Mutagenesis of Segment 487Phe-Ser-Arg-Asp-Lys492 of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Produces Pumps Defective in ATP Binding*" 

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The lysine residue Lys492 located in the large cytoplasmic domain of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase is implicated in nucleotide binding through affinity labeling. The contribution of segment 487Phe-Ser-Arg-Asp-Lys492 to ATP binding and pump function has been investigated through the introduction of 11 site-directed amino acid mutations. ATP binding was measured through competitive inhibition of \([\gamma\text{-}32P}]_{\text{ATP}}\) and, together with F487L and R499D/D490R, produced correspondingly smaller changes in ATP-mediated activities. The ATP dependence of ATPase activity of these five mutants showed deviations from the wild-type profile in the low, intermediate, and high concentration ranges, suggesting defects in ATP-dependent conformational changes. Mutations S488A and D490A had no effect on ATP binding (\(K_p = 0.4 \mu M\)) or ATP-mediated activities. None of the mutations significantly affected phosphorylation from \(P_i\) or acetyl phosphate-supported Ca\(^{2+}\) transport. Mutations F487L and F487S, and not those at residue 492, increased the \(K_p\) for Ca\(^{2+}\) activation of transport 2- and 8-fold, respectively. The results implicate Phe487, Arg489, and Lys492 in binding ATP in both a catalytic and a regulatory mode.

The sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase belongs to a family of cation transport P-type ATPases that are phosphorylated by ATP on an aspartyl residue during the catalytic cycle. Ca\(^{2+}\) translocation occurs in the first part of the cycle and is activated by Ca\(^{2+}\) and MgATP binding to separate high affinity sites leading to phosphorylation and Ca\(^{2+}\) occlusion within the protein (1–5). Phosphorylation appears to be facilitated by an ATP-induced conformational change which may align enzymatic groups in the transition state (6). A nucleotide-dependent conformational change in the presence of Ca\(^{2+}\) is recorded by probes attached to Lys674 (7–10) and is seen also by the increased reactivity of the latter (8, 11) and the exposure of a critical, reducible disulfide (12). A further conformational change of the phosphoenzyme permits Ca\(^{2+}\) release to the lumen (13, 14). The cycle is completed with dephosphorylation and the associated transport of H\(^{+}\) counterions (15).

The pump cycle is regulated by ATP. Ca\(^{2+}\) binding (16–20), Ca\(^{2+}\) release to the lumen and the E1P to E2P transition (13, 21–23), and dephosphorylation (24–27) are accelerated by ATP in different concentration ranges. These modulations result in a complex dependence of ATP hydrolysis on ATP concentration (1, 23, 28, 29). The regulatory ATP binding site has been the subject of intense study over many years, and there is increasing evidence, mainly from results obtained with probes covalently attached at the catalytic site (27, 30–32), that at least part of the effect is due to ATP rebinding at or close to the catalytic site following phosphoenzyme formation and ADP dissociation.

The identification and role of residues complexing ATP, in either a catalytic or a regulatory mode, are being elucidated by a variety of approaches. Chemical modification and photoaffinity labeling have implicated Lys492 (31, 33–37), Lys515 (38), Thr532 and Thr533 (39), Arg678 (33), and Lys684 (36) in high affinity ATP binding, and some of these (Lys492 and Lys515) appear to be involved in binding ATP in a regulatory capacity following phosphorylation. However, some doubt has been cast on the role of Lys492 in ATP binding because derivatization with 7-amino-4-methylcoumarin-3-acetic acid succinimidyl ester apparently has no effect on ATPase activity (37). The function of Lys492 may be complex, because derivatization with TNP-8N\(_3\)-ATP partially uncoupled Ca\(^{2+}\) transport from hydrolysis of the tethered nucleotide (32) and cross-linking Lys492 to Arg778 with glutaraldehyde permitted ATP-dependent Ca\(^{2+}\) occlusion but completely blocked Ca\(^{2+}\) release to the lumen (14).

Site-directed mutagenesis has been used to probe several conserved amino acids and segments for involvement in ATP binding. Defective ATP binding following mutation of Lys515, wild type; CrATP, 8-y-bidentate chromium(III) complex of ATP; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay.
has been inferred from lowered Ca\(^{2+}\) transport activity (40), and the role of this residue may be complex because mutation K515A inhibited both ATP- and acetyl phosphate-supported transport (41). Changes to Lys\(^{492}\) in the hinge domain inhibited phosphorylation from both ATP and P\(_i\), although the mutants appeared to bind nucleotides normally as judged from unaltered nucleotide-dependent inhibition of an intramolecular cross-link formed with glutaraldehyde (42). Several other residues tested in the hinge domain were also excluded from playing a crucial role in nucleotide binding (42). The conserved Asp-Pro-Asp-Arg segment is implicated in ATP binding through the lack of ATP protection of the glutaraldehyde cross-link following change D601E and the inhibition of the cross-link itself with change P603G (43). Lys492 has not previously been altered by mutagenesis, but changing the equivalent residue in the Na\(^{+}\)-K\(^{-}\)-ATPase either has no effect on ATP binding (measured indirectly through the induction of ouabain binding) or inhibits both ATP and P\(_i\) binding, suggesting an indirect and complex role (44).

In this study, we have introduced 11 mutations into the segment \(\text{Phe}^{487}\text{Ser}^{488}\text{Arg}^{489}\text{Asp}^{490}\text{Arg}^{491}\text{Lys}^{492}\) to assess the role of this predicted loop in ATP binding and pump function. Phe\(^{487}\) and its distance from Lys\(^{492}\) are highly conserved within the family of P-type ATPases, and Phe\(^{487}\) or its homologue has not been previously mutated in any of the pumps. We introduce a new ATP binding assay based on the specific photolabeling of Lys\(^{492}\) with TNP-S\(_2\)NP-ATP and its inhibition by ATP. The assay overcomes the technical difficulties associated with the measurement of equilibrium binding of nucleotides to mutant and wild-type Ca\(^{2+}\)-ATPases expressed in relatively low yield in COS-1 cell microsomes. The results indicate that conserved Phe\(^{487}\) and Lys\(^{492}\), and to a lesser extent Arg\(^{489}\) play a role in binding ATP in a catalytic and a regulatory mode, thereby affecting most, if not all, ATP-dependent and ATP-modulated partial reactions. The segment plays no role in phosphoryl transfer per se and has little influence on the ATP-independent pump cycle.

