{2+}$ on the wild type, there was only a minor increase in the half-life of the mutant phosphoenzyme when dephosphorylation proceeded in the presence of 5 mM Ca$^{2+}$ and ionophore, and hence the difference between the dephosphorylation rates of wild type and mutant amounted to more than 40-fold under these conditions. This is consistent with the insensitivity of the ATPase activity of the mutant to back inhibition by accumulated Ca$^{2+}$ as described above.

The dephosphorylation of $E_2P$ was in addition examined at pH 7.0 in the absence of K$^+$ and Ca$^{2+}$ and presence of 15% dimethyl sulfoxide (Fig. 7D). In these experiments, the dilution medium contained 5 mM nonradioactive $P_i$ to ensure that phosphorylation from $^{32}P_i$ was efficiently terminated despite the presence of dimethyl sulfoxide. Under these conditions dephosphorylation was very slow in the wild type, in accordance with the previously reported effects of dimethyl sulfoxide (26, 27), and a 10-fold difference was seen between the half-lives of mutant and wild-type phosphoenzymes.

Yet another condition known to impede the progression of phosphoenzyme decomposition of the wild-type Ca$^{2+}$-ATPase is high pH in the presence of Mg$^{2+}$ (26, 29–31, 34). Although the dephosphorylation rate of the Lys$^{758} \rightarrow$ Ile mutant decreased considerably when the pH was increased to 8.35 in the presence of 10 mM Mg$^{2+}$, the wild type responded even stronger, and a large difference (more than 10-fold) between the dephosphorylation rates of mutant and wild type was thus manifested under these conditions (Fig. 8A). This agrees with the above described lack of inhibition of the overall turnover rate of the mutant at high pH (cf. Fig. 3).

Fig. 8B shows that addition of 1 mM Ca$^{2+}$ at pH 8.35 increased the half-life of the mutant phosphoenzyme more than 10-fold. The synergistic effects of high pH and Ca$^{2+}$ may be ascribable to the increased affinity of the luminal sites for Ca$^{2+}$ at high pH (26) and suggests that the luminal sites are still functioning and can mediate back inhibition in the mutant, although less efficiently than in the wild type. The initial fast phase of dephosphorylation seen with both the wild type and the mutant in Fig. 8B calls for some explanation. We believe that it represents the dephosphorylation occurring during the time it takes Ca$^{2+}$ present in the dilution medium to reach the inhibitory Ca$^{2+}$ sites in the vesicular lumen.

When the dephosphorylation rate of the Lys$^{758} \rightarrow$ Arg mutant was examined by the same methods as described above for Figs. 7 and 8, there was no significant difference from the wild type (results not shown).

$Ca^{2+}$-induced Back Conversion of $E_2P$ to $E_1P$Ca$^{2+}$—To examine whether a high luminal Ca$^{2+}$ concentration is able to promote conversion of $E_2P$ backwards to produce $E_1P$Ca$^{2+}$ in the Lys$^{758} \rightarrow$ Ile mutant as in the wild type, the enzyme was phosphorylated from $^{32}P_i$ in the presence of calcium ionophore, and the phosphoenzyme was subsequently diluted into a high pH medium containing 1 mM Ca$^{2+}$ and 2 mM ADP to dephosphorylate $E_1P$Ca$^{2+}$. As seen in Fig. 9, both the wild type and the mutant were able to dephosphorylate rapidly in the presence of...
Mutation Lys$^{758}$ → Ile of Sarcoplasmic Reticulum Ca$^{2+}$-ATPase

Phosphorylation was carried out at 0 °C in a medium containing 20 mM MOPS/Tris, pH 7.0, 80 mM KCl, 5 mM MgCl$_2$, 100 μM CaCl$_2$, 2 μM γ-$^{32}$P[ATP], and 1 μM calcium ionophore A23187. Following 15 s phosphorylation, 1 mM EGTA was added to terminate new formation of phosphoenzyme, and the samples were acid quenched 5 or 10 s later. The acid-quenched samples were analyzed as described under “Experimental Procedures.” To test the ADP sensitivity of the phosphoenzyme, 1 mM ADP was included with EGTA during a 5-s dephosphorylation. The percentage of the initial phosphoenzyme remaining after the dephosphorylation is shown with S.E. and number of measurements in parentheses.

