Modulatory ATP Binding Affinity in Intermediate States of E2P Dephosphorylation of Sarcoplasmic Reticulum Ca$^{2+}$-ATPase*§

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The mechanism of ATP modulation of E2P dephosphorylation of sarcoplasmic reticulum Ca$^{2+}$-ATPase wild type and mutant forms was examined in nucleotide binding studies of states analogous to the various intermediates of the dephosphorylation reaction, obtained by binding of metal fluorides, vanadate, or thapsigargin. Wild type Ca$^{2+}$-ATPase displays an ATP affinity of 4 μM for the E2P ground state analog, 1 μM for the E2P transition state and product state analogs, and 11 μM for the E2 dephosphoenzyme. Hence, ATP binding stabilizes the transition and product states relative to the ground state, thereby explaining the accelerating effect of ATP on dephosphorylation. Replacement of Phe$^{387}$ (N-domain) with serine, Arg$^{500}$ (N-domain) with leucine, or Arg$^{274}$ (A-domain) with alanine or glutamate reduces ATP affinity in all E2/E2P intermediate states. Alanine substitution of Ile$^{188}$ (A-domain) increases the ATP affinity, although ATP acceleration of dephosphorylation is disrupted, thus indicating that the critical role of Ile$^{188}$ in ATP modulation is mechanistically based rather than being associated with the binding of nucleotide. Mutants with alanine replacement of Lys$^{905}$ (A-domain) or Glu$^{439}$ (N-domain) exhibit an anomalous inhibition by ATP of E2P dephosphorylation, due to ATP binding increasing the stability of the E2P ground state relative to the transition state. The ATP affinity of Ca$_2$E$_2$P, stabilized by inserting four glycines in the A-M1 linker, is similar to that of the E2P ground state, but the Ca$^{2+}$-free E1 state of this mutant exhibits 3 orders of magnitude reduction of ATP affinity.

The sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (Ca$^{2+}$-ATPase)$^3$ is a membrane-bound P-type ATPase that translocates Ca$^{2+}$ from the cytosol to the endoplasmic reticulum, thereby allowing rapid oscillations of Ca$^{2+}$ during cellular activation events. Insight into the structural organization of the Ca$^{2+}$-ATPase has come from the elucidation of several crystal structures at atomic resolution, representing the pump in various intermediate states (reviewed in Refs. 1 and 2). The membrane-buried region of the Ca$^{2+}$-ATPase is made up of 10 membrane spanning helices and is connected to a large cytoplasmic headpiece, which is further separated into three distinct domains, denoted A ("actuator"), P ("phosphorylation"), and N ("nucleotide binding"). Ca$^{2+}$ transport is achieved by means of a reaction cycle (Scheme 1) involving the formation and decay of a phosphorylated intermediate and extensive protein conformational changes between four major states, E1, E1P, E2P, and E2. The catalytic function in E1 (autokinase activity) and E2P (autophosphatase activity) as well as the movement of Ca$^{2+}$ ions across the membrane can be understood on the basis of the sequential gathering and displacement of certain conserved amino acid motifs of the N- and A-domains relative to the catalytic site in the P-domain and the coupling of these events to rearrangements of the transmembrane helices containing the Ca$^{2+}$ sites. In the E1 and E1P states, the highly conserved 181$^{T}$GES loop of the A-domain is distant from the catalytic center containing nucleotide binding residues and the phosphorylated Asp$^{151}$ of the P-domain, which in this condition is able to react with ATP/ADP. However, during the Ca$_2$E$_1$P → E2P transition the A-domain rotates ~90° around an axis nearly perpendicular to the membrane, thereby moving the 181$^{T}$GES loop into close contact with the catalytic site, such that Glu$^{183}$ can catalyze dephosphorylation of E2P by hydrolysis (3–5). During the dephosphorylation, Glu$^{183}$ likely coordinates the water molecule attacking the aspartyl phosphoryl bond and withdraws a hydrogen.

ATP in addition to being the substrate in the phosphorylation of the Ca$_2$E1 state also functions in a non-phosphorylating mode (boxed ATP in Scheme 1), enhancing the rates of the steps involved in phosphoenzyme turnover (Ca$_2$E1P → E2P and E2P → E2) as well as the E2 → Ca$_2$E1 transition of the dephosphoenzyme (6–17). The mechanisms underlying these modulatory effects of ATP remain largely unresolved. A debated issue is whether the modulatory ATP molecule binds at the same site as the phosphorylating ATP or at a distinct, "allosteric" site (14, 18–23). During dephosphorylation the 181$^{T}$GES loop of the A-domain occupies the position in close contact with the P-domain taken up by part of the ATP and ADP in Ca$_2$E1 and Ca$_2$E1P, however, ATP may still bind to residues of the N- and
A-domains under these conditions. Recently, mutagenesis studies have pinpointed several amino acid residues in the N- and A-domains as critical for the modulatory effects of ATP and have provided evidence of an overlap between the catalytic and modulatory ATP binding sites (16, 17, 24), i.e. favoring the existence of a single site that exhibits a high degree of plasticity and flexibility, being reconfigured from slightly different positions of the P-, N-, and A-domains in the conformational states occurring during the transport cycle. The conformational transitions of the cycle likely change the affinity of the site for ATP, thus explaining the enhancing effects of ATP on the kinetics. Hence, the forward flow of a reversible reaction will be enhanced by ATP, if the product state possesses higher affinity for ATP than the ground state (23). Moreover, a relative stabilization of the transition state of a partial reaction of the cycle, leading to acceleration of this reaction, would be achieved in the presence of ATP, if ATP bound with higher affinity to the transition state than to the corresponding ground state. In the present study, we have focused on the role of ATP as a modulator of E2P dephosphorylation. We present, for the first time, direct measurements of the ATP affinity of wild type and mutants Ca2+-ATPases stabilized in states analogous to the various intermediate forms occurring during the E2P dephosphorylation reaction sequence. This was achieved by use of the metal-fluoride compounds BeF, AlF, and MgF (Scheme 2) to mimic the phosphoryl group in the ground, transition, and product states of E2P dephosphorylation, respectively (4, 5, 25, 26), and vanadate, which like AlF is thought to mimic the bipyramidal transition state of the phosphoryl group (27, 28). The ATP affinity was also determined for a stable form of the E2 dephosphorylation sequence and allows distinction between roles in nucleotide binding and in mediating the response to binding that accelerates the dephosphorylation.