**EXPERIMENTAL PROCEDURES**

SR vesicles were prepared by the method of Champeil et al. (45), with a modification to eliminate phosphorylase (14). The protein content was measured by absorbance at 290 nm in 50 mM sodium phosphate, pH 7.0, 1% (w/v) sodium dodecyl sulfate using a conversion factor of 1 absorbance unit/mg of protein, which was determined by the method of Lowry et al. (66) with bovine serum albumin as standard.

The methods used to obtain COS-1 cell microsomes with wild-type or mutated rabbit fast twitch Ca\(^{2+}\)-ATPase have been previously described in detail (40, 42, 46). In brief, oligonucleotide-directed mutagenesis was performed on a 735-base pair BamHI-SmaI fragment of the Ca\(^{2+}\)-ATPase cDNA inserted in the Bluestreak vector (Stratagene), and the presence of the desired mutation was confirmed by nucleotide sequencing. After reigation of the mutated fragment into the correct position in the full-length Ca\(^{2+}\)-ATPase cDNA, the entire piece of Ca\(^{2+}\)-ATPase cDNA was excised and cloned into vector pm2T for expression in COS-1 cells. Microsomes containing expressed wild-type or mutant Ca\(^{2+}\)-ATPase were prepared by differential centrifugation from cells grown 48–72 h after transfection with the respective cDNA in the pm2T vector. Control microsomes containing only insignificant amounts of endogenous Ca\(^{2+}\)-ATPase and no expressed exogenous Ca\(^{2+}\)-ATPase were obtained following mock-transfection with the pm2T vector without the insert. The exogenous Ca\(^{2+}\)-ATPase content of the microsomal fraction was assayed with a specific sandwich ELISA (42). The concentration of Ca\(^{2+}\)-ATPase was in the range 100–600 \(\mu\)g/ml for the expressed wild-type and all of the mutants except R491L, which was not expressed, and F487S, F488F, which was poorly expressed. The concentration of total microsomal protein in the preparations was 5–10 mg/ml.

**Nucleotide Synthesis—**Nonradiolabeled TNP-S\(_2\)NP-ATP was synthesized from ATP as described previously (30). High specific activity \(^{32}\)P\(_{\text{TNP-S}}\)ATP was prepared from S\(_2\)NP-ATP by a modification of our previous procedure (32). The \(^{32}\)P\(_{\text{TNP}}\) exchange reaction was performed for 1 h with 226 nmol of S\(_2\)NP-ATP and 1–1.5 mCi of \(^{32}\)P\(_{\text{TNP}}\) (Amersham Corp.) in 0.5 ml. The reaction mix was applied to a small column (0.5 × 2 cm) of DE52 in H\(_2\)O. It was washed with 5 ml of H\(_2\)O and acidified with 5 ml of 50% (v/v) acetic acid, washed by centrifugation in the same acid, and then subjected to HPLC purification.

**Photolabeling—**Irradiations were performed for 1 min in quartz cuvettes using a xenon lamp and tolune filters as described previously (30). The tolune was changed regularly. The standard medium was 25 mM HEPPS/tetramethylammonium hydroxide, pH 8.5, 1 mM MgCl\(_2\), 0.5 mM EGTA, 20% (w/v) glycerol, TNP-S\(_2\)NP-ATP without or with ATP at concentrations indicated in the figures, and either 5 \(\mu\)g of SR protein/ml (equal to ~20 nm ATPase) or 0.5–5 \(\mu\)g/ml Ca\(^{2+}\)-ATPase protein of COS-1 cell microsomes. The pH dependence of the reaction was examined in a similar medium except the buffers were MES (pH 6.0), MOPS (pH 7.0), HEPPS (pH 8.0), and CHES (pH 9.0 and 10.0). An aliquot of solubilizing mix (8 \(\mu\)l of 20% (w/v) sodium dodecyl sulfate, 5.4 g of 2-mercaptoethanol, and a trace of bromphenol blue) was added to the irradiated sample, and 50 \(\mu\)l were taken for Laemmli SDS-PAGE in 7.5% acrylamide for 3–4 h at 4°C. The bottom of the gel (containing the majority of the radioactivity) was cut off, and the remainder was dried. For any individual experiment, the irradiations and electrophoresis were performed in a single day because the adduct formed between the TNP-nucleotide and Lys\(^{492}\) is photo stable, exhibiting a half-life at room temperature of ~9 h (31). Autoradiography was performed with Hyperfilm-MP x-ray film (Amershams). Quantitation was performed with a Packard InstantImager 2024. The radioactivity contained in rectangles of equal area above and below the band of interest was averaged and subtracted from the test value to give the amount of labeling.

**Function assay—Ca\(^{2+}\)-transport activity was measured by Millipore filtration and assaying the filters for radioactivity (42, 46). Ca\(^{2+}\)-activated ATPase activity in the presence of calcium ionophore to prevent back-inhibition by accumulated calcium was measured spectrophotometrically by following the oxidation of NADH at 340 nm (42, 46). CrATP-induced Ca\(^{2+}\) occlusion was measured essentially as described previously (47) except for the use of a HPLC column with a larger pore size (TSK G 4000 SW, 7.5 × 300 mm, equipped with a guard column). The phosphorylation measurements were performed as described previously (46). The samples were acid quenched with 7% trichloracetic acid, washed by centrifugation in the same acid, and then subjected to SDS-PAGE at pH 6.0. The radioactivity associated with the ATPase protein was quantitated on dried gels using a Packard InstantImager.