| Condition | % phosphoenzyme |
|-----------|-----------------|
| EGTA for 5 s | 31.4 ± 6.5 (n = 6) |
| EGTA for 10 s | 10.1 ± 6.7 (n = 5) |
| ADP for 5 s | 2.6 |

Addition of ATP to the enzyme in the E$_1$Ca$_2$ form resulted in the appearance of an overshoot of phosphorylation in the Lys$^{758}$ → Ile mutant, whereas such an overshoot was not seen with the wild type under the present conditions (Fig. 10A). The overshoot observed with the mutant is composed of an initial fast phosphorylation, reaching almost the same phosphorylation level as the wild type, followed by a decline to a steady-state level of phosphorylation corresponding to 40–50% of the active site concentration, consistent with the steady-state phosphorylation data presented above (Fig. 6B). The simultaneous addition of Ca$^{2+}$, Mg$^{2+}$, and γ-$^{32}$P[ATP] to enzyme accumulated in the E$_2$ form (Fig. 10B) allows evaluation of the rate at which the enzyme becomes available for phosphorylation from ATP, i.e. the rate of the E$_2$ → E$_1$Ca$_2$ reaction.

The accelerated dephosphorylation of E$_{1}$P in the Lys$^{758}$ → Ile mutant relative to the wild type at alkaline pH is consistent with the steady-state phosphorylation data presented above (Fig. 6B).
conversion. For the wild type, this procedure resulted in a slightly reduced rate of phosphoenzyme formation relative to the rate observed when starting from enzyme accumulated in $E_1\mathrm{Ca}_2$ form. Importantly, under these conditions, mutant Lys$^{758}\rightarrow$Ile phosphorylated at a rate that was 5–6-fold lower than that of the wild type, and no overshoot was seen. Again the mutant reached a steady-state level of phosphorylation showing an increased rate of dephosphorylation and a dephosphoenzyme described above (32, 41–43). At higher concentrations, the rate enhancement in the wild type is primarily due to the effect of ATP on the rate of $E_2$ to $E_1\mathrm{Ca}_2$ transition of the dephosphoenzyme described above (32, 41–43). At higher concentrations, ATP modulates the rates of the $E_1\mathrm{PCa}_2$ to $E_2P$ transition and the dephosphorylation of $E_2P$ (28, 31, 38, 42). It can be seen in Fig. 12 that the difference between the rates of ATP hydrolysis of mutant and wild type was largest at ATP concentrations below 100 $\mu$M, the activation profile of the mutant being steeper than that of the wild type between 100 $\mu$M and 1 mM ATP.

Inhibition by Vanadate—From the above described data showing an increased rate of dephosphorylation and a decreased rate of the Ca$^{2+}$-binding $E_2$ to $E_1\mathrm{Ca}_2$ transition in the Lys$^{758}\rightarrow$Ile mutant, it may be rationalized that an increased amount of mutant enzyme should accumulate in the $E_2P$ form at steady state. To examine the level of $E_2$ intermediate present at steady state, vanadate inhibition of ATPase activity was investigated. Vanadate, acting as a transition state analog of the phosphoryl group, binds to the enzyme in the $E_2$ conformation, thereby impeding the continuation of the enzyme cycle (25, 44).

From the result shown in Fig. 13 it is clear that the mutant is inhibited by vanadate with much higher apparent affinity than the wild type. $K_{0.5}$ values being 0.5 and 30 $\mu$M for the mutant and wild type, respectively. This finding is consistent with the hypothesis that the mutant accumulates in the $E_2P$ form (or an "$E_2P$-like" form) at steady state to a greater extent than the wild type.
The basic and ATP-modulated rates of the ATP hydrolysis of the wild type (□) and the Lys758 → Ile mutant (▼) were measured at 37 °C as 100%. The rates of ATP hydrolysis of the wild type and mutant were normalized separately taking the maximal values at 5 mM ATP as 100%. The lines are theoretical curves computed on the basis of the reaction cycle in Fig. 1, with ATP-induced rate enhancements incorporated corresponding to the $E_1$ to $E_1Ca_2$ transition, the $E_2PCa_1$ to $E_2P$ transition, and the dephosphorylation of $E_2P$. The basic and ATP-modulated rates of the $E_2$ to $E_1Ca_2$ transition were, respectively, 30- and 12-fold reduced in the mutant relative to the wild type, and the affinity of the $E_2P$ form for ATP was 10-fold reduced in the mutant ($K_{\text{ATP}}$ values of 500 and 50 μM for mutant and wild type $E_2$ forms, respectively), whereas the ATP affinities of the other intermediates in the enzyme cycle were identical in mutant and wild type.

