Asparagine 706 and Glutamate 183 at the Catalytic Site of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Play Critical but Distinct Roles in E2 States

Johannes D. Clausen, David B. McIntosh, David G. Woolley, Anne Nyholm Anthonisen, Bente Vilsen, and Jens Peter Andersen

From the Department of Physiology, Institute of Physiology and Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark and the Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Observatory, Cape Town 7925, South Africa

Mutants with alteration to Asn\(^{706}\) of the highly conserved TGDGVND\(^{707}\) motif in domain P of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase were analyzed for changes in transport cycle kinetics and binding of the inhibitors vanadate, BeF\(^{2-}\), AlF\(^{3-}\), and MgF\(^{2-}\). The fluorides likely mimic the phosphoryl group/P\(_i\) in the respective transition, ground, and product states of phosphoenzyme hydrolysis (Danko, S., Yamasaki, K., Daiho, T., and Suzuki, H. (2004) J. Biol. Chem. 279, 14991–14998). Binding of BeF\(^{2-}\), AlF\(^{3-}\), and MgF\(^{2-}\) was also studied for mutant Glu\(^{183}\) \(\rightarrow\) Ala, where the glutamate of the 181TGES\(^{184}\) motif in domain A is replaced. Mutations of Asn\(^{706}\) and Glu\(^{183}\) have in common that they dramatically impede the function of the enzyme in E2 states, but have little effect in E1. Contrary to the Glu\(^{183}\) mutant, in which E2P slowly accumulates (Clausen, J. D., Vilsen, B., McIntosh, D. B., Einholm, A. P., and Andersen, J. P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2776–2781), E2P formation was not detectable with the Asn\(^{706}\) mutants. Differential sensitivities of the mutants to inhibition by AlF\(^{3-}\), MgF\(^{2-}\), and BeF\(^{2-}\) made it possible to distinguish different roles of Asn\(^{706}\) and Glu\(^{183}\). Hence, Asn\(^{706}\) is less important than Glu\(^{183}\) for gaining the transition state during E2P hydrolysis but plays critical roles in stabilization of E2P ground and E2-P product states and in the major conformational changes associated with the Ca\(^{2+}\)E1P \(\rightarrow\) E2P and E2 \(\rightarrow\) Ca\(^{2+}\)E1 transitions, which seem to be facilitated by interaction of Asn\(^{706}\) with domain A.

The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (1) is an energy-transducing enzyme of the P-type that couples hydrolysis of ATP to translocation of Ca\(^{2+}\) from the cytosol to the endoplasmic reticulum. In this control of cytosolic Ca\(^{2+}\) concentration, the Ca\(^{2+}\)-ATPase plays a vital role in cellular activation events, such as muscle contraction, hormone secretion, immune responses, cell migration, and protein synthesis. Ca\(^{2+}\) transport is coupled to ATP hydrolysis by a reaction cycle (Scheme 1), in which the enzyme is transiently phosphorylated at a conserved aspartic acid residue and undergoes major conformational changes (2, 3).

In recent years, several high resolution crystal structures of the Ca\(^{2+}\)-ATPase, each thought to represent a particular intermediate state in the pump cycle, have been solved (4–9). The Ca\(^{2+}\)-ATPase consists of a membrane-spanning domain of ten helical segments and a large cytoplasmic head piece, comprising three distinct domains, named “N” (nucleotide binding), “P” (phosphorylation), and “A” (actuator). By combining crystallographic data with functional changes in site-specific mutants, an increasingly detailed picture of the mechanisms of energy interconversion and ion translocation in the Ca\(^{2+}\)-ATPase is emerging. Thus, the catalytic function in E1 (autokinase activity) and E2 forms (autophosphatase activity), the movement of Ca\(^{2+}\) ions across the membrane, as well as the major rate-limiting conformational changes of the cycle, i.e. E2 \(\rightarrow\) E1 and E1P \(\rightarrow\) E2P, can all be understood on the basis of the sequential gathering and displacement of certain conserved amino acid motifs in domains N and A relative to the catalytic site in domain P and the coupling of these events to rearrangements of the transmembrane helices containing the high affinity Ca\(^{2+}\) sites.

In the present study, we address the role of Asn\(^{706}\) at the catalytic site of Ca\(^{2+}\)-ATPase and revisit a previously examined mutant, E183A (10). Asn\(^{706}\) and Glu\(^{183}\) reside in the conserved TGDGVND\(^{707}\) (domain P) and 181TGES\(^{184}\) (domain A) motifs, respectively, and both residues are found in all known P-type ATPases (11). In fact, Asn\(^{706}\) is highly conserved even in the superfamily of phosphohydrolases and phosphotransferases (the HAD superfamily), which, given the similarities in reaction mechanism, protein sequence, and structural architecture of the catalytic site, are believed to share a common evolutionary ancestor with the phosphorylation domain of the P-type ATPases (12–14). The side chains of Asn\(^{706}\) and Glu\(^{183}\) are both centrally located at the catalytic site in the E2 forms of Ca\(^{2+}\)-ATPase, close to the phosphorylated aspartate, Asp\(^{551}\) (6, 7, 9). In E1 conformations, Glu\(^{183}\) has departed the phosphorylation site, whereas Asn\(^{706}\) retains its close proximity to Asp\(^{551}\) (4, 5, 7, 8). In our previous study of Glu\(^{183}\) (10), we demonstrated that substitution of the glutamate with alanine leads to a much reduced rate of both E2P hydrolysis and of the reverse phosphorylation of E2 with P\(_i\), suggesting that Glu\(^{183}\) is critical for E2P transition state stabilization and catalysis. This proved to correlate well with the subsequently published E2AlF\(^2\) crystal structure, in which Glu\(^{183}\) seems to coordinate and likely activates the attacking water molecule in the transition...
Asn$^{706}$ and Glu$^{183}$ of SR Ca$^{2+}$-ATPase

SCHEME 1. SERCA reaction cycle. E1, enzyme form with cytoplasmically facing high affinity Ca$^{2+}$ binding sites; E1P, ADP-sensitive phosphoenzyme intermediate containing Ca$^{2+}$ in the occluded state; E2, enzyme form with low affinity for Ca$^{2+}$; E2P, ADP-insensitive phosphoenzyme intermediate with luminaly facing low affinity Ca$^{2+}$ binding sites.

state complex (6). Here we show that Asn$^{706}$ likewise plays a crucial role in phosphorylation of E2 with $P_i$, as well as in the major protein conformational changes involved in phosphoenzyme and dephosphoenzyme processing. We describe here, for the first time, measurements of the apparent binding affinities of mutants for the phosphoryl analogs AIF and MgF recently used for crystallization, as well as BeF, assumed to be an analog of phosphate in the E2P ground state (15). These measurements suggest different roles for Asn$^{706}$ and Glu$^{183}$ during catalysis of the E2P $\leftrightarrow$ E2 reaction.