**Calculations—**The TNP-S\(_2\)NP-ATP photolabeling of SR Ca\(^{2+}\)-ATPase data and ATP-supported phosphorylation data were fitted to the Hill equation

\[
Y = Y_{\text{max}} \left[ S \right]^{n} / K_{0.5} + \left[ S \right]^{n}
\]

where \(Y\) = relative counts or cpm, \(Y_{\text{max}}\) = relative maximal counts or cpm, \([S]\) = ligand concentration, \(K_{0.5}\) = concentration of TNP-S\(_2\)NP-ATP or ATP at which half-maximal labeling or phosphorylation is obtained, and \(n\) = Hill coefficient. The mutant labeling data were fitted to the same equation plus a linear component, \(m\)-TNP-S\(_2\)NP-ATP, where \(m\) = slope.

The data obtained from ATP inhibition of TNP-S\(_2\)NP-ATP photolabeling...
ing was fitted to the binding algorithm

\[ Y = \left[ 100 \cdot K_{D,ATP}/(K_{D,ATP} + [ATP]) \right] + \text{offset} \quad (\text{Eq. 2}) \]

where \( Y \) = 100 (counts in the presence of ATP) and \( K_{D,ATP} \) = concentration of ATP at half-maximal inhibition. The "true" dissociation constant for ATP binding was obtained from the equation

\[ K_{D,ATP} = K_{D,ATP}/[1 + ([\text{TNP-8N}_3\text{-ATP}] / K_{D,\text{TNP-8N}_3\text{-ATP}})] \quad (\text{Eq. 3}) \]

ATP-driven Ca\(^{2+}\) transport was fitted to the above Hill equation extended to kinetic measurements

\[ v = V_{max} \cdot [\text{Ca}^{2+}] / K_{D,\text{s}} + [\text{Ca}^{2+}] \]  
\[ y = A_1 \cdot e^{-kt} + A_2 \cdot e^{-0.04t} \quad (\text{Eq. 4}) \]

The data describing the Ca\(^{2+}\)-dependent hydrolysis of the tethered TNP-nucleotide were fitted to a biexponential decay function

\[ y = A_1 \cdot e^{-kt} + A_2 \cdot e^{-0.04t} \quad (\text{Eq. 5}) \]

where the value 0.04 min\(^{-1}\) is the rate constant for the slower phase of hydrolysis, was obtained from the best fit of Ca\(^{2+}\)-dependent hydrolysis data of SR Ca\(^{2+}\)-ATPase obtained over 15 min (not shown).

**RESULTS**

Characteristics of Photolabeling of SR Ca\(^{2+}\)-ATPase and the Wild-type Ca\(^{2+}\)-ATPase Expressed in COS-1 Cell Microsomes—It has been shown previously that the Ca\(^{2+}\)-ATPase in the native SR membrane is labeled by TNP-8N\(_3\)-ATP at Ly\(_5\) with an efficiency such that ~50% of the active ATPase is derivatized per irradiation period (30, 31). The main reason for not obtaining a higher efficiency is the production of tight binding photolysis products that compete for the catalytic binding site (30). For example, the pH dependence of labeling of Ca\(^{2+}\)-dependent ATPase expressed in COS-1 cell microsomes is different from that of SR membranes. ATPase in COS-1 cell microsomes is different from that in SR membranes. There is strong background labeling of Ca\(^{2+}\)-ATPase seen above 3 mM ATP, as indicated. The samples were subjected to SDS-PAGE and autoradiographed. The position of the Ca\(^{2+}\)-ATPase is shown by the arrowhead.

![Fig. 1. Photolabeling of microsomes isolated from COS-1 cells transfected with the pm72 vector without insert (control), with wild-type Ca\(^{2+}\)-ATPase cDNA, or with the cDNA-encoding mutant K492L. The microsomes were photolabeled in standard medium (i.e. pH 8.5) with varying concentrations of [γ\(^{32}\)P]TNP-8N\(_3\)-ATP in the absence or presence of 1 mM ATP, as indicated. The samples were subjected to SDS-PAGE and autoradiographed. The position of the Ca\(^{2+}\)-ATPase is shown by the arrowhead.](http://www.jbc.org/)

![Fig. 2. pH dependence of photolabeling of Ca\(^{2+}\)-ATPase in SR vesicles and wild-type Ca\(^{2+}\)-ATPase in COS-1 cell microsomes. Photolabeling was performed under standard conditions with 5 μg of SR protein/ml or ~200 μg of COS-1 cell microsomal protein/ml (containing wild-type Ca\(^{2+}\)-ATPase) with 2 μM [γ\(^{32}\)P]TPN-8N\(_3\)-ATP. Only the relevant portions of the autoradiographs following SDS-PAGE are shown.](http://www.jbc.org/)

(30) Fig. 2 shows that the pH dependence of labeling of Ca\(^{2+}\)-ATPase expressed in COS-1 cell microsomes is different from that in SR membranes. There is strong background labeling of the COS-1 cell membranes at pH 7 and below. It seems that the amount of nonspecific labeling of the Ca\(^{2+}\)-ATPase (as well as other proteins) in the foreign membrane is much more pH dependent than in the natural SR membrane and becomes overwhelming below pH 7.0. It is possible that due to electrostatic differences the TNP-nucleotide partitions more readily into the COS-1 cell microsomal membrane than the SR membrane at acidic pH. Hence, a pH of 8.5 was chosen to provide maximal specific labeling, but as seen above, and will be seen below, in most cases this still yielded more nonspecific labeling than was obtained in a SR preparation.