Figure 12. ATP dependence of Ca\textsuperscript{2+}-ATPase activity. The rates of ATP hydrolysis of the wild type (□) and the Lys758 → Ile mutant (▼) were measured at 37 °C at the indicated concentrations of ATP (present almost entirely as MgATP) in the presence of 25 mM TES, pH 7.5, 100 mM KCl, 100 μM CaCl\textsubscript{2}, 1 mM free Mg\textsuperscript{2+}, and 1 μM calcium ionophore A23187, using the NADH-coupled assay. The molecular turnover rates for wild type and mutant were normalized separately taking the maximal values at 5 mM ATP as 100%. The lines are theoretical curves computed on the basis of the reaction cycle in Fig. 1, with ATP-induced rate enhancements incorporated corresponding to the $E_1$ to $E_1Ca_2$ transition, the $E_2PCa_1$ to $E_2P$ transition, and the dephosphorylation of $E_2P$. The basic and ATP-modulated rates of the $E_2$ to $E_1Ca_2$ transition were, respectively, 30- and 12-fold reduced in the mutant relative to the wild type, and the affinity of the $E_2P$ form for ATP was 10-fold reduced in the mutant ($K_{\text{ATP}}$ values of 500 and 50 μM for mutant and wild type $E_2$ forms, respectively), whereas the ATP affinities of the other intermediates in the enzyme cycle were identical in mutant and wild type.

Figure 13. Titration of inhibition by vanadate of Ca\textsuperscript{2+}-ATPase activity. The rates of ATP hydrolysis of the wild type (□) and the Lys758 → Ile mutant (▼) were measured by the Baginski method at 37 °C in the presence of 50 mM TES/Tris, pH 7.0, 100 mM KCl, 7 mM MgCl\textsubscript{2}, 5 mM ATP, 1 mM EGTA, 1.1 mM CaCl\textsubscript{2}, 1 μM calcium ionophore, and the indicated concentrations of vanadate. The molecular turnover rates for wild type and mutant were normalized separately taking the maximal values determined in the absence of vanadate as 100%, and the data were fitted to the Hill equation with $K_{0.5}$ values of 30 and 0.5 μM for the wild type and mutant, respectively.

and dephosphorylation studies from which the result in Fig. 13 was predicted were carried out at 0 °C, demonstrating that the temperature is of minor importance for the general conclusions.

**DISCUSSION**

The present study is the first to describe a mutant that displays a higher rate of dephosphorylation of $E_2P$ than the wild type. An increased dephosphorylation rate of the Lys758 → Ile mutant relative to that of the wild type was seen independent of variation of pH, K\textsuperscript{+}, and Ca\textsuperscript{2+} concentration. Even in the presence of 15% dimethyl sulfoxide, which is a very potent inhibitor of dephosphorylation (26, 27), the dephosphorylation rate of the mutant was much faster than that of the wild type.

The Lys758 → Ile mutant, furthermore, exhibited a conspicuous reduction of the rate of the Ca\textsuperscript{2+}-binding $E_2$ to $E_1Ca_2$ conversion of the dephosphoenzyme (5-6-fold at neutral pH, low ATP concentration, 0 °C), as seen in the measurements of the phosphorylation rate with enzyme preaccumulated in the $E_2$ form. The $E_2$ to $E_1Ca_2$ conversion comprises several substeps, including the sequential dissociation of two or three protons, the sequential binding of two calcium ions, and associated (or intervening) conformational changes (2, 45), and one or more of these substeps may be affected by the Lys758 → Ile mutation. Like the increased dephosphorylation rate, the reduced rate of $E_2$ to $E_1Ca_2$ conversion in the Lys758 → Ile mutant represents a new feature not previously reported for any Ca\textsuperscript{2+}-ATPase mutant. Together these two changes of partial reaction rates may explain most of the phenotypic characteristics of the mutant.

