Interaction of Nucleotides with Asp$^{351}$ and the Conserved Phosphorylation Loop of Sarcoplasmic Reticulum Ca$^{2+}$-ATPase*

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The nucleotide binding properties of mutants with alterations to Asp$^{351}$ and four of the other residues in the conserved phosphorylation loop, DKTGTLT$^{357}$, of sarcoplasmic reticulum Ca$^{2+}$-ATPase were investigated using an assay based on the 2'-O-(2,4,6- trinitrophenyl)-8-azidoadenosine triphosphate (TNP-8N$_3$-ATP) photolabeling of Lys$^{492}$ and competition with ATP. In selected cases where the competition assay showed extremely high affinity, ATP binding was also measured by a direct filtration assay. At pH 8.5 in the absence of Ca$^{2+}$, mutations removing the negative charge of Asp$^{351}$ (D351N, D351A, and D351T) produced pumps that bound MgTNP-8N$_3$-ATP and MgATP with affinities 20–156-fold higher than wild type (K$_D$, as low as 0.006 μM), whereas the affinity of mutant D351E was comparable with wild type. Mutations K352R, K352Q, T355A, and T357A lowered the affinity for MgATP and MgTNP-8N$_3$-ATP 2–1000- and 1–6-fold, respectively, and mutation L356T completely prevented photolabeling of Lys$^{492}$. In the absence of Ca$^{2+}$, mutants D351N and D351A exhibited the highest nucleotide affinities in the presence of Mg$^{2+}$ and at alkaline pH (E1 state). The affinity of mutant D351A for MgATP was extraordinarily high in the presence of Ca$^{2+}$ (K$_D$ = 0.001 μM), suggesting a transition state like configuration at the active site under these conditions. The mutants with reduced ATP affinity, as well as mutants D351N and D351A, exhibited reduced or zero CrATP-induced Ca$^{2+}$ occlusion due to defective CrATP binding.

The Ca$^{2+}$-ATPase of sarcoplasmic reticulum is a 10-trans-

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1 The abbreviations used are: TNP-8N$_3$-ATP, 2'-O-(2,4,6-trinitrophenyl)-8-azido adenosine triphosphate; TNP, trinitrophosphoryl; AMP-PCP, adenylyl β,γ-methylene triphosphate; CrATP, β,γ-hydehydratemagnesium(III) complex of ATP; E1 and E2, major conformational states of Ca$^{2+}$-ATPase; MOPS, 3-(N-morpholino)propanesulfonic acid; HEPPS, N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid; HPLC, high performance liquid chromatography.
been covalently attached to Lys492 by light activation (14–16). Tethering the nucleotide still permits Ca\(^{2+}\)-dependent hydrolysis in the forward direction of catalysis, proving direct interaction with Asp351, and yet has little effect on P\(^{2-}\)-dependent phosphorylation in the absence of Ca\(^{2+}\), showing that the nucleotide, or at least a portion of it, shifts position with respect to the aspartyl residue upon Ca\(^{2+}\) binding.

The phosphorylated aspartate and adjoining residues on the COOH-terminal side, segment 351DKGTGTLT357, termed the phosphorylation loop in this study, are highly conserved in P-type ATPases (17), and previous mutational analysis has documented their functional importance (18, 19). Besides Asp351, also Lys352, Thr355, Leu356, and Thr357 are critical to Ca\(^{2+}\) transport as well as phosphorylation (19), and further clarification of the distinct roles of these residues in nucleotide binding, phosphoryl transfer, and long range interaction with the Ca\(^{2+}\) sites may aid understanding of energy transduction in the pump. In this study, we assess the effects on nucleotide binding of mutations to the phosphorylation loop residues that previously were shown to result in severely impaired or inactive pumps (18, 19). ATP binding is measured mainly through inhibition of TNP-8N\(_3\)-ATP photolabeling of Lys492, which has recently been successfully applied to Ca\(^{2+}\)-ATPase mutated in segment 487FSRDRK495 (16). In selected cases where the affinity is extremely high, binding is also measured by a direct filtration assay. In addition, the effects of the mutations on Ca\(^{2+}\) occlusion induced with CrATP are determined. The results show that most of the mutations in the phosphorylation loop affect nucleotide binding and disrupt CrATP-induced Ca\(^{2+}\) occlusion. Our analysis of mutations to Asp351 reveals high intrinsic nucleotide binding energies when the negative charge is removed, particularly in the presence of Mg\(^{2+}\) and Ca\(^{2+}\), i.e. in the E1Ca\(_{a}\) state (KD = 0.001 \(\mu\)M for mutant D351A). These favorable interactions may be utilized to gain the transition state and to provoke conformational changes that communicate with the transport sites.

**EXPERIMENTAL PROCEDURES**

The mutant Ca\(^{2+}\)-ATPase cDNAs used in this study were the same as those described previously (18, 19) but were shuffled to vector pMT2 (20) to obtain higher expression levels in COS-1 cells (18, 21). COS-1 cell microsomes containing expressed wild-type or mutated Ca\(^{2+}\)-ATPase were isolated by differential centrifugation 48–72 h after transfection (18). The exogenous Ca\(^{2+}\)-ATPase content of the microsomal fraction was assayed with a specific sandwich enzyme-linked immunosorbent assay (21).