**Validation of the ATP Binding Assay**—The concentration dependence of labeling of Ca\(^{2+}\)-ATPase of SR membranes and that expressed in COS-1 cell microsomes (wild type) and the inhibition by ATP, performed in the absence and in the presence of thapsigargin, are compared in Fig. 3. Thapsigargin has been shown to decrease the affinity of the SR Ca\(^{2+}\)-ATPase for ATP ~2 orders of magnitude without affecting TNP-ATP binding (48). Photolabeling was performed over two overlapping
concentration ranges of TNP-8N₃-ATP. In the native membrane, the Ca²⁺-ATPase is labeled in a manner that is closely modeled by a single hyperbolic function, with $K_{0.5} = 0.7 \mu M$. There is no extra labeling in the 10–30 µM range. In contrast, the Ca²⁺-ATPase expressed in the COS-1 cell microsomes exhibited biphasic labeling with significant extra labeling occurr-
ring in the higher concentration range. Fitting the data to the sum of a hyperbolic and a secondary linear function yields \( K_{0.5} = 1 \mu M \) for the former process, which is very similar to that of the Ca\(^{2+}\)-ATPase in the native membrane. In the presence of thapsigargin, the \( K_{0.5} \) values for SR and wild type were 1.2 \( \mu M \). The \( K_{0.5} \) values are listed in Table I.

ATP concentration dependence of inhibition of TNP-8N\(_3\)-ATP labeling at a concentration of TNP nucleotide at close to 3 \( \times \) \( K_{0.5} \) yielded data that could be fitted satisfactorily to a simple binding function, with an offset in the case of the COS-1 cell microsomes to account for the small amount of secondary labeling at this relatively low concentration of TNP-8N\(_3\)-ATP. Table I lists the “true” \( K_{0.5} \) values for ATP binding calculated from the apparent values according to the equation for reversible competitive inhibition (see “Experimental Procedures”). The values are very similar for the ATPase in the two membranes (0.4 and 0.5 \( \mu M \) for SR and COS-1 cell microsomes, respectively, pH 8.5) and compare favorably with values obtained by direct binding measurements (e.g., 1.3 \( \mu M \), pH 8.0 (49)). Thapsigargin induced a pronounced shift in the inhibitory curve for both SR and COS-1 cell microsomes (\( K_{0.5} = 147 \) and 195 \( \mu M \), respectively). The values are similar to those found by DeJesus et al. (48) for SR vesicles (90 ± 50 and 260 ± 130 \( \mu M \), depending on the method).

We conclude that the photolabeling assay is capable of measuring ATP binding affinity and changes therein with reasonable accuracy and that there is no difference in this respect between the Ca\(^{2+}\)-ATPase in SR and that expressed in COS-1 cell microsomes.

**Mutations—** An alignment of several P-type ATPases around segment 48Phe-Ser-Arg-Asp-Phe-Lys492, including representatives of the four main groups, is shown in Fig. 4A. Alignment of the bacterial pumps in this region is ambiguous, and only three of the more homologous ones are included. The 11 amino acid replacements chosen to probe this segment in the present investigation are shown in Fig. 4B. The most conserved amino acid is Phe487, and most mutations were introduced here, testing aromaticity, hydrophobicity, the lack of charge, and distance from Lys492. Hydrophobic amino acids seem to be prohibited in the next position, prompting the S488A mutation, and the same reasoning applies to the R491L mutation. The central arginine and aspartate are more variable, but acidic residues are absent in the first position and basic ones are rare in the second position, suggesting the positional swap Lys489 → Arg500, and the same reasoning applies to the R491L mutation. The central arginine and aspartate are more variable, but acidic residues are absent in the first position and basic ones are rare in the second position, suggesting the positional swap Lys489 → Arg500, and the same reasoning applies to the R491L mutation. The central arginine and aspartate are more variable, but acidic residues are absent in the first position and basic ones are rare in the second position, suggesting the positional swap Lys489 → Arg500, and the same reasoning applies to the R491L mutation.

**Nucleotide Binding to Mutant ATPases—** Using the experimental design validated above, the nucleotide binding to the expressed mutant Ca\(^{2+}\)-ATPases was studied. Mutants F487L, F487S, positional swap F487/S488F, S488A, R491L, D490A, and K492Y were well labeled by TNP-8N\(_3\)-ATP, and the cont-
centration dependences of labeling are shown in Fig. 5 for these mutants. The corresponding \(K_{0.5}\) values are listed in Table I. Some of the mutants exhibited slightly higher apparent affinities for TNP-8N₃-ATP compared with wild-type Ca²⁺-ATPase; these included F487L, F487S, positional swap F487S/S488F, and K492Y. The apparent affinities displayed by mutants S488A and D490A were indistinguishable from that of the wild type. Mutants F487E and positional swap R489D/D490R were poorly labeled (results not shown), probably due to a low affinity for the nucleotide (cf. the functional studies reported below).

As already described above, mutant K492L was not labeled above background, even in the 3–30 μM range (Fig. 1).

Because of the variation in apparent affinity for TNP-8N₃-ATP, studies of ATP inhibition of the photolabeling had to be carried out at label concentrations that were individually designed for each mutant and the wild type to achieve the same degree of saturation with the label in the absence of ATP in each case. Fig. 6 shows for each mutant and the wild type the ATP concentration dependence of inhibition by ATP of TNP-8N₃-ATP labeling at a concentration of TNP-8N₃-ATP close to 3 times the respective \(K_{0.5}\) value. The changes in \(K_{0.5}\) caused by the mutations can be separated into three groups, all represented in the autoradiographs shown in Fig. 6, inset. Mutations S488A and D490A had little or no effect on \(K_{0.5}\), whereas F487L, R489L, and K492Y reduced the affinity 16–100-fold, and F487S and swap F487S/S488F showed no inhibition by ATP in the concentration range studied. The \(K_{0.5}\) values calculated from the apparent values as described above for the wild type are listed in Table I.