**EXPERIMENTAL PROCEDURES**

The cDNA encoding the mutant Ca2+-ATPases studied in the present work was the same as that applied in our previous studies (16, 17, 33–35). The cDNA was inserted into the expression vector pMT2 (36). To express wild type or mutant cDNA, COS-1 cells were transfected using the calcium phosphate precipitation method (37). Microsomal vesicles containing either expressed wild type or mutant Ca2+-ATPase were isolated by differential centrifugation (38). SR vesicles isolated from rabbit hind leg muscles (prepared as described in Refs. 39 and 40) were a gift from Dr. Philippe Champeil (Saclay, France). The concentration of expressed Ca2+-ATPase was determined by an enzyme-linked immunosorbent assay (41) and by measurement of the maximum capacity for phosphorylation with ATP or Pi ("active site concentration" (42)). As previously described, the expression levels of the mutants were similar to that of the wild type (16, 17, 33, 34). The amount of endogenous endoplasmic reticulum Ca2+-ATPase present in the preparation is less than 1% that of the exogenous expressed enzyme, hence labeling corresponding to endogenous Ca2+-ATPase is negligible (cf. Fig. 1 of Ref. 33, compare "wild type" with "control").

Formation of the complexes of SR or expressed wild type or mutant Ca2+-ATPase in the E2 state with Tg, MgF, AlF, vanadate, and BeF prior to photolabeling was achieved by pre-equilibration of the enzyme for 30 min at 25 °C in 25 mM MOPS/tetramethyl ammonium hydroxide (pH 7.0), 80 mM KCl, 2 mM EGTA, and the concentrations of inhibitors and co-factors indicated as follows: Tg, 1 μM; MgF, 5 mM MgCl2 and 5 mM NaF; vanadate, 0.1 mM orthovanadate and 5 mM MgCl2; AlF, 0.5 mM AlCl3, 2 mM NaF, and 0.2 mM MgCl2; BeF, 0.1 mM BeSO4, 2 mM NaF, and 0.2 mM MgCl2. The enzyme-inhibitor complexes were formed immediately prior to the initiation of the photolabeling experiments and kept on ice throughout (<1 h). The inhibited state of wild type and mutants at the inhibitor concentrations applied, as well as the stability of the enzyme-inhib-
**ATP Binding to Intermediate States of Ca\(^{2+}\)-ATPase**

![Scheme 3. TNP-8N\(_3\)-ATP photolabeling reaction.](image)

When the azide of TNP-8N\(_3\)-ATP is exposed to ultraviolet light, it forms a highly reactive but short-lived nitrene that can initiate reactions with neighboring reactive groups, such as the amino group of the Lys\(^{992}\) side chain, thereby forming a stable covalent bond between the nucleotide and the protein (20, 45, 46). R\(_1\) represents the TNP-ATP moiety of TNP-8N\(_3\)-ATP (see supplemental Fig. S4), and R\(_2\)-NH\(_2\) is the Lys\(^{992}\) side chain of the Ca\(^{2+}\)-ATPase.

The synthesis of the [\(^{32}\)P]TNP-8N\(_3\)-ATP photolabel, its application as a specific photolabel of the Ca\(^{2+}\)-ATPase (Scheme 3), the competitive inhibition by ATP of [\(^{32}\)P]TNP-8N\(_3\)-ATP photolabeling, and the quantification of [\(^{32}\)P]ATP were carried out by acid quenching followed by acid SDS-polyacrylamide gel electrophoresis and quantification of the radioactivity associated with the Ca\(^{2+}\)-ATPase band, using the previously established procedures (16, 17, 43). To study the ATP concentration dependence of phosphorylation, microsomes were incubated for 15 s at 0 °C in 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl\(_2\), 100 \(\mu\)M CaCl\(_2\), and varying concentrations of [\(^{32}\)P]ATP. For studies of the ATP dependence of dephosphorylation of E2P, phosphorylation with 0.5 mM [\(^{32}\)P]ATP was carried out for 10 min at 25 °C in 100 mM MES/Tris (pH 6.0), 10 mM MgCl\(_2\), 2 mM EGTA, and 30% (v/v) dimethyl sulfoxide. The phosphorylated sample was chilled in ice water, and dephosphorylation was followed at 0 °C by a 19-fold dilution into ice-cold medium containing 50 mM MOPS/Tris (pH 7.0), 2 mM EGTA, 10 mM EDTA, 5 mM H\(_3\)PO\(_4\), and various concentrations of ATP.

The data were analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.). The analysis of the TNP-8N\(_3\)-ATP photolabeling data were based on the hyperbolic function,

\[
Y = Y_{max} \times \frac{[\text{TNP-8N}_3\text{-ATP}]/(K_{0.5} + [\text{TNP-8N}_3\text{-ATP}])}{1 + [\text{TNP-8N}_3\text{-ATP}]/(K_{0.5} + [\text{ATP}]^{n})}
\]

The analysis of the ATP inhibition of TNP-8N\(_3\)-ATP photolabeling was based on the Hill equation modified to describe inhibition, where \(n\) is the Hill coefficient (varying between 0.6 and 1.1 for the present data). The “true” dissociation constant, \(K_{DP}\), for ATP binding was calculated from the measured \(K_{0.5}\) values using the validated equation for competitive inhibition (33).

The analysis of the ATP dependence of phosphorylation from [\(^{32}\)P]ATP was based on the Hill equation, \(EP = EP_{max} \times [\text{ATP}]^{n}/[K_{0.5} + [\text{ATP}]^{n})\). For analysis of the modulatory effect of ATP on the rate of E2P dephosphorylation, the ATP concentration dependence of the rate constant was analyzed according to the hyperbolic function, \(k_{obs} = k_0 + (k_{max} - k_0) \times [\text{ATP}] / (K_{0.5} + [\text{ATP}])\), in which \(k_{obs}\) is the rate constant observed at the indicated ATP concentration, \(k_0\) is the rate constant in the absence of ATP, and \(k_{max}\) is the extrapolated value of the rate constant corresponding to infinite ATP concentration (17).