The synthesis of [\(^{32}\)P]TNP-8N\(_3\)-ATP, photolabeling of COS-1 cell microsomes, the inhibition by ATP, quantification of labeled bands by electronic autoradiography (“imaging”) following SDS-polyacrylamide gel electrophoresis, curve fitting equations and calculations of the “true” KD,ATP have been described previously (16, 22). For fitting of the TNP-8N\(_3\)-ATP labeling data, the Hill equation with or without a linear component was used, and the Hill coefficient was set to 1. The concentration of free Ca\(^{2+}\) was set with 5 mM EGTA and variable amounts of total CaCl\(_2\) calculated according to Fabiato and Fabiato (23) taking the Mg\(^{2+}\) concentration and pH into consideration. CrATP-dependent Ca\(^{2+}\) occlusion was measured as before (16, 24).

Equilibrium ATP binding to mutants D351N and D351A was also measured by filtration. COS-1 cell microsomes (1 \(\mu\)l of stock microsomes in 1 ml; approximately 0.5 pmol of Ca\(^{2+}\)-ATPase protein/ml) were incubated with [\(^{32}\)P]ATP, 1 mM [\(^{2}\)H]lucose, and other components as indicated in the Fig. 7 legend for 1 min at 25 °C, and the sample was filtered on Millipore GS 0.22-\(\mu\)m filters under mild vacuum. The radioactivity of the filter was measured by liquid scintillation counting. The wet volume of the filter was determined from the tritium radioactivity (range: 28–42 \(\mu\)l), allowing determination of the radioactivity of unbound nucleotide, which was subtracted from the total \(^{32}\)Pcpm to obtain the amount of ATP bound to the microsomes.

The formation of a slowly dissociating CrATP complex with the Ca\(^{2+}\)-ATPase in the presence of Ca\(^{2+}\) was followed through the inhibition of [\(^{32}\)P]TNP-8N\(_3\)-ATP photolabeling. Microsomes containing expressed wild-type or mutated Ca\(^{2+}\)-ATPase were incubated at 37 °C with CrATP for up to 1 h. Aliquots were taken at timed intervals and diluted 50-fold into irradiation medium with 0.5 \(\mu\)M [\(^{32}\)P]TNP-8N\(_3\)-ATP. The samples were irradiated for 1 min and subjected to SDS-polyacrylamide gel electrophoresis, and the radioactivity was quantified by electronic autoradiography as described previously (16).

### RESULTS

Twenty-four mutations have previously been introduced into the conserved phosphorylation loop of the Ca\(^{2+}\)-ATPase between Ile348 and Thr357 (18, 19). All the mutants with alteration to the aspartic acid residue Asp351 receiving the phosphoryl group during catalysis are inactive, and so are the mutants with alterations to Lys352 even in the case of the most conservative replacement of Lys352 with arginine. Activity is not affected by conservative replacements of Thr355 or Thr357 with serine, but replacement with alanine reduces the Ca\(^{2+}\) transport activity as well as the level of phosphoenzyme. Replacement of Leu356 with isoleucine is without effect on activity, but mutation to threonine inactivates the pump. Hence, these residues (nine mutants in all; see Table I) were selected for the present study of nucleotide binding properties.

The assay for nucleotide binding, which is based on specific [\(^{32}\)P]TNP-8N\(_3\)-ATP photolabeling of Lys492 and nucleotide competition, has been validated previously (16). Results obtained under optimum labeling conditions at pH 8.5 demonstrated that this assay is able to produce highly accurate values for TNP-8N\(_3\)-ATP and ATP binding affinities of Ca\(^{2+}\)-ATPase expressed in COS-1 cell microsomes (16). In assessing the results to be described below, it is furthermore useful to know that TNP-8N\(_3\)-ATP is a substrate of the Ca\(^{2+}\)-ATPase, albeit a slow one, whether untethered or tethered to Lys492 by photolabeling (15, 16). This means that the position of the \(\gamma\)-phosphoryl group of the bound nucleotide must be similar, although probably not identical, to that of bound ATP.

The concentration dependence of TNP-8N\(_3\)-ATP photolabeling of wild-type and mutant Ca\(^{2+}\)-ATPases at pH 8.5 in the presence of Mg\(^{2+}\) and absence of Ca\(^{2+}\) (presence of EGTA) is shown in Fig. 1A. The data could be fitted satisfactorily to the sum of a simple hyperbolic binding function and a linear component, the latter representing nonspecific labeling as predicted by filtration.

### Table 1

| Mutant    | KD\(_{S,\text{TNP-8N}_{3}\text{-ATP}}\) | KD\(_{\text{ATP}}\) | Ca\(^{2+}\) occlusion |
|-----------|-----------------------------------|---------------------|----------------------|
| Wild type | 0.98                              | 0.54                | ++                   |
| D351N     | 0.0063                            | 0.0065              | --                   |
| D351T     | 0.053                             | 0.012               | ND                   |
| D351A     | 0.040                             | 0.025               | --                   |
| D351E     | 1.3                               | 0.88                | ND                   |
| K352R     | 1.0                               | 7.1                 | +                    |
| K352Q     | 4.9                               | ~500                | --                   |
| T355A     | 0.74                              | 0.93                | +                    |
| L356T     | ~500                              | no specific labeling | --                   |
| T357A     | 6.4                               | ~20                 | --                   |

\(^{a}\) The “true” KD\(_{S}\) calculated under the assumption of competitive inhibition as described in Ref. 16; the concentration of TNP-8N\(_3\) was 3 × KD\(_{\text{S}}\) except for mutants K352Q and T357A where it was equal to the KD\(_{\text{S}}\).
For most of the mutants, the linear component was small and insignificant, but as seen in Fig. 1 the linear component was rather prominent for mutant K352Q, for unknown reasons. The derived $K_{0.5}$ values corresponding to the hyperbolic component are listed in Table I. It can be seen that removal of the negative charge on Asp351, as shown by mutants D351N, D351A, and D351T, led to a pronounced increase in TNP-8N3-ATP affinity, with D351N exhibiting the largest increase of 156-fold. By contrast, mutation D351E, which conserves the negative charge, did not significantly affect the TNP-8N3-ATP binding affinity. The concentration of Ca$^{2+}$-ATPase in the irradiation assay was approximately 0.4 nM for the tightly binding mutants and approximately 2 nM for the rest to ensure a reasonably high ratio of free to bound nucleotide, thereby allowing the total concentration to be equated with the free concentration.