In interpreting the data it should be kept in mind that different reaction steps may become rate-limiting for the overall Ca\textsuperscript{2+}-ATPase cycle depending on the experimental conditions, including temperature, pH, K\textsuperscript{+}, Mg\textsuperscript{2+}, ATP concentration, and the Ca\textsuperscript{2+} concentration on the two sides of the membrane (29, 38, 39). In the wild-type Ca\textsuperscript{2+}-ATPase, the ATPase activity measured at saturating substrate concentration in the presence of K\textsuperscript{+} at neutral pH and 37 °C increases 2-3-fold upon addition of the calcium ionophore A23187, due to relief of the back inhibition of the $E_2PCa_1$ to $E_2P$ transition by accumulated Ca\textsuperscript{2+} present at millimolar concentration inside the microsomes. This effect was not seen with the mutant enzyme despite its ability to accumulate Ca\textsuperscript{2+}, but on the contrary the presence of the calcium ionophore slightly inhibited the ATPase activity of the mutant. Absence of ionophore-mediated activation was previously observed with the Tyr\textsuperscript{763} → Gly mutant and was shown to be a consequence of uncoupling of Ca\textsuperscript{2+} transport from ATP hydrolysis induced by the Tyr\textsuperscript{763} → Gly mutation (16). However, because Ca\textsuperscript{2+} accumulation in the vesicles could be observed with the Lys758 → Ile mutant, and the ratio between Ca\textsuperscript{2+} transport and ATP hydrolysis was the same as in the wild type, the lack of activation by ionophore cannot in the present case be explained by uncoupling. Moreover, because we demonstrated that addition of 1 mM Ca\textsuperscript{2+} at alkaline pH in the presence of calcium ionophore reduced the rate of dephosphorylation of $E_2P$ considerably and led to back conversion of $E_2P$ into ADP-sensitive phosphoenzyme (Figs. 8 and 9), the luminal inhibitory Ca\textsuperscript{2+}-binding sites seem to function in the mutant, and the increased rate of dephosphorylation is probably not accomplished through an alternative reaction path in which dephosphorylation can occur with Ca\textsuperscript{2+} remaining bound to the phosphoenzyme. The two fundamental kinetic effects of the mutation, the increased rate of dephosphorylation of $E_2P$ (without bound Ca\textsuperscript{2+}) and the reduced rate of the $E_2$ to $E_1Ca_2$ conversion, may, however, both be contributing to the absence of ionophore-induced activation of the ATP hydrolysis in the Lys758 → Ile mutant, as can be demonstrated by computer simulation of the reaction cycle. An increased rate of dephosphorylation of $E_2P$ effectively increases the Ca\textsuperscript{2+} concentration required to drive the $E_2PCa_1$ to $E_2P$ interconversion backwards, and inhibition of the $E_2$ to $E_1Ca_2$ conversion renders this step more rate determining for the overall reaction than the $E_2PCa_1$ to $E_2P$ conversion sensitive to luminal Ca\textsuperscript{2+}. The 3-fold lower maximum turnover rate of the mutant relative to the wild type seen at 37 °C at neutral pH (Fig. 2) shows clearly that rate limitation in the mutant is imposed by the $E_2$ to $E_1Ca_2$ conversion under these conditions. The slight inhibitory effect of the calcium ionophore on ATPase activity in the mutant is also noteworthy. A23187 causes strong inhibition of the $E_2$ to $E_1Ca_2$ conversion when added in a much larger dose...
than that applied in the present study to make the membrane leaky (46), and it can be predicted that even the low concentration used here should exert some inhibition of the $E_2$ to $E_1$ conversion. Any effect of this inhibition on ATPase activity would be masked in the wild type due to the simultaneous relief of the back inhibition of the rate-determining $E_2'$PCa$_2$ to $E_2$P conversion, but if the $E_2$ to $E_1$ conversion limits the overall rate of the enzyme cycle in the mutant, the inhibitory effect of the ionophore should be manifested as a decrease of the ATPase activity in this case, consistent with the experimental finding.

The Lys$^{758}$→Ile mutant displayed a pH dependence of ATPase activity which was alkaline shifted, so that the turnover rate of the mutant was increased relative to that of the wild type at high pH. This can be explained by the effect of the mutation on the dephosphorylation of $E_2$P. In the wild type, alkaline pH inhibits the dephosphorylation severely in the presence of Mg$^{2+}$ (26, 29–31, 34, 39), but in the mutant the dephosphorylation was less strongly inhibited under these conditions. In addition, the rate of the $E_2$ to $E_1$Ca$_2$ transition increases with pH (32, 35), so that this step becomes less limiting for the rate of the overall cycle compared with the situation at neutral pH.