The experiments were conducted at least twice on independent microsomal preparations, and average values are shown.

**RESULTS AND DISCUSSION**

TNP-8N\(_3\)-ATP Photolabeling of Wild Type Ca\(^{2+}\)-ATPase in E2-Tg, E2-MgF, E2-Vanadate, E2-AIF, and E2-BeF States—To study the interaction of nucleotides with the wild type Ca\(^{2+}\)-ATPase in stable analog forms of the intermediate states occurr-
ring during E2P dephosphorylation, SR vesicles or microsomes containing expressed enzyme were incubated with saturating concentrations of Tg, MgF, vanadate, AlF, or BeF and subjected to nucleotide binding analysis by TNP-8N3-ATP photolabeling of Lys492 as previously described for the E1 form (33). The formation of the complex with metal fluoride or vanadate took place in the presence of Mg2+, whereas subsequent photolabeling was carried out in medium without free Mg2+ (EDTA added), considering that the substrate that binds to E2P with reasonable affinity and accelerates E2P dephosphorylation is metal-free ATP (10, 14, 15). Enzyme with Tg bound and enzyme in the E1 form was photolabeled either in the absence or presence of Mg2+. In the latter case, EGTA was present to specifically chelate Ca2+, because it was essential to remove Ca2+ to prevent enzyme activation and consequent hydrolysis of the photolabel and ATP. Photolabeling was carried out at a rather high pH of 8.5 to prevent unspecific labeling (33) and to ensure that even in the absence of Ca2+ the enzyme without Tg or metal fluoride bound resides predominantly in the E1 state rather than E2 (44). We were concerned that the enzyme-inhibitor complexes remained stable during photolabeling, and by studying the time course of reactivation following addition of Ca2+, evidence was obtained that all five enzyme-inhibitor complexes were very stable under the photolabeling conditions, despite the high pH of the medium (supplemental Fig. S2).

The time dependence of photolabeling of expressed wild type Ca2+-ATPase pre-equilibrated with or without inhibitor is shown in Fig. 1A. With the current irradiation setup, photolabeling proceeded at a rate of ~0.3 min−1 irrespective of the enzyme conformational state/inhibitor bound. The photolabeling rate of ~0.3 min−1 is comparable with the rate of photolysis of the azido group in TNP-8N3-ATP of ~2 min−1, determined using the same irradiation setup, cf. supplemental Fig. S4, implying that for Ca2+-ATPase with or without bound fluoride complex or vanadate the rate-limiting step in the labeling reaction is the formation of the reactive nitrene (cf. Scheme 3). The subsequent chemical reaction between the nitrene of the photoactivated nucleotide and Lys492 of the Ca2+-ATPase (Scheme 3) is likely much faster, given the typical short lifetime and high reactivity of nitrene intermediates (45, 46). Based on the time dependence of TNP-8N3-ATP photolabeling of the Ca2+-ATPase (Fig. 1A) as well as the time dependence of TNP-8N3-ATP photolysis (supplemental Fig. S4), a pre-steady state irradiation time of 35 s was chosen for all subsequent photolabeling experiments.

The maximum levels of photolabeling (corresponding to saturation with photolabel) of the expressed wild type Ca2+-ATPase as well as SR were ~3.5-fold higher in the E2-vanadate, E2-AlF, and E2-MgF states and ~1.7-fold higher in the E2-BeF state than in E2-Tg or E1 (Fig. 1). The labeling stoichiometry corresponding to the highest labeling levels indicated as 100% in Fig. 1 can be roughly estimated to be ~0.7 mol of label incorporated per mol of Ca2+-ATPase present in the microsomal membrane, assuming that no label or protein is lost during gel electrophoresis. The higher maximum levels of photolabeling in the vanadate- and metal-fluoride-complexed states as compared with E2-Tg or E1 do not result from additional labeling of other residues than Lys492, because no labeling of the mutant K492L was seen in any of the enzyme states examined (Table 1 and supplemental Fig. S5). Rather, the increased labeling levels may reflect a conformational change resulting in shortening of the interaction distance between Lys492 and the azido group of TNP-8N3-ATP and/or desolvation of the intervening space thereby removing competing water molecules and increasing the nucleophilicity of the amino group, resulting in increased efficiency of the coupling reaction between Lys492 and the reactive nitrene of the photolabel following UV irradiation (cf. Scheme 3). Apart from this clue, little detail is known about the interactions of the photolabel in the various conformational states. Although competition of the photolabel with ATP for binding indicates that there is at least a partial overlap of binding sites, it is also clear from the difference in mutational effects on affinities for photolabel and ATP that the photolabel must bind rather differently from ATP (see below, compare Tables 1 and supplemental Fig. S5).
TABLE 1

Affinity for TNP-8N3-ATP of SR and expressed wild type Ca2+-ATPase and mutants in various stabilized states