EXPERIMENTAL PROCEDURES

Mutagenesis, Expression, and Assays of the Overall Reaction—Oligonucleotide-directed mutagenesis of cDNA encoding the rabbit fast-twitch muscle Ca$^{2+}$-ATPase (SERCA1a isomoph) was carried out as described previously (16). The cDNA encoding the Ca$^{2+}$-ATPase mutant E183A was the same as that applied in two previous studies (10, 17). For expression, the wild-type or mutant cDNA, inserted in the pMT2 vector (18), was introduced into COS-1 cells (19) by transfection using the calcium phosphate precipitation method (20). The microsomal fraction containing wild-type or mutant Ca$^{2+}$-ATPase was isolated by differential centrifugation (21). The concentration of expressed Ca$^{2+}$-ATPase was quantified by a specific enzyme-linked immunosorbent assay (22, 23). Transport of $^{45}$Ca$^{2+}$ into the microsomal vesicles was measured by filtration, and the ATPase activity was determined by measuring the amount of $P_i$ liberated in the presence of $P_i$ and MgF$_2$. In experiments involving AlF, BeF, or ADP-AIF, the concentration of Mg$^{2+}$ (a cofactor of the reaction with the inhibitor (30–32)) was kept as low as 200 $\mu$M during the inhibition step to avoid formation of MgF$_2$. Following incubation with the inhibitor for 30 min at 25 °C and subsequent cooling for 10 min at 0 °C, the amount of inhibitor-free enzyme was determined by phosphorylation for 10 s at 0 °C with 5 $\mu$M $[^{32}P]ATP$. In the experiments where inhibition was carried out with enzyme pre-equilibrated in the absence of Ca$^{2+}$, excess Ca$^{2+}$ was added prior to the phosphorylation step (~5 s before the addition of ATP). The data presented in the supplemental material (Figs. SI and SII) validate the above described method. Fig. SI shows the time course of inhibitor binding for vanadate, AIF, BeF, MgF$_2$, and ADP-AIF. Fig. SII shows the time course of the dissociation of the enzyme-inhibitor complexes upon addition of Ca$^{2+}$. Note in Fig. SII that dissociation of the complexes of Ca$^{2+}$-ATPase with vanadate, AIF, BeF, and MgF$_2$ is very slow at 0 °C, demonstrating that the 5-s incubation with Ca$^{2+}$ prior to phosphorylation does not cause significant dissociation of enzyme-inhibitor complex. However, because the enzyme-MgF$_2$ complex was more Ca$^{2+}$-sensitive than the complexes with AIF and MgF, we used a free Ca$^{2+}$ concentration of only 100 $\mu$M for activation of phosphorylation in the experiments with BeF, whereas the Ca$^{2+}$ concentration was 500 $\mu$M in the experiments with AIF and MgF$_2$.

As a consequence of the high affinity of Mg$^{2+}$ to Ca$^{2+}$-ATPase, the photolabeling of COS-1 cell microsomes containing wild-type or mutant Ca$^{2+}$-ATPase, the inhibition of photolabeling by ATP, and the quantification of labeled bands by radioimaging following SDS-polyacrylamide gel electrophoresis were carried out as described previously (33–35).

Calculations and Data Analysis—Experiments were conducted at least twice, and the complete set of data was analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.) or by computer using the SimZyme program (27, 36). The analysis of ligand concentration dependences was based on the Hill equation, $EP = EP_{max} \cdot [L]^n/(K_{0.5}^n + [L]^n)$, or, for the concentration dependences of inhibitory ligands, on the Hill equation for inhibition, $EP = EP_{max} \cdot (1 - [L]^n)/(K_{0.5}^n + [L]^n)$. The "true" dissociation constant for ATP binding in [32P]TNP-8N$_5$-ATP labeling experiments was calculated using the validated equation for competitive inhibition (33).

RESULTS

Expression, Overall Activity, and Phosphorylation of Ca$^{2+}$ Pumps with Alterations to Asn$^{706}$—Asn$^{706}$ of the Ca$^{2+}$-ATPase was replaced by either alanine, cysteine, or serine, these mutations being chosen to either completely remove the side-chain carboxamide function (alanine) or remove the nitrogen, while retaining an oxygen in the side chain (serine), or introduce minimal negative charge (cysteine, the ionized fraction depending on the pH and pK, in all cases with only minor changes of the volume of the side chain. The mutant proteins were expressed in COS-1 cells, and the expression level of the mutants was similar to that obtained with wild-type Ca$^{2+}$-ATPase, as evaluated by

Assays for Binding of Vanadate and Fluorides—We used the previously described assay for vanadate binding (29), and this assay, which is based on the inability of the inhibitor-bound Ca$^{2+}$-ATPase to be phosphorylated by ATP, was modified to study also the binding of AIF, BeF, MgF, and ADP-AIF to wild-type and mutant Ca$^{2+}$-pumps. Microsomes were pre-equilibrated at 25 °C and pH 7, either in the absence of Ca$^{2+}$ to allow accumulation of the E2 state or in the presence of Ca$^{2+}$ for accumulation of Ca$^{2+}$. The reaction with inhibitor was then initiated by addition of varying concentrations of AlCl$_3$, BeSO$_4$, or MgCl$_2$ at a fixed concentration of NaF with or without ADP. In experiments involving AIF, BeF, or ADP-AIF, the concentration of Mg$^{2+}$ (a cofactor of the reaction with the inhibitor (30–32)) was kept as low as 200 $\mu$M during the inhibition step to avoid formation of MgF$_2$. Following incubation with the inhibitor for 30 min at 25 °C and subsequent cooling for 10 min at 0 °C, the amount of inhibitor-free enzyme was determined by phosphorylation for 10 s at 0 °C with 5 $\mu$M $[^{32}P]ATP$. In the experiments where inhibition was carried out with enzyme pre-equilibrated in the absence of Ca$^{2+}$, excess Ca$^{2+}$ was added prior to the phosphorylation step (~5 s before the addition of ATP). The data presented in the supplemental material (Figs. SI and SII) validate the above described method. Fig. SI shows the time course of inhibitor binding for vanadate, AIF, BeF, MgF, and ADP-AIF. Fig. SII shows the time course of the dissociation of the enzyme-inhibitor complexes upon addition of Ca$^{2+}$. Note in Fig. SII that dissociation of the complexes of Ca$^{2+}$-ATPase with vanadate, AIF, BeF, and MgF$_2$ is very slow at 0 °C, demonstrating that the 5-s incubation with Ca$^{2+}$ prior to phosphorylation does not cause significant dissociation of enzyme-inhibitor complex. However, because the enzyme-MgF$_2$ complex was more Ca$^{2+}$-sensitive than the complexes with AIF and MgF$_2$, we used a free Ca$^{2+}$ concentration of only 100 $\mu$M for activation of phosphorylation in the experiments with BeF, whereas the Ca$^{2+}$ concentration was 500 $\mu$M in the experiments with AIF and MgF$_2$.

As a consequence of the high affinity of Mg$^{2+}$ to Ca$^{2+}$-ATPase, the photolabeling of COS-1 cell microsomes containing wild-type or mutant Ca$^{2+}$-ATPase, the inhibition of photolabeling by ATP, and the quantification of labeled bands by radioimaging following SDS-polyacrylamide gel electrophoresis were carried out as described previously (33–35).

Calculations and Data Analysis—Experiments were conducted at least twice, and the complete set of data was analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.) or by computer using the SimZyme program (27, 36). The analysis of ligand concentration dependences was based on the Hill equation, $EP = EP_{max} \cdot [L]^n/(K_{0.5}^n + [L]^n)$, or, for the concentration dependences of inhibitory ligands, on the Hill equation for inhibition, $EP = EP_{max} \cdot (1 - [L]^n)/(K_{0.5}^n + [L]^n)$. The "true" dissociation constant for ATP binding in [32P]TNP-8N$_5$-ATP labeling experiments was calculated using the validated equation for competitive inhibition (33).