**ATPase Activity and Secondary Activation by ATP**—The ATP dependences of ATP hydrolysis catalyzed by the mutants (open circles) are compared with that of the wild-type Ca²⁺-ATPase expressed in COS-1 cells (closed circles) in Fig. 7. The wild type exhibits the typical complex profile displayed by the Ca²⁺-ATPase in the native sarcoplasmic reticulum membrane (42). First, there is a basal activation below 10 μM MgATP, reflecting the saturation of the catalytic site in the enzyme form stabilized by Ca²⁺ binding at high affinity sites. This basal activation of ATP hydrolysis was not studied in any detail in the present experiments due to the relatively low signal to background ratio at the subsaturating ATP concentrations. Second, a further increase in ATPase activity is seen in the 10–100 μM range, and finally a 2-fold activation in the millimolar range (23, 29). Activation of the wild-type Ca²⁺-ATPase in the latter two ranges is due to ATP acceleration of conformational changes that are rate-determining for the overall reaction. Mutants S488A and D490A exhibited activation profiles similar to that of the wild type, whereas the activation profiles of all the rest differed from the wild type. The difference between duplicate determinations was usually less than 5%, so that even the smaller alteration to the profile observed for mutant F487L is significant. Mutants F487S and F487E were only activated in the millimolar range. The profiles for R489L, positional swap R489D/D490R, K492L, and K492Y were similar to each other, exhibiting reduced activity at 16 μM, little or no activation in the 16–100 μM range and clear evidence of saturation of the catalytic site corresponding to the basal activation, and activation in the millimolar range but without the saturation above 2 mU seen for the wild type. Modeling the data in the millimolar ATP concentration range suggests that there is a reduction in the affinity for ATP in these mutants of ~3-fold. Hence, it is evident that there are several ATP-associated defects.

**ATP-driven Ca²⁺ Transport**—To assess the influence of the mutations on Ca²⁺ binding, the Ca²⁺ dependence of Ca²⁺ transport in the presence of 5 mM MgATP was investigated, and the results are shown in Fig. 8 for the wild-type and selected mutants. Mutants with alterations to Phe⁴⁸⁷ showed a small but significant lowering of apparent Ca²⁺ binding affinity and cooperativity (see figure legend for binding parameters). Mutations to Lys⁴⁹² and positional swap R489D/D490R had no effect on these parameters. The mutants showing a reduced rate of ATP hydrolysis at 5 mM MgATP (cf. Fig. 7) exhibited a similar extent of reduction of the maximal rate of Ca²⁺ transport at 5 mM MgATP. Mutants S488A and D490A displayed Ca²⁺-activation profiles indistinguishable from that of the wild type (not shown).

**Phosphorylation from ATP**—The acid-stable phosphoenzyme intermediate formed from ATP during steady-state ATP hydrolysis was measured at pH 7.0 and 0°C (to promote its accumulation). Like the wild type, all the expressed mutants were capable of forming the phosphorylated intermediate in the presence of micromolar Ca²⁺ and a sufficiently high ATP concentration, whereas no phosphoenzyme was observed in the absence of Ca²⁺ (presence of excess EGTA). The ATP concen-
Phosphorylation from Pi—Concentration dependences of Ca\(^{2+}\)-dependent phosphorylation of wild-type and selected mutants are shown in Fig. 9. The \(K_{0.5}\) values of all the mutants analyzed are listed in Table I. The wild type displays a very high apparent ATP affinity \((K_{0.5} \approx 0.1 \mu M)\) in this assay due to a favorable ratio between the rate constants for phosphorylation and dephosphorylation at 0°C. Mutants F487S and F487E failed to saturate below 20 \(\mu M\) ATP, and the data for these two mutants suggested huge changes in ATP binding affinity. On the other hand, the changes in \(K_{0.5}\) produced by the other mutations were relatively small. Of these, the largest was the 10-fold change for mutant R489L, which correlates reasonably well with the 16-fold change in ATP binding affinity determined in the ATP/TNP-8N\(_3\)-ATP competition assay described above. The correlations between the assays were poorer with mutants K492Y (5-fold compared with 98-fold) and F487L (3-fold compared with 22-fold). Therefore, the measurements were repeated with 6–10 different microsomal preparations of each of the latter two mutants and wild type, with the same result. In addition, to mimic the conditions used for photolabeling the ATP concentration dependence of phosphorylation was determined for the wild type and mutant F487L at pH 8.5 in the presence of 20% (v/v) glycerol. Even under these conditions the difference between the \(K_{0.5}\) values was about 3-fold (not shown).

**Phosphorylation from P\(_1\).**—Like the wild-type Ca\(^{2+}\)-ATPase, the mutants were able to be phosphorylated from P\(_1\) in the absence of Ca\(^{2+}\), whereas this reaction was inhibited in the presence of micromolar Ca\(^{2+}\). The phosphorylation from P\(_1\) in the absence of Ca\(^{2+}\) is shown in Table II. Possible affinity changes for P\(_1\) were analyzed by performing the phosphorylation assay at limiting and saturating concentrations of P\(_1\) in the presence or absence of dimethyl sulfoxide (which is well known to increase the apparent affinity of wild-type Ca\(^{2+}\)-ATPase for P\(_1\)) so that saturation is obtained at submillimolar concentrations of P\(_1\)) and at two different pH values. As seen from Table II, the mutations had no significant effect on the P\(_1\) phosphorylation levels under these conditions.