The titration of steady-state phosphorylation at 0°C as a function of ATP concentration below 10 μM showed only a slight reduction of apparent affinity for ATP in the Lys$^{758}$→Ile mutant relative to the wild type (Fig. 6B). Moreover, on the basis of the wild-type-like phosphorylation rate observed at 2 μM ATP, when the mutant enzyme had been preaccumulated in the $E_1$Ca$_2$ form (Fig. 10A), it seems unlikely that the intrinsic ATP affinity of the catalytic site in $E_1$Ca$_2$, or the rate constant for phosphorylation, was significantly lower in the mutant than in the wild type. The presence of a phosphorylation overshoot and a low steady-state level of phosphorylation in the mutant can be accounted for by the low rate of $E_2$ to $E_1$Ca$_2$ conversion in combination with a high rate of dephosphorylation.

In the Lys$^{758}$→Ile mutant, as well as in the wild type, titration of the ATP concentration dependence of steady-state ATP hydrolysis activity at 37°C (Fig. 12) showed a complex activation profile indicative of secondary activation by ATP that binds with different affinities to the various intermediates, as described previously for the wild type (10, 38, 42). The difference between the ATPase activities of mutant and wild type was largest at ATP concentrations below 100 μM, where the secondary activation seen in the wild type predominately reflects the modulation by ATP of the $E_2$ to $E_1$Ca$_2$ conversion (32, 41–43). At higher ATP concentrations the rise in activity was steeper in the mutant than in the wild type, suggesting that a modulatory effect of ATP exerted between 100 μM and 1 mM ATP was partly able to compensate for the intrinsically low rate of $E_2$ to $E_1$Ca$_2$ transition in the mutant. We have carried out a series of computer simulations of the enzyme cycle with ATP-induced rate enhancements incorporated corresponding to the $E_2$ to $E_1$Ca$_2$ transition, the $E_2$P'Ca$_2$ to $E_2$P transition, and the dephosphorylation of $E_2$P (for further discussion of these modulatory effects, see Ref. 10, 38, and 42). To reproduce the data in Fig. 12, it was necessary to assume not only that the basic rate of the $E_2$ to $E_1$Ca$_2$ conversion at low ATP concentrations is severalfold reduced in the mutant relative to that of the wild type, but also that the ATP-induced rate enhancement of this conversion requires higher ATP concentration in the mutant than in the wild type (see legend to Fig. 12). Hence, it is possible that the ATP affinity of the $E_2$ form or another intermediate in the path between $E_2$ and $E_1$Ca$_2$ is lower in the mutant than in the wild type. In this connection it should be noted that a 5-fold rise in ATP concentration from 2 to 10 μM produced a proportional 5-fold increase in the $E_2$ to $E_1$Ca$_2$ conversion rate in the mutant (compare Figs. 10B and 11), indicating that the activating effect of ATP was far from being saturated in this concentration range.

The changes in phenotypic characteristics of the Ca$^{2+}$-ATPase induced by the Lys$^{758}$→Ile mutation, furthermore, included reduced apparent affinities for Ca$^{2+}$ and P$_i$, as well as an increased apparent affinity for vanadate. The reduced apparent affinity for P$_i$ follows directly as a consequence of the increased dephosphorylation rate (cf, Fig. 1) and demonstrates that the enhancement of the "off rate" for P$_i$ is not matched by a comparable enhancement of the "on rate." The 3-fold reduction in the apparent Ca$^{2+}$ affinity of the mutant relative to the wild type observed in the Ca$^{2+}$-titrations of ATPase activity and phosphorylation from ATP can be explained as a consequence of the reduced rate of the Ca$^{2+}$-binding $E_2$ to $E_1$Ca$_2$ conversion. Moreover, because vanadate binds to the $E_2$ form as a transition-state analog of the phosphorly group, the accumulation of $E_2$ at steady state may explain the increased apparent affinity for vanadate in the mutant.