RESULTS

Expression, Overall Activity, and Phosphorylation of Ca$^{2+}$ Pumps with Alterations to Asn$^{706}$—Asn$^{706}$ of the Ca$^{2+}$-ATPase was replaced by either alanine, cysteine, or serine, these mutations being chosen to either completely remove the side-chain carboxamide function (alanine) or remove the nitrogen, while retaining an oxygen in the side chain (serine), or introduce minimal negative charge (cysteine, the ionized fraction depending on the pH and pK, in all cases with only minor changes of the volume of the side chain. The mutant proteins were expressed in COS-1 cells, and the expression level of the mutants was similar to that obtained with wild-type Ca$^{2+}$-ATPase, as evaluated by
Asn706 and Glu183 of SR Ca\textsuperscript{2+}-ATPase

Nucleotide Binding—In a recent mutagenesis study (35) we investigated the importance of several other conserved domain P residues for the nucleotide binding properties of the Ca\textsuperscript{2+}-ATPase by studying the nucleotide concentration dependence of TNP-8N\textsubscript{5}-[\gamma\textsuperscript{32}P]ATP photo-labeling and ATP/MgATP competitive inhibition thereof. Asp\textsuperscript{703} and Asp\textsuperscript{707}, which are close to Asn\textsuperscript{706} both in the primary and the three-dimensional structure, proved of importance for ATP/MgATP binding. Thus, charge-removal from the side chains of these aspartates enhanced ATP binding up to 14-fold in the absence of Mg\textsuperscript{2+}, and inhibited ATP binding up to 8-fold in the presence of Mg\textsuperscript{2+}. Table 1 shows the results of similar experiments carried out with the Asn\textsuperscript{706} mutants. It is clear that Asn\textsuperscript{706} is not a critical residue for TNP-8N\textsubscript{5}-ATP or ATP binding, neither in the absence nor presence of Mg\textsuperscript{2+}, as the reduction of affinity was maximally 2-fold relative to wild type. It is noteworthy, however, that the largest reduction of affinity was seen in the presence of Mg\textsuperscript{2+} and for N706C, which also showed a significant reduction of apparent Mg\textsuperscript{2+} affinity in the titration of steady-state phosphorylation described above.

**Time Course of ATP Phosphorylation of Ca\textsuperscript{2+}-saturated Enzyme**—Fig. 3A shows the results of rapid kinetic measurements at 25 °C of the time course of phosphorylation from 5 μM MgATP of enzyme pre-equilibrated with Ca\textsuperscript{2+}. Compared with wild type, the phosphorylation rate was significantly reduced for N706A (1.8-fold) and N706C (4.4-fold) but unaltered for N706S (Fig. 3A). Because a subsaturating ATP concentration was used, the measured phosphorylation rate depends not only on the rate constant as deduced from the fit, it phosphorylation was performed for 15 s at 0 °C in 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl\textsubscript{2}, 1 mM EGTA, 1 μM Ca\textsuperscript{2+}, and 5 mM Mg\textsuperscript{2+}. Asp\textsuperscript{707}\textsuperscript{0} and Glu\textsuperscript{183} of SR Ca\textsuperscript{2+}-ATPase

their immunoreactivity in a specific enzyme-linked immunosorbent assay (data not shown). To obtain an initial overview of the functional consequences of the mutations, we measured the overall rates of \textsuperscript{45}Ca\textsuperscript{2+} transport into the microsomal vesicles and ATP hydrolysis at 37 °C and saturating substrate conditions. In either assay, the three mutants displayed no significant activity above the background level obtained with control microsomes harvested from mock-transfected COS-1 cells. For N706A, the lack of ATPase activity confirms a previously published result (37).

We then proceeded to test the phosphorylation of the mutants, from \([\gamma\textsuperscript{32}P]ATP in the presence of Ca\textsuperscript{2+}, as well as from [\gamma\textsuperscript{32}P]ATP in the backward direction of the normal reaction cycle (Scheme 1). The steady-state level of phosphoenzyme formed in the presence of 5 μM [\gamma\textsuperscript{32}P]ATP at 0 °C was wild-type-like for all three mutants (Fig. 1, upper gel). However, no significant phosphoenzyme was formed in the mutants in the presence of 500 μM \textsuperscript{32}P\textsubscript{i} (Fig. 1, lower gel), under conditions where E2P accumulates for wild type (25 °C, presence of Me\textsubscript{2}SO, absence of Ca\textsuperscript{2+}, and incubation for 10 min). Under these conditions, the concentration of P\textsubscript{i}, giving half-maximal phosphorylation of wild-type enzyme is ~10 μM, and with 500 μM P\textsubscript{i}, the reaction reaches steady-state within ~10 s (10). Thus, it is clear from Fig. 1 that the replacement of Asn\textsuperscript{706} affects the ability to form the E2P state dramatically. It should be noted that this finding is at variance with a recent study (38) reporting that mutant N706A phosphatases with P\textsubscript{i} to ~50% of the extent seen for wild-type Ca\textsuperscript{2+}-ATPase. The latter study (38) also reported 19% labeling and ATP/MgATP competitive inhibition thereof. Asp\textsuperscript{703} and Asp\textsuperscript{707}, which are close to Asn\textsuperscript{706} both in the primary and the three-dimensional structure, proved of importance for ATP/MgATP binding.

**Ca\textsuperscript{2+} and MgATP Dependence of Phosphorylation from ATP**—The ability of the mutants to form a phosphoenzyme from ATP allowed us to further study the partial reactions of the pump cycle in phosphorylation experiments. First, we tested the Ca\textsuperscript{2+} concentration dependence of steady-state phosphorylation from [\gamma\textsuperscript{32}P]ATP (Fig. 2A). Small, but significant, deviations from the wild-type enzyme were found with the mutants, with the activation curve of N706S being slightly left-shifted relative to wild type and that of N706C being right-shifted. Similar effects were seen for the MgATP dependence of phosphorylation (Fig. 2B), corresponding to a 3-fold increase of affinity for N706S and a 4-fold reduction for N706C, relative to wild type. The observed changes of apparent affinity for Ca\textsuperscript{2+} as well as MgATP seem to be kinetic effects, resulting from a reduced rate of phosphoenzyme turnover in combination with various degrees of slowing of the phosphorylation rate (see below and "Discussion").
TABLE 1

| Binding affinities for ATP and MgATP determined by inhibition of [γ-32P]TNP-8N2, ATP photolabeling |
|-----------------------------------------------|
| | EDTA** | MgATP**/EGTA** |
| | K_D (TNP-8N2-ATP) | K_D (ATP) | K_D (TNP-8N2-MgATP) | K_D (MgATP)** |
| Wild type | 0.19 | 27 | 0.79 | 0.91 |
| N706A | 0.095 | 17 | 0.82 | 0.74 |
| N706C | 0.12 | 21 | 1.86 | 0.86 |
| N706S | 0.11 | 16 | 0.99 | 0.55 |

**Medium: 25 mM EPPS/TMAH (pH 8.5), 20% (v/v) glycerol, 1 mM MgCl2, 0.5 mM EGTA.
** The “true” K_D, calculated from the measured K_D values under the assumption of competitive inhibition as described previously (33). In the inhibition experiments, the concentration of [γ-32P]TNP-8N2, ATP was 3× K_D.