**Acetyl Phosphate-supported Ca\(^{2+}\) ATPase** with the nonphosphorylating substrate analogue CrATP in the presence of Ca\(^{2+}\) causes CrATP and Ca\(^{2+}\) to become very tightly bound at their respective sites (51–54). A similar Ca\(^{2+}\) occluded form of the pump with hindered access of Ca\(^{2+}\) to the medium is probably an intermediate of the normal pump cycle (3). CrATP-induced 46Ca\(^{2+}\) occlusion can be measured in Ca\(^{2+}\)-ATPase expressed in COS-1 cell microsomes by subjecting the detergent-solubilized preparations to size exclusion HPLC and determining the amount of radioactivity associated with the soluble monomeric Ca\(^{2+}\)-ATPase (47). The results obtained with the wild-type and selected mutants are shown in Fig. 10. To be able to evaluate the occlusion semiquantitatively, equivalent amounts of wild-type and mutant Ca\(^{2+}\)-ATPase protein, as determined by ELISA, were applied to the column. Mutant F487L (shown in Fig. 10) as well as mutants S488A, R489L, D490A, and positional swap mutant R489D/D490R (not shown) displayed levels of Ca\(^{2+}\) occlusion not distinguishable from wild type, whereas mutants K492L and K492Y (shown in Fig. 10) exhibited diminished abilities to occlude Ca\(^{2+}\), and mutants F487S and F487E (shown in Fig. 10) failed to occlude Ca\(^{2+}\), probably as a consequence of the defective nucleotide binding.

**Hydrolysis of Tethered TNP-Nucleotide**—Addition of Ca\(^{2+}\) to an SR preparation prephotolabeled with [\(\gamma\)-\(^32\)P]TNP-8N\(_3\)-ATP in EGTA activates hydrolysis of the tethered nucleotide at acidic pH, resulting in the liberation of the \(\gamma\)-phosphate (32). Because this reaction reflects the correct positioning of the \(\gamma\)-phosphoryl group of the label in the catalytic site, it was of interest to determine to what extent the rate of hydrolysis was affected by the mutations. For this we chose one of the more severely impaired mutants in terms of ATP binding (but not of TNP-8N\(_3\)-ATP binding), namely F487L, and one in which the labeled amino acid itself was substituted, namely K492Y. The results are shown in Fig. 11. SR Ca\(^{2+}\)-ATPase and wild type exhibited closely similar rate constants for Ca\(^{2+}\)-activated hydrolysis. Neither of the mutants exhibited any inhibition of hydrolysis; instead, the hydrolysis appeared to be accelerated.

**Acetyl Phosphate-supported Ca\(^{2+}\) Transport**—Acetyl phosphate is a nonnucleotide substrate for the SR Ca\(^{2+}\)-ATPase which serves well as energy donor for active Ca\(^{2+}\) accumulation. It appears to be utilized through a similar reaction pathway to ATP, with the exception of steps modulated by ATP...
including phosphorylation and, at higher concentrations of nucleotide, Ca$^{2+}$ binding and the E1P to E2P transition (23). The ability of wild-type Ca$^{2+}$-ATPase and selected mutants to transport Ca$^{2+}$ using acetylphosphate as substrate is shown in Fig. 12. With acetyl phosphate as substrate, there was no significant difference between the Ca$^{2+}$ transport rates measured for wild type and mutants, meaning that the functional differences described above must reflect changes in the rate or equilibrium constants associated with ATP binding and ATP-dependent reaction pathways.

**DISCUSSION**

In this study, we report for the first time on SR Ca$^{2+}$-ATPase mutants which are defective in ATP binding and all measured ATP-dependent functions but exhibit minimal changes in ATP-independent reactions. The mutations were introduced into segment 487Phe-Ser-Arg-Asp-Arg-Lys492 in the large cytoplasmic loop, between membrane segments M4 and M5, which incorporates the putative phosphorylation, nucleotide binding, and hinge domains. Lys492 has previously been implicated in ATP binding through affinity labeling (31, 32, 34–37) and ATP inhibition of a glutaraldehyde cross-link between Lys492 and Arg578 (33). The present results support a role for Lys492 in ATP binding, show that it is involved in ATP-dependent reactions and modulations, and also implicate conserved Phe487 and possibly Arg489 in these processes.

Measurement of ATP binding to the minute amounts of Ca$^{2+}$-ATPase expressed in COS-1 cell microsomes cannot be performed directly and is not straightforward. The method used here depends on ATP inhibition of TNP-8N3-ATP photolabeling of Lys492 or its substitute. We show that it is possible to selectively and strongly label the expressed Ca$^{2+}$-ATPase. The specificity of the photolabeling reaction is demonstrated by (i) virtually only the expressed wild-type Ca$^{2+}$-ATPase being labeled in the COS-1 cell microsomes at concentrations of nucleotide sufficient to just saturate the catalytic site (3 μM, Fig. 1), (ii) the complete inhibition of labeling by low concentrations of ATP, and (iii) the lack of labeling of mutant K492L. The pH dependence of photolabeling revealed that the COS-1 cell microsomes are more prone to nonspecific labeling than are SR vesicles, especially at low pH and high concentrations of nucleotide. This extra labeling was rigorously quantitated and only became a problem for the mutants that exhibited a low affinity for TNP-8N3-ATP. Fortuitously, most of the mutants showed an apparent higher affinity for the nucleotide compared with wild type.

Another concern was whether photolabeling, which is not an equilibrium reaction, could produce accurate values for ATP binding affinity from inhibition curves. To resolve this, we compared the photolabeling and inhibition characteristics of the ATPase in the natural membrane and in COS-1 cell microsomes, in the absence and in the presence of thapsigargin. The latter tight-binding inhibitor substantially lowers the affinity of the pump for ATP but not for TNP-8N3-ATP. Fortuitously, most of the mutants showed an apparent higher affinity for the nucleotide compared with wild type.
hydrophilicity and charge of Ser488 and Asp490 respectively are counteracting the serious consequences of change F487S. The Ca$^2+$ places the equilibrium in favor of E2 or an “E2-like” conformation, seen for instance from the effect of thapsigargin which disrupts the binding affinity changes, approximately 10-fold smaller than the shifts in $K_v$ values for ATP binding. The $K_v$ could, thus, serve as a useful rough indicator of binding changes for those mutants that were poorly photolabeled. It is apparent that mutant F487E binds ATP very poorly and that mutants K492L and K492Y are poorly photolabeled. It is of interest to compare the ATP binding measurements with the apparent ATP affinities detected in the functional assays, to see how accurately the latter predict the binding changes.