Mutation K352Q, which removes the positive charge of Lys352, lowered the affinity for TNP-8N3-ATP at least 5-fold, whereas the more conservative replacement with arginine, K352R, was without significant effect. Mutation T357A exhibited a low level of labeling that was linear with increasing concentrations of TNP-8N3-ATP up to 30 $\mu$M. This indicates that either Lys492 was not being labeled or the affinity was extremely poor ($K_{0.5}$ estimated to be $>50$ $\mu$M).

The inhibition of photolabeling by ATP under the same buffer conditions is shown in Fig. 1B, and the derived true $K_D$ values assuming competitive inhibition are listed in Table I. Usually, the concentration of TNP-8N3-ATP was fixed at $3 \times K_{0.5}$ for each mutant Ca$^{2+}$-ATPase (see Table I), except for T357A and K352Q, where it was equal to the $K_{0.5}$ and ATP was included at the concentrations shown. In two cases, namely mutations K352Q and T357A, additional Mg$^{2+}$ was included at 1 and 3 mM ATP to a total concentration of 2 and 4 mM, respectively. The data were fitted to a simple binding function with an offset representing nonspecific labeling. The $K_{0.5,\text{ATP}}$ values calculated from the derived $K_{0.5}$ values as described (16) are listed in Table I. "●", wild type; "●", D351N; "●", D351T; "●", D351A; "●", D351E; "●", K352R; "○", K352Q; "□", T355A; "♀", T357A.
induced Ca\(^{2+}\) by

other hand, mutation D351E had little effect on the binding of both nucleotides, with D351N being the most dramatic. On the

equiv amount of wild-type and mutant Ca\(^{2+}\) shown in Table I. The microsomes were incubated at 37 °C in 50 mM MOPS/TMAH, pH 7.0, 100 mM NaCl, 5 mM MgCl\(_2\), 40 \(\mu\)M CaCl\(_2\), and 1 mM CrATP. The graphs show the size exclusion HPLC elution profiles of \(^{45}\)Ca associated with solubilized protein originating from microsomes harvested from cells transfected with wild-type or mutant Ca\(^{2+}\)-ATPase cDNA and from control microsomes from cells transfected with the expression vector without insert. The difference between the test and control curves corresponding to the elution time of the monomeric Ca\(^{2+}\)-ATPase at 14–15 min provides a measure of the occluded Ca\(^{2+}\). Equivalent amounts of wild-type and mutant Ca\(^{2+}\)-ATPase protein (as determined by enzyme-linked immunosorbent assay) were applied to the column in the experiments shown. A summary of the results is shown in Table I.

inhibition to be reached, the choice of the offset of the binding curve was somewhat uncertain in these cases, resulting in corresponding uncertainties with respect to the exact \(K_{D_{DATP}}\) values.

Hence, the results shown in Fig. 1 indicate that the most disruptive mutation in terms of nucleotide binding is L356T, followed by K352Q, T357A, and K352R. The latter three mutations affected ATP binding much more than the binding of TNP-nucleotide, similar to the situation with mutations close to Lys\(^{351}\) (16). All of the mutants in which the negative charge on Asp\(^{351}\) was removed exhibited a large increase in affinity for both nucleotides, with D351N being the most dramatic. On the other hand, mutation D351E had little effect on the binding of either nucleotide.

Nucleotide binding and the coupling between the catalytic and transport sites can be assessed by measuring CrATP-induced Ca\(^{2+}\) occlusion. CrATP slowly forms a complex with Ca\(^{2+}\)-ATPase expressed in COS-1 cell microsomes (as determined by enzyme-linked immunosorbent assay) and from control microsomes from cells transfected with the expression vector without insert. The amount of control microsomes in mg of total membrane protein corresponds to that chromatographed in the case of the wild type. The distinct peak for mutants D351N and D351A, and hence these mutants did not occlude Ca\(^{2+}\). Mutants K352R and T355A showed partial occlusion, and mutants K352Q, L356T, and T357A also failed to occlude Ca\(^{2+}\).

Thus, for some mutations, notably those that slightly or grossly lower the affinity for ATP, the effect of CrATP-induced Ca\(^{2+}\) occlusion seems to be correlated with the change in ATP binding affinity. However, mutations to Asp\(^{351}\) which caused a huge increase in affinity for ATP also appeared to prevent CrATP-induced Ca\(^{2+}\) occlusion. In order to elucidate whether this was due to defective CrATP complexation at the catalytic site or an uncoupling of CrATP complexation and Ca\(^{2+}\) occlusion, we devised an assay wherein the microsomes were incubated with CrATP for up to 1 h at 37 °C and then diluted substantially prior to photolabeling with TNP-8N\(_3\)-ATP. The affinity of the Ca\(^{2+}\)-ATPase for CrATP is not high, and a concentration of CrATP in the millimolar range is required to obtain saturation so that occlusion occurs in a reasonable period of time. To minimize the competitive binding of contaminant ATP that might be present in the CrATP preparation, the samples were irradiated under conditions where the affinity for TNP-8N\(_3\)-ATP is reasonably high and that for ATP is fairly low (pH 8.5 in the presence of EDTA). Also with this in mind, a concentration of TNP-8N\(_3\)-ATP of approximately 10\(\times\) \(K_{0.5}\) was