The functional properties found with the Lys$^{758}$→Ile mutant resemble at least partially those of the non-muscle isoform of the Ca$^{2+}$-ATPase (SERCA3). Hence, the functional differences found between SERCA3 and the wild type of SERCA1 (the isoform used in the present study) included a decreased apparent affinity for Ca$^{2+}$, an increased inhibition by vanadate, and an alkaline shift in pH optimum (47). The phosphorylation from P$_i$ and the dephosphorylation rate of $E_2$P have not been characterized for SERCA3. The functional differences between SERCA3 and SERCA1 cannot be associated to Lys$^{758}$, which is present in both isoforms, but there are multiple amino acid differences between SERCA3 and SERCA1 in the cytoplasmic region just N-terminal to S5 (for sequence comparison, see Ref. 48). Interestingly, the literature contains other examples of functional perturbations of the type seen with the Lys$^{758}$→Ile mutant. Hence, both nonylphenol (49) and jasmonate, a component of peppermint oil (50, 51), caused enhanced dephosphorylation of $E_2$P as well as a reduced rate of $E_2$ to $E_1$Ca$_2$ conversion, when incorporated into Ca$^{2+}$-ATPase membranes. The group of sesquiterpene lactones, to which thapsigargin belongs, as well as 2,5-di(tert-buty1)-1,4-benzohydroquinone, also appear to stabilize an $E_2$-like state of the Ca$^{2+}$-ATPase with low ATP affinity and destabilize $E_2$P (10, 52–54). Other hydrophobic amphiphilic molecules such as C$_{12}$E$_{6}$ (38) and procarine (39) exert the opposite effect, combining enhancement of the $E_2$ to $E_1$Ca$_2$ transition with inhibition of $E_2$P dephosphorylation. Hence, there seems to exist an obligatory linkage between the stabilization/destabilization of $E_2$ and $E_2$-like states and the destabilization/stabilization of $E_2$P, suggesting that the above mentioned perturbations all exert their effects by displacing a conformational equilibrium of the protein. The present results define amino acid residue at position 758 as a significant factor in the control of this conformational equilibrium.