Rate of the Ca2+ -binding Transition in the Presence or Absence of ATP—Fig. 3B shows the time course of phosphorylation determined under conditions similar to those corresponding to Fig. 3A, except that the enzyme was pre-equilibrated in the absence of Ca2+ (presence of EGTA), and phosphorylation was initiated by mixing with a buffer containing [γ-32P]ATP and excess Ca2+. For wild type, the rate obtained under these conditions is 1.8-fold lower than that obtained following pre-equilibration with Ca2+, reflecting the rate-limiting nature of the Ca2+ binding transition (i.e. E2 → E1 conformational change and accompanying Ca2+ binding) preceding the phosphorylation reaction (27). The three mutants displayed 4- to 6-fold lower rates of phosphorylation when starting from Ca2+-deprived enzyme as compared with Ca2+-pre-equilibrated enzyme (Fig. 3, compare A and B). Relative to wild type, the Ca2+ binding transition was 4.4-, 7.4-, and 3.2-fold slowed in N706A, N706C, and N706S, respectively (Fig. 3B).

In the experiment corresponding to Fig. 3B, 5 μM MgATP was present during the course of the Ca2+ binding transition. Because even micromolar concentrations of ATP accelerate E2 → E1 (40), we speculated whether the inhibition of the Ca2+ binding transition in the Asn706 mutants could result from defective ATP modulation of Ca2+ in the Asn706 mutants. We therefore examined the rate of the Ca2+ binding transition using an assay in which MgATP is absent during Ca2+ binding (Fig. 4A). Enzyme pre-equilibrated with EGTA was mixed with an excess amount of Ca2+ and incubated for varying time intervals (t in the mixing protocol at the top of Fig. 4A). The amount of phosphorylatable Ca2+1 accumulated during the Ca2+ incubation step was then determined for each time interval by a further 34-ms incubation with 5 μM [γ-32P]ATP and 5 mM Mg2+, followed by acid quenching. Because the temperature and buffer conditions during the Ca2+ binding transition in this assay were identical to those applied in the experiment corresponding to Fig. 3B, except for the absence of MgATP, the rate constants obtained in the two assays can be compared directly, and their ratio reflects the modulatory influence of MgATP on the Ca2+ binding transition. For both wild type and mutants, the Ca2+ binding transition was ~2-fold slower in the absence of MgATP as compared with its presence, implying that MgATP modulation of the E2 → E1 transition is unaffected by the Asn706 mutations. Hence, relative to wild type the Ca2+ binding transition in the absence of MgATP was 6.6-, 8.6-, and 4.5-fold slowed in N706A, N706C, and N706S, respectively (Fig. 4A).

Ca2+ Dissociation from the High Affinity Binding Sites in Ca2+1—The function of the two high affinity Ca2+ sites in E1 was studied by measurement of the rate of Ca2+ dissociation from Ca2+1, taking advantage of the fact that only the E1 form with two bound Ca2+ ions can be phosphorylated by ATP (41). As illustrated by the mixing protocol at the top of Fig. 4B, enzyme pre-equilibrated with Ca2+ was mixed with excess EGTA and incubated for varying time intervals. The amount of phosphorylatable Ca2+1 remaining after the EGTA incubation step was then determined for each time interval by a further 34-ms incubation with 5 μM [γ-32P]MgATP, followed by acid quenching to the indicated time intervals. The lines show the best fits of a monoexponential function giving the following rate constants: wild type, 20 s⁻¹; N706A, 4.5 s⁻¹; N706C, 2.7 s⁻¹; N706S, 6.3 s⁻¹. The broken lines correspond to the wild-type curve from the upper left panel. In each case, the maximal level of phosphorylation was taken as 100%, 8, to monitor the phosphorylation of enzyme initially present in the Ca2+-bound state, microsomes suspended in a buffer containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl2, and 100 μM CaCl2, were mixed with an equal amount of the same buffer containing in addition 10 μM [γ-32P]ATP, followed by acid quenching at the indicated time intervals.

---

**FIGURE 3.** Rapid kinetics of phosphorylation from [γ-32P]ATP of enzyme pre-equilibrated with (A) or without (B) Ca2+. Quench-flow experiments were carried out using a QFM-5 quench-flow module at 25 °C with mixing protocols as illustrated above the panels. A, to monitor the phosphorylation of enzyme initially present in the Ca2+-bound state, microsomes suspended in a buffer containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl2, and 100 mM CaCl2, were mixed with an equal amount of the same buffer containing in addition 10 μM [γ-32P]ATP, followed by acid quenching at the indicated time intervals. The lines show the results of computations carried out as previously described using the SimZyme program (27, 36) with the following rate constants for the Ca-E1 → Ca-E2 transition step: wild type, 35 s⁻¹; N706A, 19 s⁻¹; N706C, 8.6 s⁻¹; N706S, 35 s⁻¹. The broken lines correspond to the wild-type curve from the upper left panel. In each case, the maximal level of phosphorylation was taken as 100%, 8, to monitor the phosphorylation of enzyme initially present in the Ca2+-deprived state, microsomes suspended in a buffer containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, and 2 mM EGTA were mixed with an equal amount of a buffer containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 10 mM MgCl2, 2.2 mM CaCl2, and 10 μM [γ-32P]ATP, followed by acid quenching at the indicated time intervals. The lines show the best fits of a monoexponential function giving the following rate constants: wild type, 20 s⁻¹; N706A, 4.5 s⁻¹; N706C, 2.7 s⁻¹; N706S, 6.3 s⁻¹. The broken lines correspond to the wild-type curve from the upper left panel. In each case, the 100% value corresponds to the phosphorylation level at infinite time as deduced from the fit.

---

**Asn706 and Glu183 of SR Ca2+-ATPase**
FIGURE 4. Kinetics of Ca$^{2+}$ binding (A) and Ca$^{2+}$ dissociation (B). A, time course of the Ca$^{2+}$ binding transition determined by measuring the appearance of ability to phosphorylate. Quench-flow experiments were carried out as illustrated by the diagram above the panel using a QFM-5 module at 25 °C. Wild-type or mutant enzyme, preincubated in 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, and 2 mM EGTA, was mixed with an equal volume of 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 10 mM MgCl$_2$, 1 mM EGTA, 10 μM [$\gamma$-32P]ATP, and 1.1 mM CaCl$_2$ was added, followed by acid quenching 34 ms later. To obtain the point corresponding to zero time, the enzyme was preincubated in a buffer containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 10 mM MgCl$_2$, 10 μM [$\gamma$-32P]ATP, and 2.2 mM CaCl$_2$, followed by acid quenching 34 ms later. The lines show the best fits of a monoexponential function with an initial offset, giving the rate constants indicated in parentheses:

- circles: wild type (11.2 s$^{-1}$); squares: N706A (1.7 s$^{-1}$); triangles pointing upward, N706C (2.0 s$^{-1}$); triangles pointing downward, N706S (2.9 s$^{-1}$).

In each case, the phosphorylation level corresponding to zero time was taken as 100%.

