Functional Consequences of Alterations to Amino Acids Located in the Hinge Domain of the Ca$^{2+}$-ATPase of Sarcoplasmic Reticulum*

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Site-specific mutagenesis of the sarcoplasmic reticulum Ca$^{2+}$-ATPase was used to investigate the functional roles of 18 amino acid residues located at or near the "hinge domain," a highly conserved region of the cation-transporting ATPases. Mutation of Lys$^{684}$ to arginine, alanine, histidine, and glutamine resulted in complete loss of calcium transport function and ATPase activity. For the Lys$^{684} \rightarrow$ Ala, histidine, and glutamine mutants, this coincided with a loss of the ability to form a phosphorylated intermediate from ATP or P. The Lys$^{684} \rightarrow$ Arg mutant retained the ability to phosphorylate from ATP with normal apparent affinity, demonstrating the importance of the positive charge. On the other hand, no phosphorylation was observed with P$_i$ as substrate in this mutant. Examination of the partial reactions after phosphorylation from ATP in the Lys$^{684} \rightarrow$ Arg mutant demonstrated a reduction of the rate of transformation of the ADP-sensitive phosphoenzyme intermediate (E,P) to the ADP-insensitive phosphoenzyme intermediate (E,P), which could account for the loss of transport function. Once accumulated, the E,P intermediate was able to decompose rapidly in the presence of K$^+$ at neutral pH. These results may be interpreted in terms of a preferential destabilization of protein phosphate interactions in the E,P form of this mutant.

The Asp$^{703} \rightarrow$ Ala and Asn$^{797} \rightarrow$ Ala-Ala mutants were completely inactive and unable to form phosphoryl enzyme intermediates from ATP or P. In these mutants as well as in the Lys$^{684} \rightarrow$ Ala mutant, nucleotides were found to protect with normal affinity against intramolecular cross-linking induced with glutaraldehyde, indicating that the nucleotide binding site was intact. Mutation of Glu$^{446}$, Glu$^{447}$, Asp$^{508}$, Asp$^{560}$, Glu$^{608}$, Asp$^{595}$, Glu$^{606}$, Glu$^{154}$, and Glu$^{732}$ to alanine did not affect the maximum rates of calcium transport and ATP hydrolysis or the apparent affinities for calcium and ATP.

Mutation of the 2 highly conserved proline residues, Pro$^{681}$ and Pro$^{709}$, as well as Lys$^{728}$, to alanine resulted in partially inhibited Ca$^{2+}$-ATPase enzymes with retention of the ability to form a phosphoenzyme intermediate from ATP or P, and with normal apparent affinities for ATP and calcium. The proline mutants retained the biphasic ATP concentration dependence of ATPase activity, characteristic of the wild-type, and therefore the partial inhibition of turnover could not be ascribed to a disruption of the low affinity modulatory ATP site.

The data seem to exclude a critical role of the hinge domain in the formation of calcium and nucleotide binding sites but point to a role of the hinge domain in the binding and transfer of phosphate to the protein and in the transport-associated conformational changes of the phosphorylation site.

The Ca$^{2+}$-ATPase of sarcoplasmic reticulum utilizes the energy derived from the hydrolysis of ATP to transport Ca$^{2+}$ ions across the membrane against a concentration gradient (Hasselbach and Makinose, 1961). It belongs to a family of transport ATPases for which it is an obligatory step to form a phosphorylated intermediate during the course of cation-transport ("P-type ATPases"). The cloning and sequencing of CDNA encoding the Ca$^{2+}$-ATPase of rabbit muscle sarcoplasmic reticulum have resulted in the proposal of a structural model for the enzyme in which two cytoplasmic regions are joined to 10 predicted transmembrane $\alpha$-helices (M$_1$-M$_{10}$) by a pentalilical stalk region (MacLennan et al., 1985; Brandl et al., 1986). The largest cytoplasmic region connecting the putative membrane-spanning helices M$_i$ and M$_{i+1}$ contains on its N-terminal side the phosphorylation domain with the aspartyl residue (Asp$^{357}$) being phosphorylated from ATP, in its central region the nucleotide binding domain containing the lysine labeled by fluorescein isothiocyanate (Lys$^{675}$), and on its C-terminal side a relatively short segment (64 residues, beginning with Cys$^{675}$), which has been referred to as the "hinge domain," since, by analogy to several kinases of known structure, this segment was suggested to interact in a hinge bending move with the phosphorylation domain (Green et al., 1986; Taylor and Green, 1989). Although the hinge domain constitutes one of the most highly conserved regions of the P-type ATPases (Serrano, 1988), there is little precise knowledge about its functional role and spatial arrangement relative to the other extramembranous domains. It has been proposed to form a more integral part of the ATP binding site (Portillo and Serrano, 1988; Serrano, 1989) or to form part of a second (low affinity) ATP site (Shull and Greeb, 1988). The hinge domain contains a number of acidic residues and could also be directly implicated in the binding of Ca$^{2+}$, in line with the model by Tanford et al. (1987), explaining the Ca$^{2+}$ activation
of the transfer of γ-phosphate from ATP by a jaw-closing mechanism.