**Fig. 2.** CrATP-dependent Ca\(^{2+}\) occlusion. Oclusion of Ca\(^{2+}\) by wild-type or mutant Ca\(^{2+}\)-ATPase expressed in COS-1 cell microsomes was measured following incubation for 1 h at 37 °C in 50 mM TES/Tris, pH 7.0, 100 mM NaCl, 2 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), and without (open symbols) or with (solid symbols) 1 mM CrATP. At the indicated times, aliquots were diluted 50-fold into irradiation medium containing 29 mM HEPES/TMAH, pH 8.5, 2 mM EDTA, 20% (w/v) glycerol, and 0.5 \(\mu\)M \([^{32}\)P\]TNP-8N\(_3\)-ATP. The samples were irradiated and subjected to SDS-polyacrylamide gel electrophoresis, and the radioactivity was quantified. Circles, wild type; squares, D351N; triangles, D351A.

**Fig. 3.** CrATP inhibition of TNP-8N\(_3\)-ATP photolabeling. The microsomes were incubated at 37 °C in 50 mM MOPS/TMAH, pH 7.0, 100 mM NaCl, 2 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), and without (open symbols) or with (solid symbols) 1 mM CrATP. At the indicated times, aliquots were diluted 50-fold into irradiation medium containing 29 mM HEPES/TMAH, pH 8.5, 2 mM EDTA, 20% (w/v) glycerol, and 0.5 \(\mu\)M \([^{32}\)P\]TNP-8N\(_3\)-ATP. The samples were irradiated and subjected to SDS-polyacrylamide gel electrophoresis, and the radioactivity was quantified. Circles, wild type; squares, D351N; triangles, D351A.
chosen. As seen in Fig. 3, photolabeling of the wild-type Ca\textsuperscript{2+}-ATPase was inhibited by CrATP in a time-dependent manner, indicative of a gradual and effectively irreversible complexation of CrATP at the catalytic site. In mutants D351N and D351A, CrATP failed to inhibit photolabeling, showing that the irreversible binding of CrATP had not occurred. This explains the lack of CrATP-induced Ca\textsuperscript{2+} occlusion in these mutants.

The buffer conditions in the above binding experiments with TNP-8N\textsubscript{3}-ATP and ATP (pH 8.5, in the presence of Mg\textsuperscript{2+} and absence of Ca\textsuperscript{2+}) largely favor accumulation of the E1 state of the wild-type Ca\textsuperscript{2+}-ATPase, whereas the E2H and E2H\textsubscript{3} forms (cf. Scheme 1) would predominate at neutral and acid pH in the absence of Ca\textsuperscript{2+} (7). To better understand the huge increase in nucleotide affinity induced by mutations D351N and D351A and the implications for the catalytic mechanism, we investigated the influence of pH, Mg\textsuperscript{2+}, and Ca\textsuperscript{2+}, as well as thapsigargin, a tightly binding inhibitor that appears to lock the enzyme into E2 or an "E2-like" state (28). Results of these studies are presented in Figs. 4–6 and summarized in Table II in terms of derived dissociation constants.

As previously noted (16), the efficiency of the specific incorporation of TNP-8N\textsubscript{3}-ATP photolabel into the Ca\textsuperscript{2+}-ATPase expressed in COS-1 cell membrane is almost as high at neutral pH as the buffer was MOPS, pH 7.0. Additional MgCl\textsubscript{2} was added at high ATP concentrations as in Fig. 1. The dashed line in A shows the curve obtained after subtraction of the linear component. The insets show portions of the autoradiograms of the SDS-polyacrylamide gels at the level of the expressed wild-type Ca\textsuperscript{2+}-ATPase. In A, there are 10 lanes with concentrations of TNP-8N\textsubscript{3}-ATP as follows: 0.03, 0.1, 0.3, 0.6, 1, 3, 6, 10, 20, and 30 \textmu M; in B, there are also 10 lanes with concentrations of ATP of 0, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 \textmu M.
$K_{\text{D,ATP}}$ of the wild-type enzyme in the presence of Ca$^{2+}$ has been estimated on the basis of rapid kinetic measurements of association and dissociation rate constants (8). The inability of mutants D351N and D351A to phosphorylate offered a unique possibility for studies of the effect of Ca$^{2+}$ on equilibrium ATP binding to these mutants. As seen in Figs. 5 and 6 and Table II, we determined the affinities for TNP-8N3-ATP as well as ATP in the presence of Mg$^{2+}$ and Ca$^{2+}$ at both pH 8.5 and pH 7.0.

For mutant D351N, Ca$^{2+}$ binding caused a significant 4-fold reduction of the affinity for ATP at pH 8.5, whereas, surprisingly, at pH 7.0 the affinities for both nucleotides were increased (10-fold for TNP-8N3-ATP and 20-fold for ATP). For mutant D351A, Ca$^{2+}$ binding caused a tremendous increase in ATP affinity, resulting in a $K_D$ of about 0.001 μM at both pH values (300-fold increase in affinity at pH 7.0 and 23-fold at pH 8.5). Also, the affinity for the TNP-nucleotide increased very much in the presence of Ca$^{2+}$ in this mutant (10-fold at pH 8.5 and 40-fold at pH 7.0).