| Wild type | 100 × 14 (n = 4) | 100 × 12 (n = 6) | 100 × 10 (n = 8) | 100 × 9 (n = 9) | 100 × 14 (n = 7) | 100 × 18 (n = 5) |
|-----------|----------------|----------------|----------------|----------------|----------------|----------------|
| E2 (Tg)   | (62 ns)        | (10.5 ns)      | (9.6 ns)       | (11.6 ns)      | (54 ns)        | (290 ns)       |
| E2-P (MgF)| EDTA           | EDTA           | EDTA           | EDTA           | EDTA           | EDTA           |
| E2-P (vanadate) | 74 ± 7 (n = 4) | 69 ± 10 (n = 3) | 255 ± 7 (n = 2) | 255 ± 7 (n = 2) | 795 ± 27 (n = 2) | 73 |
| E2-P (AlF) | 100 ± 10 (n = 2) | 100 ± 16 (n = 2) | 122 ± 18 (n = 2) | 68 ± 12 (n = 2) | 13 (n = 2) | 80 |
| E2-P (BeF) | 76 ± 5 (n = 2) | 69 ± 6 (n = 2) | 19 ± 2 (n = 2) | 68 |
| E2 (Tg)   | 90 ± 16 (n = 2) | 89 ± 15 (n = 3) | 101 ± 1 (n = 2) | 81 ± 1 (n = 2) | 88 ± 1 (n = 2) | 25 |
| E2-P (MgF)| EDTA           | EDTA           | EDTA           | EDTA           | EDTA           | EDTA           |
| E2-P (vanadate) | 108 ± 3 (n = 2) | 120 ± 2 (n = 2) | 702 ± 109 (n = 2) | 85 |
| E2-P (AlF) | No labeling    | No labeling    | No labeling    | No labeling    | No labeling    | No labeling    |
| E2-P (BeF) | 81 ± 2 (n = 2) | 52 ± 8 (n = 2) | 100,000 (n = 2) | 100 |
| E2 (Tg)   | 965 ± 107 (n = 3) | 116 ± 12 (n = 2) | 108 ± 3 (n = 2) | 120 ± 2 (n = 2) | 702 ± 109 (n = 2) | 85 |
| E2-P (MgF)| EDTA           | EDTA           | EDTA           | EDTA           | EDTA           | EDTA           |
| E2-P (vanadate) | 96 ± 6 (n = 3) | 52 ± 6 (n = 4) | 60 ± 6 (n = 3) | 60 ± 6 (n = 3) | 78 |
| E2-P (AlF) | No labeling    | No labeling    | No labeling    | No labeling    | No labeling    | No labeling    |
| E2-P (BeF) | 52 ± 8 (n = 2) | 100,000 (n = 2) | 100 | 120 |
| E2 (Tg)   | 151 ± 23 (n = 2) | 83 ± 2 (n = 2) | 96 ± 5 (n = 2) | 106 ± 2 (n = 2) | 83 ± 7 (n = 2) | 96 ± 17 (n = 2) |
| E2-P (MgF)| EDTA           | EDTA           | EDTA           | EDTA           | EDTA           | EDTA           |
| E2-P (vanadate) | 95 ± 10 (n = 3) | 81 ± 1 (n = 2) | 64 ± 2 (n = 3) | 138 ± 5 (n = 3) | 1250 |
| E2-P (AlF) | No labeling    | No labeling    | No labeling    | No labeling    | No labeling    | No labeling    |
| E2-P (BeF) | 52 ± 8 (n = 2) | 100,000 (n = 2) | 100 | 120 |

a EDTA refers to the condition without free Mg2+ present described under "Experimental Procedures."

b EDTA/Mg2+ refers to the condition with free Mg2+ present (E1 state) described under "Experimental Procedures." Except in the case of mutant 4Gi-46/47, the data shown in this column have been published previously (16, 17, 33, 34) and are included for comparison.

d and 2, the difference is particularly striking for mutants R489L, K205A, L562F, and R489L.

Nucleotide Affinity of Wild Type Ca2+-ATPase in E2-Tg, E2-MgF, E2-Vanadate, E2-AlF, and E2-BeF States—We then proceeded to study the TNP-8N3-ATP concentration dependence of photolabeling of SR and expressed wild type Ca2+-ATPase in the E2-Tg, E2-MgF, E2-vanadate, E2-AlF, and E2-BeF states (Fig. 2, left panels). The TNP-8N3-ATP affinity of the expressed wild type Ca2+-ATPase with thapsigargin bound was 62 nm (Fig. 2, upper left panel, and Table 1). A similar affinity of 54 nm (although, as noted above, with a 1.7-fold higher maximum labeling level) was obtained for the E2-BeF state of the expressed wild type. In contrast, the TNP-8N3-ATP affinities of the E2-MgF, E2-vanadate, and E2-AlF states of the expressed wild type were significantly higher, in the 9–12 nm range (Table 1), possibly due to the same conformational change that results in the ~3.5-fold higher maximal labeling levels described above. In comparison, the affinity for TNP-8N2-EATP of the uncomplexed wild type in the presence of Mg2+ (E1 state) was 290 nm (Table 1). The TNP-8N3-ATP affinities of SR with bound Tg, MgF, vanadate, AlF, and BeF were rather similar to Aarhus were 0.29 and 0.14 μM (Tables 1 and 2), respectively, the previously published values being 0.79 and 0.51 μM, respectively. Similarly, in the E2-Tg state, the affinities of the expressed wild type for TNP-8N3-ATP and ATP measured in Aarhus were 62 nm and 10.8 μM (Tables 1 and 2), respectively, the previously published values being 150 nm and 20 μM, respectively (16). We do not presently know the exact reason for the difference.
those obtained with the expressed wild type (compare Fig. 2, upper left and lower left panels).

Fig. 2, right panels, illustrates the inhibition by ATP of TNP-8N₃-ATP photolabeling, showing also that the ATP affinity of the E₂/vanadate, E₂/AlF, and E₂/MgF forms of expressed wild type Ca²⁺-ATPase or SR Ca²⁺-ATPase is much higher (Kₐ₁ ~ 1 μM) than that of E₂/Tg (Kₐ₁ 11 μM), although not nearly as high as the affinity of the E₁ state (Kₐ₁ 0.14 μM).⁴ In E₂/BeF, the affinity for ATP was of an intermediate magnitude (Kₐ₁ 4 μM). These data are summarized in Table 2. The higher ATP affinity of the E₂P transition state analogs E₂/vanadate and E₂/AlF, as compared with the E₂P ground state analog, E₂/BeF, and the E₂ dephosphoenzyme, makes it conceivable that stimulation of E₂P dephosphorylation by ATP is accomplished by increasing the stability of the transition state of E₂P dephosphorylation, thereby lowering the energy barrier for formation of the transition state. The binding of the modulatory ATP may lead to a more compact packing of the A-, P-, and N-domains and to an optimal positioning of Glu¹₈³ in the ¹₈¹TGES motif of the A-domain for coordinating the attacking water molecule during dephosphorylation (3–5) (cf. Fig. 3). In addition, stabilization of the product state E₂Pi, as evidenced by the high ATP affinity of the E₂-MgF complex, could be of importance for the modulatory effect on the dephosphorylation.