The ATP dependence of ATPase activity provides valuable insights into other consequences of the binding defects. The complex dependence of wild type activity on ATP concentration encompasses several ATP-mediated effects, including ATP-deocclusion and ATP-induced Ca$^{2+}$ occlusion sites were defective. A rather good correlation is found between the ATPase activity at 16°C and at low temperature (two conditions that both stabilize the E1 form) would thus tend to report on the ATP binding affinity of the catalytic site in E1 as well as on the rate constants of phosphorylation and dephosphorylation, whereas the ATP/TNP-8N$_3$-ATP competition assay (carried out in the absence of Ca$^{2+}$ to avoid ATP hydrolysis) reports on the true ATP binding affinities of E1 and E2 forms as well as the equilibrium constant for their interconversion in the absence of Ca$^{2+}$. Nevertheless, the ATP dependence of Ca$^{2+}$-activated phosphorylation did detect all of the ATP affinity changes reported by the binding assay, although the shifts in $K_v$ were, at least for the intermediate binding changes, approximately 10-fold smaller than the shifts in $K_v$ values for ATP binding. The $K_v$ could, thus, serve as a useful rough indicator of binding changes for those mutants that were poorly photolabeled. It is apparent that mutant F487E binds ATP very poorly and that mutants K492L and K492Y probably exhibit intermediate affinities for ATP. The Ca$^{2+}$-induced Ca$^{2+}$ occlusion assay likewise detected the severely defective mutants F487S and F487E and also registered the defects in mutants K492L and K492Y. Since these mutants were capable of Ca$^{2+}$ transport at Ca$^{2+}$ concentrations in the micromolar range, it seems to be excluded that their Ca$^{2+}$ occlusion sites were defective. A rather good correlation is found between the ATPase activity at 16 μM MgATP and the severity of the binding changes. The activity at high ATP is less predictive of the binding changes.

The ATP dependence of ATPase activity provides valuable insights into other consequences of the binding defects. The complex dependence of wild type activity on ATP concentration encompasses several ATP-mediated effects, including ATP-dependent acceleration of a conformational change leading to phosphorylation (6–12), ATP activation of Ca$^{2+}$ deocclusion and conversion from E1P to E2P (21–23), ATP activation of E2P hydrolysis (24–27), and ATP acceleration of conformational changes associated with Ca$^{2+}$ binding (16–20). Bodley and Jencks have analyzed the ATP dependence of ATPase activity, to see how accurately the latter predict the binding changes. The specific activity calculated per μg of Ca$^{2+}$-ATPase protein is shown as a percentage of that of the wild type measured at 30 μM free Ca$^{2+}$. The lines show the best fit to the Hill equation. The symbols, with $K_v$ (Ca) and $n_v$ in parentheses, are: ● (0.17 μM, 1.4), wild-type Ca$^{2+}$-ATPase; ○ (0.30 μM, 1.1), F487L; ◇ (1.22 μM, 0.92), F487S; □ (0.15 μM, 1.4), R489D/D490R; ◆ (0.18 μM, 1.6), R492L; □ (0.21 μM, 1.4), R492Y.

![Fig. 8. Ca$^{2+}$ dependence of oxalate-supported ATP-driven Ca$^{2+}$ uptake activity.](http://www.jbc.org/)
The effect of ATP on the E2 to E1 conversion and Ca\(^{2+}\) of phosphoenzyme (23). It is likely to be related to the activation of Phe487, Arg489, and Lys492 in the ATP-mediated conformational activation but with intermediate binding changes, excepting F487L, exhibited this effect of ATP on the E1P to E2P conversion (23). Again, all the mutants with intermediate binding changes, excepting F487L, failed to show increases in ATPase activity in the 10–100 \(\mu\)M range.

### TABLE II

**Phosphorylation by P1**

| % of phosphorylation | \(\text{MeSO}^3\) | \(\text{MeSO}^3\) |
|----------------------|-------------------|-------------------|
| \(0.25\) mM P1 | 0.025 mM P1 |
| 0.25 mM P1 | 0.025 mM P1 |

- *Phosphorylation was determined at equilibrium after 10 min of incubation with \(^{32}\)P, at 25 °C. The specific phosphorylation (calculated per \(\mu\)g of Ca\(^{2+}\)-ATPase protein determined by ELISA) is given as the percentage of the maximum specific phosphorylation of the wild type.
- Measured in a medium containing 50 mM TES/Tris, pH 7.0, 5 mM MgCl\(_2\), 1 mM EGTA, and 30% (v/v) dimethyl sulfoxide (MeSO).
- Measured in a medium containing 50 mM MES/Tris, pH 6.0, 5 mM MgCl\(_2\), and 2 mM EGTA. After acid quenching and washing by centrifugation in 7% (v/v) trichloroacetic acid, the samples were subjected to SDS-PAGE at pH 6.0. The radioactivity associated with dried gels was quantitated using a Packard InstantImager.
- ND, not determined; NE, not expressed.

activity in terms of three activation phases with \(K_0.5\) values of 2.9, 65, and 2860 \(\mu\)M (23). The lowest value describes unactivated "basal" velocities, and their data in this range fit well to a rectangular hyperbola (more complicated kinetics have also been reported (29)). Our data show that several mutants with measured or predicted intermediate binding changes display reduced levels of activity at 16 \(\mu\)M MgATP despite clear evidence of saturation. In the wild-type Ca\(^{2+}\)-ATPase, rate limiting of the unactivated cycle, under our conditions (37 °C, pH 7.5, 100 mM KCl, 1 mM MgCl\(_2\)), is principally due to slow Ca\(^{2+}\) deocclusion and the E1P to E2P conversion (23). This step of the unactivated cycle is not affected by the mutations as shown by the lack of inhibition of the acetyl phosphate-supported Ca\(^{2+}\) transport. Consequently, the low basal ATPase activity of some of the mutants must be due to low steady state levels of E1P with occluded Ca\(^{2+}\). This, in turn, can come about only if an equilibrium constant, specifically a forward rate constant, of one (or more) of the steps leading to phosphorylation is reduced. A step that is most likely to be inhibited is the rate-limiting ATP-dependent conformational change leading to phosphorylation seen by kinetic (6) and other means (7–12).