The important implications of these Ca$^{2+}$-dependent changes in ATP binding and the extremely high affinities for ATP prompted us to try to verify the photolabeling results by measuring ATP binding directly by filtration. This method is not generally applicable to expressed wild-type or mutant proteins, since each data point requires several μg of protein when the $K_D$ is in the micromolar range or above, and also there could be a problem with the specificity under these conditions considering the large number of proteins in the COS cell microsomes. Fig. 7 shows the results obtained at pH 7.0, and as can be seen extremely tight [γ-32P]ATP binding to mutants D351A and D351N could indeed be measured in the presence of Ca$^{2+}$. The $K_D$ values (0.0012 and 0.030 μM, respectively) are the same or very similar to those found by photolabeling (0.0014 and 0.028 μM, respectively). The binding in the absence of Ca$^{2+}$ was much weaker, and the data points are compatible with the $K_D$ values obtained by photolabeling (0.42 and 0.57 μM, respectively, Table II). It could be noted that the filtration experiments were performed in the absence of glycerol, and the similarity of the photolabeling and filtration measurements indicates that the cosolvent is not affecting the $K_D$ values significantly under these conditions. Using the filtration assay, we also obtained a few data points at pH 8.5, confirming that the addition of Ca$^{2+}$ increases the ATP affinity of mutant D351A and decreases the ATP affinity of mutant D351N (results not shown).

The increase in the affinity for TNP-8N3-ATP upon Ca$^{2+}$ binding to the transport sites of mutants D351N and D351A at
TABLE II
Effect of Mg²⁺, Ca²⁺, pH, and thapsigargin on nucleotide binding parameters of wild-type Ca²⁺-ATPase and mutants D351N and D351A

| Conditions | Ca²⁺-ATPase | Kᵣ₀/TNP-8N₃-ATP | Kᵣ₁(TNP) |
|------------|-------------|------------------|-----------|
| pH 8.5     |             |                  |           |
| MgCl₂      | WT          | 0.98             | 0.54      |
| EGTA*      | D351N       | 0.0063           | 0.0065    |
|            | D351A       | 0.040            | 0.025     |
| Thapsigargin* | WT        | 1.2              | 195       |
|            | D351N       | 0.096            | 5.0       |
|            | D351A       | 0.47             | 21        |
| CaCl₂      | WT          | 0.0058           | 0.028     |
|            | D351N       | 0.0035           | 0.0011    |
| EDTA*      | WT          | 0.11             | 18        |
|            | D351N       | 0.014            | 1.3       |
|            | D351A       | 0.050            | 1.1       |
| pH 7.0     |             |                  |           |
| MgCl₂      | WT          | 0.48             | 8.4       |
| EGTA*      | D351N       | 0.060            | 0.57      |
|            | D351A       | 0.21             | 0.42      |
| CaCl₂      | WT          | 0.0062           | 0.028     |
|            | D351N       | 0.0050           | 0.0014    |
| EDTA*      | WT          | 0.35             | 73        |
|            | D351N       | 0.037            | 1.1       |
|            | D351A       | 0.17             | 4.3       |

a Standard medium: 25 mM buffer, 20% (w/v) glycerol, 1 mM MgCl₂, 0.5 mM EGTA.
b WT, wild type.
c Standard medium with 0.01–0.09 μM thapsigargin (approximately 10 mol of thapsigargin/mol of Ca²⁺-ATPase).
d Medium: 25 mM buffer, 20% (w/v) glycerol, 1 mM MgCl₂, 50 μM CaCl₂.
e Medium: 25 mM buffer, 20% (w/v) glycerol, 2 mM EDTA.

Our results document the close interaction of bound nucleotides with Asp³⁵¹ and other residues in the conserved phosphorylation loop of sarcoplasmic reticulum Ca²⁺-ATPase. Elimination of the charge on Asp³⁵¹, as in mutations D351N, D351A, and D351T, enhanced the affinity for ATP and TNP-8N₃-ATP up to 156-fold in the absence of Ca²⁺ and even more in the presence of Ca²⁺, suggesting strong electrostatic effects between the γ-phosphate and the aspartate in the wild-type pump. In contrast, fairly conservative changes to Lys³⁵², Thr³⁵⁵, Leu³⁵⁶, and Thr³⁵⁷ impaired nucleotide binding to various extents, suggesting that these residues either directly ligate nucleotide or influence those that do. The removal of the positive charge of Lys³⁵² in mutant K352Q gave rise to a very pronounced decrease in ATP affinity of at least 1000-fold, again revealing the importance of electrostatic interactions at the active site. The disruptive mutations, as well as D351N and D351A, reduced or eliminated Cr₃ATP-induced Ca²⁺-ATPase activity.