### ATP Binding to Intermediate States of Ca²⁺-ATPase

**Nucleotide Binding to E₂-Tg-AlF**—Thapsigargin has been widely applied in crystallization studies of the Ca²⁺-ATPase either without (30, 47) or with additional inhibitors such as MgF (5), AlF (4, 48), or BeF (48). To address the issue whether thapsigargin binding influences the nucleotide affinity of the metal fluoride complex, we measured TNP-8N₃-ATP and ATP binding to enzymes complexed by both Tg and AlF (Fig. 4). SR vesicles were incubated under optimal conditions for forming the E₂/AlF complex, followed by supplementation and further incubation with 1 μM Tg. Alternatively, incubation was first carried out with Tg and then with AlF. As seen in Fig. 4, the result was independent of which complex was formed first. The affinity of E₂-Tg-AlF for TNP-8N₃-ATP was 17–18 nM, i.e. ~2-fold lower and ~3-fold higher than the TNP-8N₃-ATP Fig. 2. TNP-8N₃-ATP (left panels) and ATP (right panels) concentration dependences of photolabeling of wild type Ca²⁺-ATPase stabilized in the intermediate states occurring during E₂P dephosphorylation. Expressed wild type Ca²⁺-ATPase (upper panels) or SR (lower panels) was incubated with Tg, MgF, vanadate, AlF, or BeF as described under “Experimental Procedures,” and subjected to TNP-8N₃-ATP photolabeling at the indicated concentrations of TNP-8N₃-ATP without ATP (left panels), or at 3 X the Kₐ₁ for TNP-8N₃-ATP with the indicated concentrations of ATP (right panels). In each case, the maximum level of specific labeling was defined as 100%. Symbols for all panels are indicated in the upper left panel.

**Fig. 3.** Structural arrangement of the nucleotide binding site in Ca²⁺-ATPase crystallized in the E₂-AlF-AMPPCP state. The Protein Data Bank accession code corresponding to the structure shown is 3B9R (26). Amino acid side chains are shown for residues discussed in the text. Carbon and aluminum atoms are shown in gray, nitrogen in blue, oxygen in red, phosphorous in orange, and fluoride in cyan.
affinity of the $E2$-AlF and $E2$-Tg complexes, respectively (Fig. 4, left panel). The affinity of $E2$-Tg-AlF for ATP was on the other hand very similar to that of $E2$-AlF but 12- to 15-fold higher than that of $E2$-Tg (Fig. 4, right panel), implying that AlF binding dominates over Tg binding with respect to influencing the conformation of the ATP binding site in the $E2$-Tg-AlF complex. Also with respect to the maximal labeling levels seen in Fig. 4 did the $E2$-Tg-AlF complex resemble $E2$-AlF more than $E2$-Tg (cf. Fig. 1). Thus, the maximum labeling level of $E2$-Tg-AlF was ~3.5-fold higher than that of $E2$-Tg. It can be concluded that phosphorylation and nucleotide binding sites are fully flexible in the Tg-bound state, readily able to bind the AlF complex, and subsequently be photolabeled by TNP-8N$_3$-ATP with a $K_a$ for the photolabel, a $K_a$ for the inhibition by ATP of the photolabeling, and a maximal photolabeling level similar to that of the AlF-complexed, but Tg-free, enzyme. Hence, the inhibitory effect on catalysis of thapsigargin binding between transmembrane helices M3, M5, and M7 (30) is a local effect in the membrane, leaving the cytoplasmic domains free to bind nucleotide and adopt the various conformations characteristic of the transitional states of E2P dephosphorylation.

**ATP Affinity of Mutant Ca$^{2+}$-ATPases in $E2$-Tg, $E2$-MgF, $E2$-Vanadate, $E2$-AlF, and $E2$-BeF States**—Studies of the ATP dependence of the rate of E2P dephosphorylation in mutants (16, 17, 24) have pinpointed certain residues as critical for ATP modulation of E2P dephosphorylation, including Glu$^{439}$, Phe$^{487}$, and Arg$^{560}$ in the N-domain and Arg$^{174}$, Ile$^{198}$, and Lys$^{205}$ in the A-domain (Fig. 3). To understand how the effect of ATP on E2P dephosphorylation is brought about, a critical question to answer for each of these residues is whether the residue is directly involved in binding of the modulatory nucleotide, or its role is instead associated with mediating the response to binding. By applying the photolabeling assay to determine the ATP affinity of the various states of the E2P dephosphorylation reaction sequence in mutants, it is possible to distinguish between mutational effects on ATP modulation caused by direct interference with ATP binding and effects caused by interference with the consequences of the binding. This analysis was carried out with a series of mutants previously shown to be defective in ATP modulation of E2P dephosphorylation as observed in functional studies (R174A, R174E, I118A, I118F, K205A, E439A, F487S, and R560L, cf. Refs. 16 and 17) and with selected mutants previously found defective in ATP binding at the catalytic site in the E1 conformation (F487S, F487L, R489L, R560L, and L562F, cf. Refs. 33 and 34). The binding data are displayed in Fig. 5 for ATP and under supplemental Fig. S6 for TNP-8N$_3$-ATP, and the resulting affinity constants are listed in Tables 1 and 2.
Mutants with alterations to N-domain residues Phe487, Arg489, Arg560, and Leu562 (cf. Fig. 3) were previously shown to be severely defective with respect to ATP binding at the catalytic site in the E1 conformation (33, 34) (Table 2, right column). In functional studies mutations F487S and R560L were, furthermore, found to reduce the apparent affinity for ATP modulation of E2P dephosphorylation as much as >50- and 30-fold, respectively (16), which on the basis of the binding data in Fig. 5 and Table 2 can be ascribed to a deficiency of binding of the modulatory ATP throughout the E2P dephosphorylation reaction sequence. Hence, ATP binding affinity in the five E2/E2P states was reduced 11–23-fold for mutant F487S and 12–38-fold for mutant R560L, relative to wild type. Phe487 and Arg560 are in close proximity to the nucleotide in the E1:AMPPCP, E2-Tg-AMPPCP, E2-MgF-AMPPCP/ATP/ADP, and E2-AIF-AMPPCP crystal structures, with Phe487 apparently interacting with the adenine ring and Arg560 with the ribose and/or the β-phosphate (5, 26, 47–50), and the present data support the notion that the catalytic ATP binding site in the E1 state and the modulatory ATP binding site responsible for stimulation of E2P dephosphorylation are overlapping. Mutation F487L, retaining the bulk and hydrophobicity of the side chain, was, however, much less detrimental to ATP binding than F487S, in the E2P-like analog states stabilized with MgF, vanadate, AIF, and BeF. Hence, a wild-type-like affinity for ATP was seen for mutant F487L in E2-MgF, E2-vanadate, and E2-AIF states, and in E2-BeF the ATP affinity was only moderately (3.5-fold) reduced. The effect of the F487L mutation was much more pronounced in the E1 and E2-Tg states (22- and 16-fold reduction of ATP affinity, respectively, cf. Table 2). In most of the crystal structures with bound nucleotide the adenine ring is interposed between Phe487 and Leu562. In the Ca2E1 state, the aromatic ring of the phenylalanine side chain is nearly parallel to the adenine ring, indicating a π-stacking interaction that explains the marked effect of the leucine substitution in this state. In the E2-MgF and E2-AIF crystal structures the phenylalanine and adenine rings are more angled toward each other, suggesting less efficient π-stacking, which might be the reason that despite the lack of aromaticity the leucine is able to substitute quite well for phenylalanine in these states. In the E2-Tg state, however, mutation F487L was just as detrimental to ATP binding as in E1, despite a non-parallel orientation of the phenylalanine ring and the adenine ring in the E2-Tg-AMPPCP crystal structures, thus implying that positioning of the adenine ring in the native enzyme in the E2-Tg state differs somewhat from that seen in the E2-Tg-AMPPCP crystal structures.