FIGURE 5. Dephosphorylation of phosphoenzyme formed from [$\gamma$-32P]ATP. Wild-type or mutant enzyme was phosphorylated for 15 s at 0 °C in a buffer containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 5 mM MgCl$_2$, 1 mM EGTA, 0.955 mM CaCl$_2$ (giving a free Ca$^{2+}$ concentration during phosphorylation of 10 μM), 2 μM calcium ionophore A23187, and 5 μM [$\gamma$-32P]ATP. To measure dephosphorylation, the phosphoenzyme was chased at 0 °C by addition of 10 μM EGTA to remove Ca$^{2+}$, either without (open symbols) or with (solid symbols) 1 mM ADP, and acid quenching was performed at the indicated time intervals. The lines show the best fits of a monoexponential decay function, giving the rate constants indicated in parentheses: open circles, wild type (0.113 s$^{-1}$); open squares, N706A (0.019 s$^{-1}$); open triangles pointing upward, N706C (0.0078 s$^{-1}$); open triangles pointing downward, N706S (0.020 s$^{-1}$). The broken lines correspond to the wild-type curve from the upper left panel. In each case, the phosphorylation level at zero time was taken as 100%.

quenching. Because only the Ca$^{2+}$-bound enzyme fraction phosphorylates, the rate of disappearance of ability to phosphorylate reflects the rate of Ca$^{2+}$ dissociation. As seen in Fig. 4B, there were only minor differences between the Ca$^{2+}$ dissociation rates of the wild-type and the Asn$^{706}$ mutants, apparently excluding a role for Asn$^{706}$ in Ca$^{2+}$ binding or in the conformational changes involved in Ca$^{2+}$ dissociation from the high affinity sites.

The Ca$_{E1P}$ → E2P Conformational Transition of the Phosphoenzyme—To investigate the processing of the phosphoenzyme, the enzyme was phosphorylated with [$\gamma$-32P]ATP under conditions where Ca$_{E1P}$ accumulates as the major steady-state intermediate in the wild-type enzyme (0 °C, presence of K$^+$, neutral pH). Phosphoenzyme decay in the forward direction of the pump cycle was examined by addition of excess EGTA to terminate phosphorylation by removing Ca$^{2+}$, followed by acid quenching at varying time intervals. As shown in Fig. 5 (open symbols), all three mutants displayed reduced rates of phosphoenzyme turnover relative to wild type (2.4-, 8.8-, and 2.9-fold for N706A, N706C, and N706S, respectively).

For wild type, the conformational change of the phosphoenzyme, the Ca$_{E1P}$ → E2P transition, is rate-limiting for the overall ATPase reaction, whereas the ensuing dephosphorylation of E2P (cf. Scheme 1) is much faster in the presence of K$^+$ at neutral pH. The Ca$_{E1P}$ state is characterized by being able to donate its phosphoryl group back to ADP, forming ATP, whereas E2P is ADP insensitive and dephosphorylates only by hydrolysis of the acyl phosphate. To determine whether the Ca$_{E1P}$ → E2P step or E2P → E2 is rate-limiting in the mutants, we measured the ADP sensitivity of the accumulated phosphoenzyme. The dephosphorylation solution was supplemented with 1 mM ADP, and phosphoenzyme decay was again followed (Fig. 5, solid symbols). For the three mutants, as well as the wild type, all phosphoenzyme disappeared completely within 5 s of the addition of ADP, demonstrating that the accumulated phosphoenzyme was entirely ADP-sensitive Ca$_{E1P}$. Similar experiments were carried out under buffer conditions where a high level of E2P accumulates at steady state in wild type (pH 8 and K$^+$).
replaced by Li⁺, cf. Ref. 10), and again no significant level of E2P was detected in the Asn⁷⁰⁶ mutants (data not shown). The fact that no ADP-insensitive E2P had accumulated shows that the low rate of phosphoenzyme processing in the mutants, corresponding to the open symbols in Fig. 5, results from a block of the Ca²⁺E1P → E2P transition, and that E2P hydrolysis is not grossly slowed in the mutants.

Affinity of E2 for Vanadate—As demonstrated above (Fig. 1), none of the Asn⁷⁰⁶ mutants showed any phosphorylation from inorganic phosphate, even after incubation for a long time at a concentration of Pi, 50-fold higher than the concentration required for half-maximal phosphorylation of wild type. Because the mutants could easily undergo phosphorylation from ATP, the binding of the phosphate analog vanadate could be examined by taking advantage of the competition between ATP and vanadate, as previously described (29). Vanadate, which often binds to the phosphoryl group, binds to the Asn⁷⁰⁶ mutants (data not shown). The fact that no ADP-phosphate, even after incubation for a long time at a concentration of Pi, 50-fold higher than the concentration required for half-maximal hydrolysis is not grossly slowed in the mutants. Fig. 5, results from a block of the Ca²⁺E1P → E2P transition, and that E2P hydrolysis is not grossly slowed in the mutants.

![FIGURE 6. Vanadate binding to E2 determined by inhibition of phosphorylation from [γ-32P]ATP. Microsomes were incubated for 1 h at 25 °C and subsequently 15 min at 0 °C in a buffer containing 40 mM MOPS/Tris (pH 7.0), 80 mM KCl, 2 mM EGTA, 5 mM MgCl₂, and the indicated concentration of orthovanadate. The level of inhibition was then tested at 0 °C by sequential addition of 2.5 mM CaCl₂ and 5 μM [γ-32P]ATP, followed by acid quenching 15 s later. The maximum level of phosphorylation obtained in the absence of vanadate was taken as 100%. The lines show the best fit of the Hill equation for inhibition (see “Experimental Procedures”) to the data, giving the K₁/₂ values indicated in parentheses: open circles, wild type (0.16 μM); squares, N706A (>100 μM); triangles pointing upward, N706C (>100 μM); triangles pointing downward, N706S (>100 μM); solid circles, E183A (>100 μM).](source: image)

TABLE 2
Summary of vanadate, BeF, AIF, and MgF binding to E2 and ADP-AIF binding to Ca²⁺E1

| Enzyme state | E2 | Ca²⁺E1 |
|--------------|---|--------|
| Inhibitor    | Vanadate | BeF | AIF | MgF | ADP-AIF |
| Presumed state of phosphate represented by inhibitor | Fig. 6 | Fig. 7B | Fig. 7A | Fig. 7C (Fig. 7D) | Fig. 8 |
| Wild type    | 1 | 1 | 1 | 1 | 4.1 |
| N706A        | >1000 | 15 | 2.9 | 16 (7.4) | 1.1 |
| N706C        | >1000 | 32 | 3.5 | 8.3 (3.9) | 15 |
| N706S        | >1000 | 6.1 | 1.2 | 1.9 (1.5) | 1.6 |
| E183A        | >1000 | 9.3 | 16 | 4.1 (2.7) | 1.8 |

K₁/₂ values for inhibition are displayed relative to the values obtained with wild type (the original K₁/₂ values are listed in the legends to Figs. 6–8). For MgF, the values in parentheses refer to the experiments where the Mg²⁺ concentration was kept constant and the fluoride concentration varied.
ATPase in E2 with bound AlF or MgF (6, 7) seem to confirm the proposals by Danko et al. (15) with respect to the structure of the bound AlF and MgF complexes, whereas no crystal structure of Ca\(^{2+}\)-ATPase with bound BeF has yet been solved. Thus, the structure analogous to genuine E2P is still missing. The distinctions are important for the pumping mechanism, because the AlF and MgF protein complexes do not have an obvious passage for Ca\(^{2+}\) access from the lumen, whereas such a passage may exist in E2-BeF and E2P (15).