Recently, bacterial halocid dehalogenases have been found to be homologous with P-type ATPases (35), and the crystal structures of the dehalogenases provide insights into possible Ca²⁺-ATPase structure. A catalytic aspartate of the dehalogenase is homologous to Asp³⁵¹ of Ca²⁺-ATPase and is positioned at the end of a β-strand, which is centrally located in a β-sheet (36). The critical aspartate leads into a spiral loop and hence to a secondary domain, which acts like a cap over the aspartate and active site. In the Ca²⁺-ATPase, the nucleotide substrate must be positioned between the two domains, but it is not yet clear how it would be oriented with respect to the aspartate and the phosphorylation loop. Crude modeling suggests that Lys³⁵² may be involved in direct interaction with the nucleotide, and the disruptive influence of even K352R on ATP binding is compatible with a role for this lysine in direct ligand binding. Leu³⁵⁶ and Thr³⁵⁷ do not appear to be able to interact directly with the nucleotide but rather appear to brace Asp³⁵¹ and Lys³⁵² and lead into the hinge segment between the main phosphorylation domain and the secondary cap domain. Mutation L356T was severely disruptive and seemed to prevent photoactivation of Lys⁴⁹² (expected to be part of the cap domain and some distance away from Leu³⁵⁶). If this is occurring by shifting the position of Lys⁴⁹², it would indicate interdomain communication between critical regions of the protein and point to Leu³⁵⁶ being in a pivotal position. Alternatively, the nucleotide affinity may have been reduced as a result of displacement of Asp³⁵¹ and Lys³⁵². Thr³⁵⁵ appears to play a minor role in ATP binding, and perhaps the ATP molecule is oriented away from this residue, and its apparently crucial role in phosphorylation (19) may be catalytic, a conclusion supported by the stronger conservation of this residue in both P-type pumps and dehalogenases (35).

Mutations D351N, D351A, and D351T generally caused large increases in affinity for TNP-8N₃-ATP and ATP, suggesting the existence of a strong electrostatic repulsion between the aspartate and the γ-phosphate group of ATP, which obscures a
high intrinsic binding energy. These results are in line with the findings of Pedersen et al. (6) on renal Na\(^{+}\),K\(^{+}\)-ATPase, which demonstrated increases of up to 39-fold in the absence of Mg\(^{2+}\) following mutation of the equivalent aspartate (Asp 369) to alanine. We obtained increases of 17- and 66-fold for mutations D351A and D351N, respectively, under comparable conditions at pH 7.0 in EDTA. In many of the conditions tested, we found larger affinity increases for the asparagine substitution than for the alanine substitution, in contrast to what was found for the Na\(^{+}\),K\(^{+}\)-ATPase, where the alanine substitution appears to

| Nucleotide/Asp351 interaction energies | Δ\(\Delta G_{\text{interaction}}\)^a | kcal/mol |
|---------------------------------------|----------------------------------|---------|
| pH 8.5 (thapsigargin)                | pH 7.0                           | pH 8.5  |
| N                                     | A'                               | N       | A     | N       | A     |
| TNP-SN\(_3\)-ATP                      |                                   | 1.3     | 0.43  | 1.2     | 0.47  |
| ATP                                   |                                   | 2.5     | 1.7   | 1.6     | 1.7   |
| MgTNP-SN\(_3\)-ATP                    | 1.5                               | 0.56    | 1.2   | 0.49    | 3.0   |
| MgATP                                 |                                   | 2.2     | 1.3   | 1.6     | 1.8   |
| MgADP                                 |                                   |         | 2.6   | 1.8     |       |
| MgAMPPCP                              |                                   |         |       | 2.0     |       |
| Ca\(^{2+}\) + MgATP                   |                                   |         |       | 2.6     |       |

\(\Delta G_{\text{interaction}} = -RT \ln \frac{K_{D_{\text{mutant}}}}{K_{D_{\text{wild type}}}}, R = \text{gas constant, } T = 298 \text{ K.}\)

\(N, \text{ calculations with mutant D351N.}\)

\(A, \text{ calculations with mutant D351A.}\)

\(d, \text{ Calculated on basis of a } K_{D_{\text{pH}}} \text{ of 3 } \mu\text{M for the wild type (8).}\)

**Fig. 7.** ATP binding to mutants D351N (closed symbols) and D351A (open symbols) determined by filtration. Binding of \([\gamma^{32P}]\text{ATP}\) was measured with 0.5–0.6 pmol of Ca\(^{2+}\)-ATPase in 50 mM MOPS/TMAH, pH 7.0, 1 mM MgCl\(_2\), 1 mM \([\text{H}]\text{sucrose, either 0.1 mM CaCl}_2 (\text{circles})\) or 1 mM EGTA (triangles), and variable concentrations of \([\gamma^{32P}]\text{ATP}\) as indicated. The duplicate data points at each concentration of ATP were from separate experiments performed on different days. The data points in the presence of Ca\(^{2+}\) were fitted to the Hill equation with the Hill coefficient set to 1 and yielded \(K_D\) values of 0.030 and 0.0012 \(\mu\text{M}\) for mutants D351N and D351A, respectively. Similar \(K_D\) values were obtained on two different preparations of mutants D351N and D351A.

**Fig. 8.** Ca\(^{2+}\) dependence of TNP-SN\(_3\)-ATP photolabeling of mutants D351N (●) and D351A (△) at pH 7.0. Photolabeling was performed as in Fig. 1 except that the buffer contained 150 mM MOPS (pH 7.0), 5 mM EGTA, and variable calcium concentrations to yield the free Ca\(^{2+}\) concentrations shown. The concentration of \([\gamma^{32P}]\text{TNP-SN}_3\)-ATP was 10 and 30 nM for mutants D351N and D351A, respectively. The data were fitted to the Hill equation and offset (mutant D351N: \(K_{0.5} = 0.10 \mu\text{M, } n_H = 1.3\); mutant D351A: \(K_{0.5} = 0.05 \mu\text{M, } n_H = 1.3\)).
be the more effective. It may furthermore be noted that the Na\(^+\),K\(^-\)-ATPase D369N mutant showed no significant change (or possibly a slight decrease) in affinity for ADP relative to the wild type, whereas a 30-fold increase in affinity for MgADP was demonstrated for the corresponding Ca\(^{2+}\)-ATPase mutant. This difference may possibly be ascribed to the presence of Mg\(^{2+}\) (see below).