Mutation L562F was previously shown to reduce MgATP affinity of E1 69-fold (34) but appears much less distorting in the intermediate E2/E2P states of E2P dephosphorylation, the most marked effect being a 2-fold reduced ATP affinity in E2-Tg (Table 2), which is somewhat surprising, because the Leu562 side chain occupies almost exactly the same position relative to the nucleotide in the Ca2,E1-AMPPCP, E2-Tg-AMPPCP, E2-MgF-AMPPCP/ATP/ADP, and E2-AIF-AMPPCP crystal structures, being located 3.2–3.7 Å from the ribose and 3.3–4.9 Å from the adenine ring (26, 47–50). It is possible that because of a higher mobility of the bound ATP in the E2/E2P states, as reflected by the lower ATP affinity of wild type E2/E2P states (K_D values in the 1–11 μM range; Table 2) compared with that of E1 (K_D = 0.14 μM; Table 2), the bound nucleotide in the E2/E2P states can be correctly positioned by any large hydrophobic side chain replacing Leu562. This would not be feasible in the very tight enzyme-nucleotide complex normally seen for E1, where a phenylalanine side chain cannot be accommodated in place of the leucine without destabilization of the complex. Accordingly, the affinity of the E1 state of L562F for MgATP (~10 μM) is a bit lower than the affinity of the E2P states of L562F for ATP (0.5–5 μM) (Table 2).

Mutation R489L was equally detrimental to ATP binding in E2-Tg and E2-BeF (19- and 12-fold reduction of affinity, respectively, relative to wild type) as to MgATP binding in E1 (16-fold reduction), whereas the effect was much less pronounced for E2-MgF, E2-vanadate, and E2-AIF (2–3-fold reduced ATP affinities, relative to wild type) (Table 2). This finding provides additional evidence that ATP is bound differently in the E2 dephospho-enzyme and the E2P ground state compared with the E2P transition and product states. The minor effects seen for the E2 transition state and product state analogs accord with the crystal structures of E2-AIF-AMPPCP and E2-MgF-AMPPCP/ADP, where the distance between the Arg489 side chain guanidinium group and the nucleotide ribose-OH is somewhat larger (4–6 Å) compared with the ~3 Å seen for the various Ca2E1 crystals. In the E2-Tg-AMPPCP crystal structures the corresponding distance is 5.2 Å, again indicating that details of the positioning of the nucleotide differ somewhat from the native enzyme in the E2-Tg state, where Arg489 according to our result is an important interaction partner. Because the E2-BeF crystal structures (26, 48) do not contain bound nucleotide, there is so far no structural correlation of the marked effect of the R489L mutation on the ATP affinity of E2-BeF.

The A-domain residues Arg174, Ile188, and Lys205 are not involved in nucleotide binding in the E1 conformation, but were in our previous functional studies identified as critical for the ATP-induced acceleration of E2P dephosphorylation (17). The Arg174 side chain is rather close (~4 Å) to the adenine ring of the nucleotide in the product state (E2-MgF-AMPPCP/ATP/ADP) and transition state (E2-AIF-AMPPCP) (Fig. 3) analog crystal structures (5, 26, 49), whereas in the E2-Tg-AMPPCP crystal structure the bound nucleotide is too far away from Arg174 for any direct interaction (47). Substitution of Arg174 with alanine or glutamate leads to reduced apparent affinity for ATP modulation of E2P dephosphorylation, most markedly for R174E, in which the charge of the side chain is reversed (17). PP_i (pyrophosphate) was on the other hand found effective in stimulating dephosphorylation of mutant R174A with an affinity similar to that seen for the wild type, although the affinity of R174E for PP_i was reduced (17). Assuming that PP_i, binds at the same site as modulatory ATP, mimicking the effect of the β- and γ-phosphates of ATP, the functional data would suggest that the role of Arg174 in ATP modulation of E2P dephosphorylation is associated primarily with binding of the adenosine part of the nucleotide and not the mechanism of mediating the effect of binding. The present binding data support this concept by showing that mutation R174A reduces the affinity for ATP as much as 11-fold in the E2P ground state (E2-BeF) and
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~3-fold in the other E2/E2P-like analog states, and that R174E reduces the ATP affinity markedly (12–43-fold) in the four E2P-like analog states (Fig. 5 and Table 2). The more pronounced effect of replacement with glutamate, having a negatively charged side chain, may be a consequence of electrostatic repulsion of the phosphates of the ATP molecule (cf. Fig. 3).