To obtain more detailed information about the roles of Asn706 and Glu183 in interaction with the phosphoryl group in various E2 states, and to learn more about the differential characteristics of the fluoride complexes and vanadate, we studied the binding of AlF (Fig. 7A), BeF (Fig. 7B), and MgF (Fig. 7, C and D) to wild type and mutants, using the same method as described above for vanadate, which takes advantage of the competition at the catalytic site. This was feasible, because these phosphate analogs, like vanadate, all dissociate slowly from the Ca\(^{2+}\)-ATPase (BeF was found to dissociate at approximately the same rate as vanadate under the conditions applied, and AlF and MgF dissociated even slower, see Fig. SII in supplemental materials). To study AlF binding, Ca\(^{2+}\)-deprived enzyme was incubated for 30 min at 25 °C with 2 mM NaF, 200 μM Mg\(^{2+}\), and varying concentrations of AlCl\(_3\), followed by cooling on ice. Mg\(^{2+}\) at low concentration is required for the formation of the enzyme complex with AlF, but only 200 μM was added during the AlF binding step to avoid formation of MgF, cf Fig. 7 (C and D). The uncomplexed fraction of the enzyme was then determined by measuring the phosphorylation occurring during a 10-s incubation with 5 μM [γ\(^32\)P]ATP at 0 °C after supplementing the medium with excess Ca\(^{2+}\) and 5 mM Mg\(^{2+}\). Wild-type Ca\(^{2+}\)-ATPase displayed half-maximal inhibition of phosphorylation at 8.8 μM AlCl\(_3\) (Fig. 7A). In control experiments where no NaF had been added, 200 μM AlCl\(_3\), (probably binding at Ca\(^{2+}\) sites) was required to obtain half-maximal inhibition (broken line in Fig. 7A; data points shown only for wild type, but identical data were obtained for the mutants). The mutants with alterations to Asn706 were also very sensitive to inhibition by AlF, contrasting their lack of sensitivity to vanadate (cf. Fig. 6). Thus, the AlF inhibition profile of N706S was indistinguishable from that of the wild type, and N706A and N706C displayed 3-fold reduced apparent affinities for AlF, relative to wild type (Fig. 7A and Table 2). E183A, on the other hand, displayed very low AlF affinity (16-fold reduced relative to wild type). In fact, there was little difference between the result of the AlF titration experiment with E183A and the control experiment, where Al\(^{3+}\) was added in the absence of NaF.

We then proceeded to study the binding of BeF to E2 (Fig. 7B and Table 2). The assay was carried out as described above for AlF, except that AlCl\(_3\) was replaced by BeSO\(_4\). Wild-type Ca\(^{2+}\)-ATPase displayed half-maximal inactivation at 0.8 μM BeSO\(_4\), i.e. 11-fold higher apparent affinity than with AlCl\(_3\). The effects of the Asn706 mutations on BeF binding were markedly stronger than the effects on AlF binding. Thus, N706C displayed a 32-fold reduced affinity for BeF relative to wild type (compare with the 3.5-fold reduced affinity of N706C for AlF). A similar pattern was seen for N706A and N706S (15- and 6.1-fold reduced affinities for BeF, respectively, compare with the respective 2.9-fold reduced and wild type-like affinities for AlF). E183A was rather similar to the Asn706 mutants with respect to BeF binding, displaying 9.3-fold reduced affinity for BeF relative to wild type. As was the case with Al\(^{3+}\), Be\(^{2+}\) in the absence of NaF also inhibited the enzyme at high concentrations (broken line in Fig. 7B; data points only shown for wild type, but identical data were obtained for the mutants).

The binding of MgF to E2 was likewise examined (Fig. 7, C and D, and Table 2). The results shown in Fig. 7C were obtained as described above for AlF, except that AlCl\(_3\) was replaced by MgCl\(_2\) (and Mg\(^{2+}\) thus
apparent affinity for MgF as compared with the other fluoride complexes. In Fig. 7D, the roles of NaF and MgCl2 were reversed: the added MgCl2 concentration being kept constant at 5 mM while the added NaF concentration was varied (note the broken line, demonstrating that fluoride alone at concentrations up to 10 mM is without effect, this also serves as fluoride control in the other experiments). Irrespective of whether MgCl2 or NaF was varied, the results looked the same, consistent with the idea that the inhibitory compound is a complex between Mg2+ and F– and not any of these species in their uncomplexed state. All four mutants displayed significant affinity shifts relative to wild type, with N706A and N706C showing the most marked effects (7.4- to 16-fold and 3.9- to 8.3-fold reduction of apparent affinity, respectively), N706S (1.5- to 1.9-fold reduction of apparent affinity) being only slightly less sensitive to MgF inhibition than wild type, and E183A displaying an intermediate behavior (2.7- to 4.1-fold reduction of apparent affinity).

Affinity of Ca2_E1 for ADP-AlF—ADP and AlF bind together with high affinity in a complex with the Ca2_E1 state of wild-type Ca2+-ATPase, with concomitant tight occlusion of the two Ca2+ ions at the transport sites (31). Because the Ca2_E1 enzyme complex with ADP-AlF is believed to mimic the transition state in the transfer of the γ-phosphoryl group from ATP (5, 7), we also studied the effects of the Asn706 and Glu183 mutations on formation of this complex, using the same assay as described above, but including 100 μM Ca2+ and 1 μM ADP during complex formation (Fig. 8). The presence of Ca2+ ensures that the enzyme is in Ca2_E1. Hence, the E2 conformation reacting with AlF in the absence of ADP (cf. Fig. 7A) is depleted (as illustrated by the broken line in Fig. 8, inhibition by AlF is rather weak in the absence of ADP when Ca2+ is present). The ADP concentration of 1 μM was chosen as a compromise, being sufficient to cause marked inhibition of wild-type Ca2+-ATPase in the presence of AlCl3 and NaF, without interfering significantly with the phosphorylation of uncomplexed enzyme from MgATP (data not shown). Fig. 8 shows that the wild type displayed a K0.5 of 3.7 μM for inhibition under these conditions. N706A and N706C displayed markedly increased K0.5 values (reduced apparent affinities) relative to wild type (4.1- and 15-fold, respectively, see Table 2), whereas N706S differed only slightly from wild type. E183A likewise displayed only 1.8-fold reduced apparent affinity for ADP-AlF relative to wild type (Fig. 8 and Table 2).

**DISCUSSION**

In this study, we have explored the functional consequences of mutations of Asn706 in domain P of Ca2+-ATPase. It is informative to analyze the results obtained with the Asn706 mutants in relation to the crystal structures of the Ca2+-ATPase (Fig. 9), and furthermore to compare with the results obtained previously (10), and in the present study, with mutant E183A, where the glutamate of the conserved TGES motif of domain A is replaced. Mutations of Asn706 and Glu183 have in common that they dramatically impede the function of the enzyme in E2 forms, but have less striking effects on E1. In our previous study (10), we showed that replacement of Glu183 with alanine leads to a reduced rate of both E2P dephosphorylation and the reverse phosphorylation of E2 with P, suggesting that Glu183 is very critical for catalysis and, thus, for transition state stabilization. The functional analysis turned out to correlate well with the location of Glu183 in the subsequently published E2-AlF crystal structure (Fig. 9). In this structure, the AlF complex