The results obtained with mutant D351N in the absence of Ca\(^{2+}\) reveal that the increase in nucleotide affinity induced by the mutation (nucleotide-Asp\(^{351}\) interaction energies, ΔΔG\(_{inter}\) action, Table III) is largest at alkaline pH in the presence of Mg\(^{2+}\). Mg\(^{2+}\) increases the interaction energy (or electrostatic repulsion) between the γ-phosphoryl group and Asp\(^{351}\) at pH 8.5 but has little effect at pH 7.0. Since the E1 form of the pump is expected to prevail at pH 8.5, whereas the E2H and E2H\(_3\) forms accumulate at pH 7.0 when Ca\(^{2+}\) is absent (7), our data suggest that the Mg\(^{2+}\) effect depends on the enzyme being in the E1 form.

ATP has two negative charges on the γ-phosphoryl group at pH 8.5 and is partially protonated at pH 7.0 (pK\(_a\) = 6.63), whereas MgATP can be expected to have a single negative charge at both pH values (pK\(_a\) = 4.72) (37). Mg\(^{2+}\) might have been expected to decrease the electrostatic interaction at both pH values. Also, Mg\(^{2+}\) is expected to polarize the P–O bond, withdrawing electronegativity from the phosphorus atom, which should further reduce the repulsion. But these ameliorating effects of Mg\(^{2+}\) appear to be counteracted by an increased binding interaction and a rise in repulsion as reflected in the change in interaction energy from 1.6 kcal/mol to 2.6 kcal/mol, resulting from Mg\(^{2+}\) binding at pH 8.5 in the case of mutant D351N. This is probably the result of Mg\(^{2+}\) assisting a close approach of the γ-phosphoryl group and the carboxylate anion in the E1 state.

In the presence of Mg\(^{2+}\), the decrease in nucleotide-Asp\(^{351}\) interaction energy observed for the D351N mutant when the pH is reduced from 8.5 to 7.0 is roughly equivalent to the decrease seen upon thapsigargin binding or replacement of MgATP with MgADP. This is consistent with the hypothesis that, in the first two instances, the γ-phosphoryl group is withdrawn 3–4 Å from Asp\(^{351}\), or approximately the length of a phosphate group. Since E2 states are expected to prevail both at the lower pH and in the presence of thapsigargin, the displacement of nucleotide from the phosphorylation loop under these conditions is compatible with other findings that ATP and P\(_i\) can bind simultaneously at the active site in E2 states (10–14).

The ATP-Asp\(^{351}\) interaction energy calculated for mutant D351A at alkaline pH in the presence of Mg\(^{2+}\) is similar to the interaction energy calculated for D351N at pH 7.0 in the presence of Mg\(^{2+}\). For mutant D351A, there is furthermore little effect of reducing the Mg\(^{2+}\) concentration or pH. The reason could be that mutation D351A to some extent counteracts the influence of alkaline pH on the E1-E2 conformational equilibrium in the absence of Ca\(^{2+}\). This would be equivalent to the displacement of the equilibrium in favor of E2 reported for the D369A mutation in the Na\(^+\),K\(^-\)-ATPase (6).

A central observation in the present study is that Ca\(^{2+}\) binding to the transport sites causes a substantial increase in the intrinsic nucleotide binding energy of either of the mutants D351N and D351A at pH 7.0 and of D351A at pH 8.5. The same results were obtained both with the photolabeling assay and a direct filtration assay. In the case of mutant D351A at pH 7.0, the affinity for MgATP is increased 300-fold upon Ca\(^{2+}\) binding. The affinity of the wild-type Ca\(^{2+}\)-ATPase for MgATP in the presence of Ca\(^{2+}\) cannot be measured directly in equilibrium binding experiments due to the activation of phosphoryl

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**ASSOCIATIVE TRANSITION STATE**

\[
\text{AMP-}O-P-O'\quad \begin{array}{c}
\text{O'}
\end{array}
\quad \text{O-} \quad \text{C-CH}_2
\]

**DISSOCIATIVE TRANSITION STATE**

\[
\text{AMP-}O-P-O'\quad \begin{array}{c}
\text{O'}
\end{array}
\quad \text{O-} \quad \text{C-CH}_2
\]

**SCHEME 2**

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The Ca\(^{2+}\) activation of phosphorylation in the wild-type Ca\(^{2+}\)-ATPase and of nucleotide binding with very high affinity in the D351A and D351N mutants implies that signals originating in the Ca\(^{2+}\) transport sites in the membrane are conveyed by protein conformational changes over long distance to the catalytic site in the cytoplasmic protein domain. Signals originating at the catalytic site are likewise transmitted in the reverse direction from the cytoplasmic portion down the stalk to the Ca\(^{2+}\) sites (5, 38). Hence, in the wild-type enzyme the phosphorylation of Asp\(^{351}\) (or perhaps already the formation of the transition state) leads to occlusion of Ca\(^{2+}\). The occluded Ca\(^{2+}\) is released from the phosphoenzyme only following conformational changes that open the binding pocket toward the luminal side of the membrane (38, 39). An intriguing question was, therefore, whether mutations D351N and D351A elicited events at the catalytic site that were transmitted to the membrane domain, affecting the Ca\(^{2+}\) binding properties of the transport sites. We tested the affinity for Ca\(^{2+}\) in mutants.
D351A mutants seem to suggest that Asp351 is critical for the phosphorylated state (38). Our findings with the D351N and D351A mutants indicate that the transition state, or the stably occluded (24–27). The complex with CrATP may mimic the catalytic transition state, thereby facilitating the phosphoryl transfer. The Ca2+-induced conformational change could involve the repositioning of a positively charged residue, such as a lysine, in proximity to the γ-phosphate and Asp351 to create a salt linkage with the phosphate. This mechanism finds support in the Ca2+-dependent cross-linking of a lysyl residue to Asp351 (41) and the involvement of a lysyl residue in the catalytic mechanism of halophilic dehalogenases (35). In the Ca2+-ATPase, the events at the catalytic site elicit further conformational changes in the Ca2+ binding domain that increase the Ca2+ affinity and bring about occlusion of the Ca2+ sites.