Our previous functional analysis (17), furthermore, showed that stimulation by ATP of E2P dephosphorylation was completely abolished in mutant I188A, whereas I1188F displayed a minor, 2-fold reduction of the apparent affinity for the modulation by ATP of E2P dephosphorylation. Because of the 3–5-Å proximity of the Ile$^{188}$ side chain to the α-phosphate or adenine ring of the nucleotide in E2-MgF·ADP and E2·Al-F·AMPPCP crystal structures (Fig. 3), one might have expected Ile$^{188}$ to be directly involved in ATP binding during E2P dephosphorylation, which might explain the marked effect of mutation I1188A on ATP modulation (17). The present binding analysis showed, however, that contrary to the expected lowering of affinity, the I1188A mutation actually causes a significant 2–3-fold increase of ATP affinity in the four E2P-like analog states (Table 2, the corresponding $K_a$ values are 0.4–1.3 μM), suggesting that in fact the larger side chain of isoleucine present in the wild type is a little disturbing to the binding of the nucleotide, the alanine of the mutant accommodating the nucleotide better. In this light, the complete absence of ATP modulation of dephosphorylation of E2P in I1188A (Fig. 5 of Ref. 17) provides a clear indication of a mechanistic role of Ile$^{188}$ in mediating the stimulating effect of ATP on E2P dephosphorylation. Ile$^{188}$ is located at the start of the loop containing the TGE5 motif with Glu$^{183}$, and a slight clash between Ile$^{188}$ and the nucleotide might be instrumental in moving Glu$^{183}$ to the optimal position for catalyzing dephosphorylation. Because of the shorter side chain, alanine would not be able to fulfill this role, whereas the larger phenylalanine would (the efficiency of modulation is actually higher for I188F than for the wild type, see Table 3 in Ref. 17).

Mutants K205A and E439A are both modulated by ATP in a rather anomalous way, displaying inhibition rather than stimulation of E2P dephosphorylation by ATP (16, 17). This is illustrated for mutant E439A in Fig. 6, where more data points have been included than previously (16), showing inhibition between 0.2 and 5 mM ATP with a $K_i$ of inhibition for 3.1 mM. In comparison, wild type is stimulated by ATP with a $K_i$ of 34 μM. Phosphoenzyme decay curves for mutant E439A at various ATP concentrations are shown under supplemental Fig. S7. Detailed inhibition data for mutant K205A obtained in the same way were previously shown in Fig. 5 of Ref. 17. In the E2-MgF·AMPPCP/ATP·ADP and E2·AlF·AMPPCP (Fig. 3) crystal structures the side chain of Lys$^{205}$ in the A-domain is close (3–4 Å) to the β- and γ-phosphates of the nucleotide, whereas the side chain of Glu$^{439}$ in the N-domain is further away from the nucleotide (5–7 Å distant from the adenine ring). Indeed, the role of Glu$^{439}$ in ATP modulation of E2P dephosphorylation is likely to be of an indirect nature, relating to an interdomain hydrogen bond between Glu$^{439}$ and A-domain residue Ser$^{186}$, cf. Fig. 3 (16, 24), rather than to direct interaction with the modulatory nucleotide. The binding data in Fig. 5 provide a tentative explanation of the anomalous inhibitory effect of ATP seen for K205A and E439A. The $K_i$ values for ATP binding are for K205A, 10.2 μM in E2·BeF, 15.9 μM in E2·AlF, and 12.7 μM in E2·vanadate; and for E439A, 1.3 μM in E2·BeF, 8.4 μM in E2·AlF, and 2.4 μM in E2·vanadate, which should be compared with the wild type affinity constants of 4.1 μM in E2·BeF, 1.3 μM in E2·AlF, and 1 μM in E2·vanadate. Hence, for both mutants the ATP affinity is higher (i.e. $K_i$ lower) in the ground state (E2·BeF) of E2P compared with the transition state (E2·AlF and E2·vanadate). This is contrary to the situation seen with wild type and all the other mutants studied here, where the ATP affinity of E2·BeF is lower than that of E2·AlF and E2·vanadate. Thus, whereas the nucleotide increases stability of the E2P transition state relative to the E2P ground state in the wild type, and consequently stimulates dephosphorylation, the opposite takes place in K205A and E439A, with the nucleotide instead increasing stability of the ground state relative to the transition state, thereby inhibiting dephosphorylation.

**Nucleotide Affinity of the Stable Ca$^{2+}$E2P State of Mutant 4Gi-46/47—**For wild type Ca$^{2+}$-ATPase, the conformational transition of the phosphoenzyme, Ca$^{2+}$E1P → Ca$^{2+}$E2P, is a rate-limiting step of the overall pump cycle, and is succeeded by rapid dissociation of the two Ca$^{2+}$ ions from lumenally exposed Ca$^{2+}$ sites to the endoplasmic reticulum lumen, thus forming the Ca$^{2+}$-free E2P ground state (cf. Scheme 1). The Ca$^{2+}$E2P state is thought to be an unstable and short-lived intermediate that cannot be readily isolated. However, elongation of the A-M1 linker between the A-domain and transmembrane helix M1 by insertion of four glycines between Gly$^{46}$ and Lys$^{47}$ (mutant “4Gi-46/47”) has been shown to result in an extremely stable Ca$^{2+}$E2P state (31), in which the A-domain seems to have rotated horizontally from its position in the Ca$^{2+}$E1P state, whereas the inclining (vertical) motion of the top part of transmembrane helix M2 and the A- and P-domains has yet to take place to reach the structure corresponding to the Ca$^{2+}$-free E2P ground state (32). It is then relevant to ask whether the latter conformational change also affects the nucleotide site, or the modulatory ATP binding site of E2P has already been

![FIGURE 6. ATP dependence of the rate of dephosphorylation of E2P for mutant E439A. Dephosphorylation of the phosphoenzyme formed in the presence of 32P was followed at various ATP concentrations at pH 7.0 in the absence of Mg$^{2+}$, as described under “Experimental Procedures.” Examples of the decay curves are shown under supplemental Fig. S7. The dephosphorylation rate constants are shown here as a function of the ATP concentration. The parameters derived by fitting a hyperbolic function as described under “Experimental Procedures” are as follows (in each case, the total number of data points included in the fit is indicated in parentheses): wild type, $k_{cat} = 34 ± 9$ μM, $k_0 = 2.0$ min$^{-1}$, $k_{max} = 4.7$ min$^{-1}$ (n = 22); E439A, $K_i = 3115 ± 2171$ μM, $k_0 = 19.0$ min$^{-1}$, $k_{max} = 5.6$ min$^{-1}$ (n = 19).](image-url)
assembled from the gathering of the A-, P-, and N-domains, before the vertical tilt occurs.