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REFERENCES

1. de Meis, L., and Vianna, A. L. (1979) Annu. Rev. Biochem. 48, 275–292
2. Inesi, G., Sambilla, C., and Kirtley, M. E. (1990) Physiol. Rev. 70, 749–760
3. Andersen, J. P. (1995) Bioess. Rep. 15, 243–261
4. Mintz, E., and Guillain, F. (1997) Biochem. Biophys. Acts 1318, 52–70
5. MacLennan, D. H., Brandl, C. J., Korczak, B., and Green, N. M. (1985) Nature 316, 696–700
6. Stokes, D. L., Taylor, W. R., and Green, N. M. (1994) FEBS Lett. 346, 32–38
7. Clarke, D. M., Luo, T. W., Inesi, G., and MacLennan, D. H. (1989) Nature 339, 476–478
8. Andersen, J. P., and Vilsen, B. (1994) J. Biol. Chem. 269, 15931–15936
9. Vilsen, B., and Andersen, J. P. (1992) *J. Biol. Chem.* **267**, 25739–25743
10. McIntosh, D. B., Woolley, D. G., Vilsen, B., and Andersen, J. P. (1996) *J. Biol. Chem.* **271**, 25779–25789
11. Yonekura, K., Stokes, D. L., Sasabe, H., and Toyoshima, C. (1997) *Biophys. J.* **72**, 997–1005
12. Vilsen, B., Andersen, J. P., Clarke, D. M., and MacLennan, D. H. (1996) *J. Biol. Chem.* **271**, 25778–25789
13. Yonekura, K., Stokes, D. L., Sasabe, H., and Toyoshima, C. (1997) *Biophys. J.* **72**, 997–1005
14. Zhang, Z., Sumbilla, C., Lewis, D., Summers, S., Klein, M. G., and Inesi, G. (1995) *J. Biol. Chem.* **270**, 16283–16290
15. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) *J. Biol. Chem.* **266**, 18839–18845
16. Andersen, J. P. (1995) *J. Biol. Chem.* **270**, 908–914
17. Chen, L., Sumbilla, C., Lewis, D., Zhong, L., Strock, C., Kirtley, M. E., and Inesi, G. (1996) *J. Biol. Chem.* **271**, 10745–10752
18. Rice, W. J., and MacLennan, D. H. (1996) *J. Biol. Chem.* **271**, 31412–31419
19. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) *J. Biol. Chem.* **266**, 16157–16164
20. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
21. Baginski, E. S., Foa, P. P., and Zak, B. (1967) *Clin. Chem.* **13**, 326–332
22. Jorgensen, P. L., and Petersen, J. (1982) *Biochim. Biophys. Acta* **705**, 38–47
23. Vilsen, B. (1993) *Biochemistry* **32**, 13340–13349
24. Bers, D. M., Patton, C. W., and Nuccitelli, R. (1994) *Methods Cell Biol.* **40**, 3–29
25. Andersen, J. P., and Møller, J. V. (1985) *Biochim. Biophys. Acta* **815**, 9–15
26. de Meis, L., Martins, O. B., and Alves, E. W. (1980) *Biochemistry* **19**, 4252–4261
27. Champeil, P., Guillain, F., Venien, C., and Gingold, M. P. (1985) *Biochemistry* **24**, 69–81
28. Lund, S., and Møller, J. V. (1988) *J. Biol. Chem.* **263**, 1654–1664
29. Andersen, J. P., Lassen, K., and Møller, J. V. (1985) *J. Biol. Chem.* **260**, 371–380
30. Shigekawa, M., Dougherty, J. P., and Katz, A. M. (1978) *J. Biol. Chem.* **253**, 1442–1450
31. Wakabayashi, S., Ogurusu, T., and Shigekawa, M. (1986) *J. Biol. Chem.* **261**, 16372–16384
32. Scofano, H. M., Vieyra, A., and de Meis, L. (1979) *J. Biol. Chem.* **254**, 10227–10231
33. Inesi, G., and Hill, T. L. (1983) *Biophys. J.* **44**, 271–280
34. Bishop, J. E., and Al-Shawi, M. K. (1988) *J. Biol. Chem.* **263**, 1886–1892
35. Wakabayashi, S., Ogurusu, T., and Shigekawa, M. (1990) *Biochemistry* **29**, 10613–10620
36. Inesi, G., and de Meis, L. (1989) *J. Biol. Chem.* **264**, 5929–5936
37. Shigekawa, M., and Pearl, L. J. (1976) *J. Biol. Chem.* **251**, 6947–6952
38. Champeil, P., le Maire, M., Andersen, J. P., Guilhaud, F., Gingold, M., Lund, S., and Møller, J. V. (1986) *J. Biol. Chem.* **261**, 16372–16384
39. Henao, F., de Foresta, B., Orlofius, S., Cuenda, A., Gutierrez Merino, C., and Champeil, P. (1981) *Eur. J. Biochem.* **202**, 559–567
40. Takisawa, H., and Tonomura, Y. (1978) *J. Biochem. (Tokyo)* **83**, 1275–1284
41. Stahl, N., and Jencks, W. P. (1984) *Biochemistry* **23**, 5389–5392
42. Bodley, A. L., and Jencks, W. P. (1987) *J. Biol. Chem.* **262**, 13997–14004
43. Wakabayashi, S., and Shigekawa, M. (1990) *Biochemistry* **29**, 7309–7318
44. Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978) *J. Biol. Chem.* **253**, 7361–7368
45. Forge, V., Mintz, E., and Guillaum, F. (1995) *J. Biol. Chem.* **268**, 10953–10960
46. Hara, H., and Kanazawa, T. (1986) *J. Biol. Chem.* **261**, 16584–16590
47. Lytton, J., Westlin, M., Burk, S. E., Shull, G. E., and MacLennan, D. H. (1992) *J. Biol. Chem.* **267**, 14483–14489
48. Dode, L., Wuytack, F., Kools, P. F., Baba Aissa, F., Raemyaekers, L., Brik, F., van de Ven, W. J., and Casteels, R. (1996) *Biochem. J.* **318**, 689–699
49. Michelangeli, F., Orlofius, S., Champeil, P., East, J. M., and Lee, A. G. (1990) *Biochemistry* **29**, 3091–3101
50. Antipenko, A. Y., Spielman, A. I., and Kirchberger, M. A. (1997) *J. Biol. Chem.* **272**, 2852–2860
51. Starling, A. P., Hughes, G., East, J. M., and Lee, A. G. (1994) *Biochemistry* **33**, 3023–3031
52. Sagara, Y., Fernandez-Belda, F., de Meis, L., and Inesi, G. (1992) *J. Biol. Chem.* **267**, 12606–12613
53. Kijima, Y., Ogunbunmi, E., and Fleischer, S. (1991) *J. Biol. Chem.* **266**, 22912–22918
54. Wetome, M., Michelangeli, F., Lee, A. G., and East, J. M. (1992) *FEBS Lett.* **304**, 109–113