![Image](image-url)
(modeled as AlF₄⁻) is planar and is positioned linearly between one of the carboxylate oxygens of Asp¹⁵¹ and a water molecule, held and likely activated by Glu¹⁸₃ for nucleophilic attack on the phosphorous atom (6). It is clear from the present results that Asn⁷⁰⁶ is also a critical residue for E₂P formation. However, whereas for mutant E₁₈₃A E₂P phosphoenzyme formed from P₅, did, in fact, accumulate (though slowly relative to wild type), none of the mutants with alterations to Asn⁷⁰⁶ showed any accumulation of E₂P, even after incubation for a long time at a concentration of P₅, 50-fold higher than the concentration required for half-maximal phosphorylation of wild type (Fig. 1). The lack of ability to phosphorylate from P₅ prevented us from studying the rate of E₂P hydrolysis directly in the Asn⁷⁰⁶ mutants, but some of the data obtained with the phosphoenzyme formed from ATP suggest that the E₂P hydrolysis step is not as inhibited in these mutants as it is in E₁₈₃A. Asn⁷⁰⁶ therefore seems to play a less critical role than Glu¹⁸₃ in stabilization of the transition state in E₂P hydrolysis. Thus, virtually all phosphoenzyme accumulated at steady state following reaction of the three Asn⁷⁰⁶ mutants with ATP was ADP-sensitive Ca²⁺ (Fig. 5). In comparison, 43% of the phosphoenzyme accumulated under identical conditions with E₁₈₃A was ADP-insensitive E₂P, despite a reduced rate of the Ca₂⁺E₁P → E₂P transition in E₁₈₃A (10), thus reflecting the reduced rate of E₂P hydrolysis in the latter mutant. Even in phosphorolyis experiments with ATP carried out under buffer conditions where a high level of E₂P accumulates at steady state in wild type (i.e. pH 8 and K⁺ replaced by Li⁺), no significant level of E₂P accumulated in the mutants with alterations to Asn⁷⁰⁶. Thus, unlike E₁₈₃A, the mutations of Asn⁷⁰⁶ do not seem to impair E₂P hydrolysis markedly; in fact it may even be enhanced, due to destabilization of E₂P.

Danko et al. (15) proposed a distinction between E₂-BeF, E₂-AIF, and E₂-MgF as analogs of the ground state, transition state, and E₂P, product state, respectively, in E₂P hydrolysis. In contrast to the planar tetragonal AIF complex of the E₂-AIF crystal structure, that seems to mimic the transition state of E₂P hydrolysis, the MgF complex (i.e. MgF₂⁻) is in a tetrahedral arrangement in the E₂-MgF crystal structure (see Fig. 9 and Table 2), implying that E₂-MgF represents the E₂P, product state of E₂P hydrolysis. The E₂-BeF complex (of which no crystal structure has yet been published) shares several features with the E₂P ground state, including an increased hydrophobicity of the nucleotide site relative to E₂, E₂-AIF, E₂-MgF, and E₂-vanadate. Furthermore, E₂-BeF displays a high sensitivity to luminal Ca²⁺ (a feature not seen with E₂-AIF and E₂-MgF), as one would expect from a true E₂P ground state with luminally exposed Ca²⁺ sites (15). With respect to BeF binding (Fig. 7B), all four mutants displayed significant shifts toward lower apparent affinity relative to wild type (6- to 32-fold), suggesting that both Asn⁷⁰⁶ and Glu¹⁸₃ contribute significantly to stabilization of the E₂P ground state. The affinities for AIF and MgF differed more significantly among the four mutants. The affinity for AIF (Fig. 7A) was markedly reduced in E₁₈₃A relative to wild type, consistent with Glu¹⁸₃ being involved in stabilization of the E₂P transition state. The affinity for AIF was much less affected in the mutants with alterations to Asn⁷⁰⁶ than in E₁₈₃A, N706S, in particular, being completely wild-type-like (Fig. 7A), thus suggesting that the side chain of Asn⁷⁰⁶ contributes marginally or not at all to the stability of the E₂P transition state, in line with the results of studies of the phosphoenzyme discussed above. This is somewhat surprising considering that the side-chain nitrogen atom of Asn⁷⁰⁶ is located within 2.8 Å of AIF in the E₂-AIF crystal structure (Fig. 9) and that the side-chain oxygen of Asn⁷⁰⁶ is within bonding distance of a water molecule that coordinates the catalytic Mg²⁺ ion (indicated by orange broken lines in Fig. 9), although the link to Mg²⁺ might persist in mutant N706S, due to the oxygen in the serine side chain.

In the E₂-MgF crystal structure, thought to mimic the E₂-P, product state, the disposition of the Asn⁷⁰⁶ side chain in relation to the fluoride and the water molecule coordinating the catalytic Mg²⁺ is very similar to that seen in the E₂-AIF crystal structure (Fig. 9). Nevertheless, the inhibition data obtained with MgF (Fig. 7, C and D) showed a picture quite different from that seen for AIF. Hence, even mutation N706S lowered the affinity for MgF significantly, and the order of E₁₈₃A and N706A/N706C was reversed, such that the latter two mutants displayed the most severely reduced binding affinities of the four mutants. The MgF inhibition data suggest that the Asn⁷⁰⁶ side chain contributes significantly to stabilization of the E₂-P, product state, consistent with the E₂-MgF crystal structure.

To understand the differential effects of Asn⁷⁰⁶ mutation on the binding of AIF and MgF, it could be important that there is increased negative charge density around the phosphoryl group in an associative transition state (see schematic diagram in Ref. 34). Perhaps for this reason the role of the neutral asparagine side chain is diminished during the transition and in the E₂-AIF complex. In the E₂-AIF crystal structure, the distance from A1 to the coordinating oxygen of Asp¹⁵¹ or the water molecule positioned by Glu¹⁸₃ is only 2.1 Å, consistent with covalent bonding (6), which means that any interaction with Asn⁷⁰⁶ is relatively less important for stabilization of the E₂-AIF structure compared with E₂-MgF, where the interaction between the protein and the fluoride does not have the character of a covalent bond.

Turning now to the mutational effects on the E₁ states, it is clear from our nucleotide binding data (Table 1) that the Asn⁷⁰⁶ side chain is not critical for high affinity nucleotide binding in E₁. Thus, the mutations of Asn⁷⁰⁶ had no effect on ATP binding in the absence of Mg²⁺, and in the presence of Mg²⁺ we observed a less than 2-fold reduction of MgATP affinity, relative to wild type, for N706A and N706C, and wild type-like behavior for N706S. Likewise, the rate of phosphorylation of Ca²⁺-saturated enzyme from [γ-³²P]ATP (Fig. 3A) as well as the binding affinity for ADF-AIF (Fig. 8) were wild-type-like in N706S. The effects of the other two Asn⁷⁰⁶ mutations were larger, in particular N706C showed 4- to 5-fold reduced affinity for MgATP, consistent with a lower affinity for MgF (Fig. 5), thus suggesting that the affinity for MgF (Fig. 7A) is reduced in E₁₈₃A relative to wild type, consistent with Glu¹⁸₃ being involved in stabilization of the E₂P transition state. The affinity for AIF was much less affected in the mutants with alterations to Asn⁷⁶ than in E₁₈₃A, N706S, in particular, being completely wild-type-like (Fig. 7A), thus suggesting that the side chain of Asn⁷⁶ contributes marginally or not at all to the stability of the transition state. The side chain of Asn⁷⁶ is in a very similar position to that seen in the E₂-MgF and E₂-AIF crystal structures, i.e. within bonding distance of the fluoride and linked to the catalytic Mg²⁺ via a water molecule (cf. Fig. 9). The small effect of N706A and lack of effect of N706S on ADF-AIF binding suggest that Asn⁷⁶ plays a relatively minor role in E₁P transition state stabilization, exactly as found for the E₂P transition state. The larger effects of mutation N706C may be due to the cysteine side chain being ionized, thereby leading to electrostatic repulsion of the γ-phosphate/AIF.