To determine the nucleotide affinity of the stable Ca\textsuperscript{2+}E2P state of mutant 4Gi-46/47, phosphorylation of Ca\textsuperscript{2+}-free E2 from the mutant was carried out with inorganic phosphate, followed by supplementation of the microsomes with an excess amount of Ca\textsuperscript{2+} to saturate the luminal Ca\textsuperscript{2+} sites. These experiments were carried out in the presence of the Ca\textsuperscript{2+} ionophore A23187 to allow Ca\textsuperscript{2+} access to the luminal side of the microsomal vesicles. The data under supplemental Fig. S8 confirm the high stability of the phosphoenzyme accumulated with mutant 4Gi-46/47 under the buffer conditions used in the photolabeling assay. The TNP-8N\textsubscript{3}-ATP and ATP binding data obtained with the Ca\textsubscript{2+}E2P state of mutant 4Gi-46/47 are shown in Fig. 7A. For comparison, we furthermore, measured the TNP-8N\textsubscript{3}-ATP and ATP affinities of mutant 4Gi-46/47 in the Tg-, MgF-, vanadate-, AlF-, and BeF-complexed states (Fig. 7A), as well as the TNP-8N\textsubscript{3}-ATP and ATP affinities under E1 conditions in the absence of Ca\textsuperscript{2+} (Fig. 7B).

As shown in Fig. 7A, the Ca\textsubscript{2+}E2P state of mutant 4Gi-46/47 binds TNP-8N\textsubscript{3}-ATP and ATP with affinities that do not differ significantly from those of the Ca\textsuperscript{2+}-free E2-BeF state of the mutant, implying that any conformational change taking place during the transition to the Ca\textsuperscript{2+}-free E2P ground state does not affect the nucleotide binding site. Furthermore, there was no marked difference between nucleotide affinities of wild type and mutant 4Gi-46/47 in any of the four E2P-like analog states stabilized with MgF, vanadate, AlF, and BeF, suggesting that the mutation does not disturb conformation of the E2P ground state, transition state, or product state appreciably, in accordance with the wild type-like rate of E2P dephosphorylation of the mutant (31).

Under E1 conditions mutant 4Gi-46/47 also displayed an affinity for TNP-8N\textsubscript{3}-ATP very similar to that of wild type (Fig. 7B, left panel). Surprisingly, however, under these conditions the ATP affinity of mutant 4Gi-46/47 was reduced by more than 3 orders of magnitude, relative to wild type (right panel of Fig. 7B). To assess whether this effect of the 4Gi-46/47 mutation was caused by the absence of Ca\textsuperscript{2+} in the photolabeling medium (needed to prevent phosphorylation during labeling), we measured ATP dependence of phosphorylation of the Ca\textsuperscript{2+}-saturated E1 from [\textgamma\textsuperscript{32P}]ATP. As seen in Fig. 7C, the apparent affinity for ATP obtained with 4Gi-46/47 in the phosphorylation assay differed only 2-fold from that of the wild type enzyme. A possible explanation of these findings is that increased flexibility of the lengthened A-M1 linker in the mutant leads to detachment of the A-, P-, and N-domain interactions, resulting in stabilization in the absence of Ca\textsuperscript{2+} of an open structure similar to that seen in the crystal structure of the nucleotide-free Ca\textsubscript{2+}E1 state (51). In such a state, only the N-domain would be expected to contribute to nucleotide binding, as opposed to tight packing of the nucleotide between the N- and P-domains seen in the Ca\textsubscript{2+}E1·AMP-PCP state (50). In accordance with this hypothesis, the ATP affinity of expressed N-domain from Ca\textsuperscript{2+}-ATPase or Na\textsuperscript{+},K\textsuperscript{+}-ATPase is in the millimolar range (52–54), rather than the typical submicromolar/micromolar affinity range of the intact enzymes. The wild type-like high affinity of mutant 4Gi-46/47 for TNP-8N\textsubscript{3}-ATP may then suggest that the N-domain generally is the only critical contributor to the binding of the photolabel, thus again illustrating the notion that the photolabel binds in a way rather different from that of ATP, although the binding sites overlap.

Conclusions—By applying the TNP-8N\textsubscript{3} ATP Lys\textsuperscript{492} photolabeling method (20, 33, 34) we have measured nucleotide binding to the various intermediate states occurring during E2P dephosphorylation of wild type and mutant Ca\textsuperscript{2+}-ATPases.
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The distinct ATP affinities determined for these states in the wild type Ca\textsuperscript{2+}-ATPase suggest a certain degree of flexibility of the modulatory ATP site during E2P dephosphorylation, with a pronounced tightening of the enzyme-nucleotide interaction going from the E2P ground state to the transition state, perpetuation of this tight interaction further into the product state, and then loosening up the site again going into the E2 dephosphoenzyme. Hence, on the basis of the present results the acceleration of dephosphorylation by ATP can be understood in terms of stabilization by ATP binding of the transition and product states in the dephosphorylation reaction. Among the mutations studied here F487S, R560L, and R174A/E interfere with binding of the modulatory nucleotide, whereas I188A mutations studied here F487S, R560L, and R174A/E interfere with the binding on the optimal positioning of Glu183 for catalysis. The anomalous inhibition of E2P dephosphorylation by ATP seen for mutants K205A and E439A is caused by a reversal of the stabilities of the E2P ground and transition states. The present results fully support a model in which the adenine always stays in the gap between Phe\textsuperscript{887} and Leu\textsuperscript{662}, whether the ATP is phosphorylating the Ca\textsubscript{2}E1 form or modulating E2P dephosphorylation, and then the rest of the ATP molecule stretches or folds, as is energetically best depending on domain positions, rather like an anchor and chain.

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