The intermediate phase of activation of the wild type (\(K_0.5\) = 65 \(\mu\)M) is accompanied by an increase in the steady-state level of phosphoenzyme (23). It is likely to be related to the activating effect of ATP on the E2 to E1 conversion and Ca\(^{2+}\) binding, which occurs at quite low concentrations of ATP (13). All the mutants with intermediate binding changes, excepting F487L, failed to show increases in ATPase activity in the 10–100 \(\mu\)M range.

Activation in the high ATP concentration range occurs at constant steady-state concentration of phosphoenzyme and is probably associated with activation of Ca\(^{2+}\) deocclusion and the E1P to E2P conversion (23). Again, all the mutants with intermediate binding changes, excepting F487L, exhibited this activation but with a 3-fold lower affinity for ATP.

We conclude that the ATPase activity profiles implicate Phe487, Arg489, and Lys492 in the ATP-mediated conformational changes responsible for ATP regulation of various partial reaction steps of the enzyme cycle. This conclusion, in turn, provides support for the model of ATP regulation in which activation is brought about exclusively by ATP binding to the catalytic site in various conformational states that exhibit different ATP affinities throughout the enzyme cycle.

In contrast to the ATP-dependent reaction cycle, the mutants had small effects on the ATP-independent steps or cycle. There was no significant effect on P\(_1\) binding or P\(_1\)-dependent phosphorylation. The Ca\(^{2+}\) dependence of Ca\(^{2+}\) transport showed apparent affinity changes for mutants with alterations to Phe487 of up to 8-fold (F487S) but not for the other mutants. The apparent affinity changes observed for the Phe487 mutants may be due to defective ATP modulation of the Ca\(^{2+}\) binding steps or to a direct effect on the E1/E2 equilibrium. The fact that only the Phe487 mutants displayed the affinity shift probably points to an effect on the latter equilibrium. While this effect could contribute to the changes in ATP binding affinity for these mutants, it may not constitute a highly significant factor given the magnitude of the binding changes. It does, however, point to a functional link between the Ca\(^{2+}\) transport sites in the membrane helices and the \(487^{\text{Thr}}\)-Ser-Arg-Asp-Arg-Lys492 segment. It seems possible that the shift in ATP affinity observed for the Phe487 mutants may be the basis for the effect of thapsigargin, which binds to membrane helix M3 and/or M4 (55), could be mediated by conformational changes within the \(487^{\text{Thr}}\)-Ser-Arg-Asp-Arg-Lys492 segment.

Mutants F487S and K492Y catalyzed Ca\(^{2+}\)-activated hydrolysis of the TNP-nucleotide tethered to the amino acid residue
at position 492 with liberation of the γ-phosphate at a slightly higher rate than that pertaining to the wild type (Fig. 11). In the wild type, the rate of hydrolysis of the tethered nucleotide is probably limited by a very slow phosphorylation step, as is the case for the hydrolysis of the untethered TNP-8N3-ATP (32). Thus, the rate of hydrolysis of tethered nucleotide by the mutants may provide an indication of the effect of the mutations on the phosphorylation reaction, which seems to be accelerated rather than inhibited. Hence, the structural changes induced by the mutations may be partially overcoming the strong inhibition of the phosphorylation reaction by the TNP moiety. It is pertinent that a glutaraldehyde cross-link formed between Lys492 and Arg678 results in the acceleration of acetyl phosphate- and α-phosphoryl group could be correct. It is clear that the product of the reaction, which might be expected from a loss in salt linkage or hydrogen bond. Lys492 is labeled by several ATP analogues (31, 33–37), and the suggestion of Yamasaki et al. (35), based on labeling with 3'-O-(4,5-dinitrophenyl)-ATP, that Lys492 interacts with both the α-phosphoryl group and the base moiety. In the related Na⁺,K⁺-ATPase, the homologous lysine is labeled with 8N3-ATP (58), but the anti conformer, 2N3-ATP, labels a glycine residue alongside the fluoresein isothiocyanate-labeled lysine (59). The failure to inhibit the Ca²⁺-ATPase by derivatization of Lys⁴⁹² with 7-amino-4-methylcoumarin-3-acetic acid succinimidyld ester (36) is surprising but could mean that at high concentrations, ATP is able to partially displace the label despite its covalent attachment by virtue of it being tethered to a loosely held or mobile loop. This may explain how ATP binding to the Na⁺,K⁺-ATPase is detected following derivatization of the equivalent lysine with pyridoxal phosphate or pyridoxal-ATP (60). It might further explain how ATP binding...
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is detected following derivatization of Lys\textsuperscript{515} with fluorescein isothiocyanate (27, 61), if this predicted loop is likewise a bit floppy or the lysine tether is flexible. Our results show that Lys\textsuperscript{492} is not essential for pump function but indicate that it makes a substantial contribution to the high affinity of the native ATPase for ATP.

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Mutagenesis of Segment $^{487}\text{Phe-Ser-Arg-Asp-Arg-Lys}^{492}$ of Sarcoplasmic Reticulum Ca$^{2+}$-ATPase Produces Pumps Defective in ATP Binding

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