The conspicuous effects of the Asn⁷⁶ mutations on vanadate binding to E₂ were very similar to the effects on P, reactivity. Both vanadate and AIF have been considered phosphoryl transition state analogs, but when one compares the results in Figs. 6 and 7A, it is clear that vanadate binding was much more strongly affected than AIF binding by all four mutations studied here. In particular, N706S was almost completely insensitive to vanadate and, at the same time, wild type-like with respect to inhibition by AIF. Hence, vanadate must bind and exert its inhibitory effect differentially from AIF. As judged from the mutational effects, the E₂-vanadate state seems to be a closer mimic of the enzyme with bound phosphorolyis group than the enzyme complexes with fluoride com-
Asn\textsuperscript{706} and Glu\textsuperscript{183} of SR Ca\textsuperscript{2+}-ATPase

pounds, which may have to do with the fact that vanadate adopts a trigonal bipyramidal structure and has oxygen like phosphate, and not the strongly electronegative fluorines. The fluoride compound that resembles vanadate the most in our functional assays is BeF, with its relatively small size possibly due to its strong electronegativity. However, it appears that Asn\textsuperscript{706} is critical for stabilizing E2P and, possibly therefore, also for the Ca\textsubscript{2}E1P \rightarrow E2P transition, and that it likely loses its importance in the transition state, where charge effects are greatest, but regains consequence in E2P and E2. The latter conformation is only slowly transformed into Ca\textsubscript{2}E1 in the Asn\textsuperscript{706} mutants. Interaction of Asn\textsuperscript{706} with domain A may be an important aspect of these effects.

Acknowledgments—We thank Lene Jacobsen and Karin Kracht (University of Aarhus, Aarhus, Denmark) and Irene Mardarowicz and Joy Norman (University of Cape Town, Cape Town, South Africa) for expert technical assistance.

REFERENCES

1. Hasselbach, W., and Makino, M. (1961) Biochem. Z. 333, 518–528
2. Shigekawa, M., and Dougherty, J. P. (1978) J. Biol. Chem. 253, 1458–1464
3. de Meis, L., and Vianna, A. I. (1979) Annu. Rev. Biochem. 48, 275–292
4. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
5. Sørensen, T. L., Møller, J. V., and Nissen, P. (2004) Science 304, 1672–1675
6. Olesen, C., Sørensen, T. L., Nielsen, R. C., Møller, J. V., and Nissen, P. (2004) Science 306, 2251–2255
7. Toyoshima, C., Nomura, H., and Tsuda, T. (2004) Nature 432, 361–368
8. Toyoshima, C., and Mizutani, T. (2004) Nature 430, 529–535
9. Toyoshima, C., and Nomura, H. (2002) Nature 418, 605–611
10. Clausen, J. D., Vilsen, B., McIntosh, D. B., Einholm, A. P., and Andersen, J. P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2776–2781
11. Axelsen, K. B., and Palmgren, M. G. (1998) J. Mol. Evol. 46, 84–101
12. Arvind, L., Galperin, M. Y., and Koonin, E. V. (1998) Trends Biochem. Sci. 23, 127–129
13. Stokes, D. L., and Green, N. M. (2000) Biophys. J. 78, 1765–1776
14. Wang, W., Cho, H. S., Kim, R., Jani, J., Yokota, H., Nguyen, H. G., Grigoriev, I. V., Wemmer, D. E., and Kim, S. H. (2002) J. Mol. Biol. 319, 421–431
15. Danko, S., Yamasaki, K., Daibo, T., and Suzuki, H. (2004) J. Biol. Chem. 279, 14991–14998
16. Vilsen, B., Andersen, J. P., Clarke, D. M., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 20104–20109
17. Clarke, D. M., Loo, T. W., and MacLennan, D. H. (1990) J. Biol. Chem. 265, 14088–14092
18. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. (1989) Mol. Cell. Biol. 9, 946–958
19. Gluzman, Y. (1981) Cell 23, 175–182
20. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
21. Maruyama, K., and MacLennan, D. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3314–3318
22. Leberer, E., and Pette, D. (1986) Biochem. J. 235, 67–73
23. Vilsen, B., Andersen, J. P., and MacLennan, D. H. (1991) J. Biol. Chem. 266, 16157–16164
24. Sørensen, T., Vilsen, B., and Andersen, J. P. (1997) J. Biol. Chem. 272, 30244–30253
25. Baginski, E. S., Foa, P. P., and Zak, B. (1987) Clin. Chem. 33, 326–332
26. Andersen, J. P., Vilsen, B., Leberer, E., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 21018–21023
27. Sørensen, T. L., Dupont, Y., Vilsen, B., and Andersen, J. P. (2000) J. Biol. Chem. 275, 5400–5408
28. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
29. Clausen, J. D., and Andersen, J. P. (2003) Biochemistry 42, 2585–2594
30. Murphy, A. J., and Coll, R. J. (1992) J. Biol. Chem. 267, 5229–5235
31. Troullié, A., Girardet, J. L., and Dupont, Y. (1992) J. Biol. Chem. 267, 22821–22829
32. Murphy, A. J., and Coll, R. J. (1993) J. Biol. Chem. 268, 23307–23310
33. McIntosh, D. B., Woolley, D. G., Vilsen, B., and Andersen, J. P. (1996) J. Biol. Chem. 271, 25778–25789
34. McIntosh, D. B., Woolley, D. G., MacLennan, D. H., Vilsen, B., and Andersen, J. P. (1999) J. Biol. Chem. 274, 25427–25436
35. McIntosh, D. B., Clausen, J. D., Woolley, D. G., MacLennan, D. H., Vilsen, B., and Andersen, J. P. (2004) J. Biol. Chem. 279, 32515–32523
36. Andersen, J. P., Sørensen, T. L., Povlsen, K., and Vilsen, B. (2001) J. Biol. Chem. 276, 23312–23321
37. Inesi, G., Ma, H., Lewis, D., and Xu, C. (2004) J. Biol. Chem. 279, 31629–31637
Asn^{706} and Glu^{183} of SR Ca^{2+}-ATPase

38. Ma, H., Lewis, D., Xu, C., Inesi, G., and Toyoshima, C. (2005) *Biochemistry* **44**, 8090–8100
39. Clausen, J. D., McIntosh, D. B., Woolley, D. G., and Andersen, J. P. (2001) *J. Biol. Chem.* **276**, 35741–35750
40. Guillain, F., Champeil, P., Lacapere, J. J., and Gingold, M. P. (1981) *J. Biol. Chem.* **256**, 6140–6147
41. Petithory, J. R., and Jencks, W. P. (1988) *Biochemistry* **27**, 5553–5564
42. Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978) *J. Biol. Chem.* **253**, 7361–7368
43. Pick, U. (1982) *J. Biol. Chem.* **257**, 6111–6119
44. Smith, R. M., and Martell, A. E. (1976) *Critical Stability Constants: Inorganic Complexes*, Plenum Press, New York
45. Wang, G., Yamasaki, K., Daiho, T., and Suzuki, H. (2005) *J. Biol. Chem.* **280**, 26508–26516