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REFERENCES
1. Hasselbach, W., and Makinose, M. (1961) Biochim. Biophys. Acta 333, 518–528
2. MacLennan, D. H., Brandl, C. J., Korrak, B., and Green, N. M. (1985) Nature 316, 696–700
3. Levy, D., Seigneuriet, M., Bluzat, A., and Bigaud, J.-L. (1990) J. Biol. Chem. 265, 19524–19534
4. Degani, C., and Boyer, P. D. (1973) J. Biol. Chem. 248, 8222–8226
5. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) J. Biol. Chem. 272, 28815–28818
6. Pedersen, P. A., Rasmussen, J. H., and Jorgensen, P. L. (1996) Biochemistry 35, 16085–16093
7. Vorte, F., Minz, E., and Guilain, F. (1990) J. Biol. Chem. 265, 19535–19540
8. LeCarpentier, J.-D., and Guilain, F. (1990) Eur. J. Biochem. 211, 117–126
9. Lacapére, J.-J., Bennett, N., Dupont, Y., and Guilain, F. (1990) J. Biol. Chem. 265, 345–353
10. Pickart, C. M., and Jencks, W. P. (1984) J. Biol. Chem. 259, 1629–1643
11. Chameph, P., and Riollet, S., Orlowski, S., Guilain, F., Seebregts, C. J., and McIntosh, D. B. (1988) J. Biol. Chem. 263, 12288–12294
12. McIntosh, D. B., and Boyer, P. D. (1985) Biochemistry 24, 2867–2875
13. Andersen, J. P., and Moller, J. V. (1985) Biochim. Biophys. Acta 815, 9–15
14. McIntosh, D. B., Woolley, D. G., and Berman, M. C. (1992) J. Biol. Chem. 267, 5301–5309
15. McIntosh, D. B., and Woolley, D. G. (1994) J. Biol. Chem. 269, 21587–21595
16. McIntosh, D. B., Woolley, D. G., Vilen, B., and Andersen, J. P. (1996) J. Biol. Chem. 271, 25778–25789
17. Green, N. M. (1989) Biochem. Soc. Trans. 17, 970–972
18. Maruyama, K., and MacLennan, D. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3314–3318
19. Maruyama, K., Clarke, D. M., Fujii, J., Inesi, G., Luo, T. W., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 13038–13042
20. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. B. (1989) Mol. Cell. Biol. 9, 946–958
21. Vilen, B., Andersen, J. P., and MacLennan, D. H. (1991) J. Biol. Chem. 266, 16157–16164
22. Seebregts, C. J., and McIntosh, D. B. (1989) J. Biol. Chem. 264, 2043–2052
23. Fabiato, A., and Fabiato, F. (1979) J. Physiol. 275, 463–505
24. Vilen, B., and Andersen, J. P. (1992) J. Biol. Chem. 267, 25739–25743
25. Serperus, E. H., Kirch, U., and Schoner, W. (1992) Eur. J. Biochem. 212, 347–354
26. Vilen, B., and Andersen, J. P. (1986) Biochim. Biophys. Acta 855, 429–431
27. Vilen, B., and Andersen, J. P. (1992) J. Biol. Chem. 267, 3539–3550
28. Kajigaya, Y., Wade, J. B., and Inesi, G. (1991) J. Biol. Chem. 266, 1286–1292
29. Meissner, G. (1973) Biochim. Biophys. Acta 298, 906–926
30. Reinstein, J., and Jencks, W. P. (1993) Biochemistry 32, 6632–6642
31. Suzuki, H., and Kanazawa, T. (1996) J. Biol. Chem. 271, 5481–5486
32. Inesi, G., Kurzmack, M., Coan, C., and Lewis, D. E. (1980) J. Biol. Chem. 255, 3025–3031
33. Minz, E., Mata, A. M., Forge, V., Passafiume, M., and Guilain, F. (1995) J. Biol. Chem. 270, 27160–27164
34. Fersht, A. R. (1988) Biochemistry 27, 1577–1580
35. Arvidson, L., Palzer, K., and Koop, E. (1998) Trends. Biochem. Sci. 23, 127–129
36. Hisano, T., Hata, Y., Fujii, T., Liu, J.-Q., Kuribara, T., Ezaki, N., and Soda, K. (1996) J. Biol. Chem. 271, 20322–20330
37. Pecorara, V. L., Hermes, J. D., and Cleland, W. W. (1984) Biochemistry 23, 5262–5271
38. Vilen, B. (1995) Acta Physiol. Scand. 154, Suppl. 87, 1–146
39. McIntosh, D. B., Ross, D. C., Chample, P., and Guilain, F. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 6437–6441
40. Wyman, J. (1964) Adv. Protein Chem. 19, 223–238
41. Gutowsky-Eckel, Z., Karlheinz, M., and Baumert, H. G. (1993) FEBS Lett. 324, 314–318