The hinge domain has been shown to be the target for affinity labels directed toward the ATP binding site of Na,K-ATPase (Ohta et al., 1986; Ovchinnikov et al., 1987). Recently, Lys684 of the Ca2+-ATPase was found to be labeled with adenosine triphosphoryl-oxalate, which has proved useful for probing nucleotide sites of various enzymes (Yamamoto et al., 1988, 1989). Since the pyridoxal and γ-phosphoryl moieties are very close to each other in the adenosine triphosphoryl-oxalate molecule, these observations raise the question of whether the highly conserved Lys684 residue interacts directly with the γ-phosphate of ATP.

A specific way to identify residues important for the various partial reactions of the pump cycle is through the use of site-specific mutagenesis. This approach has proved to be valuable in the assignment of residues making up the phosphorylation site (Maruyama and MacLennan, 1988; Maruyama et al., 1989), the high affinity Ca2+-binding sites (Clarke et al., 1989a, 1989b, 1990a), and residues involved in conformational changes of the phosphoenzyme (Andersen et al., 1989; Vilsen et al., 1989; Clarke et al., 1990). In this study we describe the results of mutating 18 amino acids located close to and in the hinge domain. Of those residues, 10 are highly conserved among the P-type ATPases. We have replaced all of the carboxylic acid residues throughout the hinge domain with alanine to test for the role of the oxygen ligands in cation binding. To examine the functional significance of Lys684 and the role of its positive charge in the binding of ATP and phosphate, we mutated Lys684 to the similarly charged residue arginine as well as to alanine, glutamine, and histidine. In addition, Pro681 and Pro719, which are conserved among all the P-type ATPases, were changed to alanine. Our data point to a role of some of these residues in interaction with the γ-phosphate of ATP and with P, but do not support a critical role of the hinge domain in the binding of the nucleotide moiety of ATP or in calcium binding.

**EXPERIMENTAL PROCEDURES**

Oligonucleotide-directed Mutagenesis and Expression of Mutant DNA—The methods employed for site-specific mutagenesis have been described previously (Maruyama and MacLennan, 1988; Vilsen et al., 1989). Briefly, the method of Kunkel (1985) was used to introduce mutations into short restriction fragments of the rabbit fast twitch muscle Ca2+-ATPase cDNA cloned in the Bluescript vector (Stratagene, La Jolla, CA). The presence of the correct mutation was confirmed by sequencing, using the dideoxynucleotide chain termination method (Sanger et al., 1977). The mutated fragments were excised and religated back into their original position in the full-length Ca2+-ATPase cDNA. For expression in COS-1 cells (Gluzman, 1981) the entire ATPase cDNA containing the desired mutation was cloned into the EcoRI site of vector p91023(B) (Kaufman et al., 1989).

The plasmid DNA was transfected into COS-1 cells by the DEAE-dextran procedure described previously (Maruyama and MacLennan, 1988) or by a slightly different procedure described by Kaufman et al. (1989), permitting long time incubation of the cells in the presence of the DNA. The latter protocol enabled us to obtain the relatively high expression levels needed for measurement of ATPase activity (see below).

The microsomal fraction containing mutant Ca2+-ATPase was harvested by differential centrifugation 48–72 h after transfection of the cells (Maruyama and MacLennan, 1988). An enzyme-linked immunosorbent assay was used to quantify the expressed Ca2+-ATPase in each microsomal preparation as described by Clarke et al. (1989b), using monoclonal antibody A52 (Zubrzycka-Gaen et al., 1984) and a polyclonal goat anti-Ca2+-ATPase antibody kindly provided by Dr. H. Brogren, The National Food Agency of Denmark.

As one of our mutations (Asp396→ Ala-Ala) is located in the region identified as the epitope for the A52 antibody (Clarke et al., 1989b), we were concerned over the potential problem of using A52 in the quantitative immunosorbent assay of this mutant. Therefore, we compared the immunoreactivities of the wild-type and the Asp396→ Ala-Ala mutant on Western blots, using a different monoclonal antibody, A25, in addition to A52. We did not obtain any evidence of a preferential reduction of the immunoreactivity of the mutant Ca2+-ATPase to A52, and we were consistently able to calculate the specific phosphoesterase activity in the mutant, amounting to more than 90% of the wild-type level, using the protein concentration determined with A52 (see Table I).

**Functional Characterization of Mutants—Oxalate-supported ATP-driven uptake of calcium was measured by Millipore filtration after 20 min of the incubation of the microsomes at 27°C, in the presence of 5 mM MgATP, 100 mM K+, 5 mM Ca2+, 50 mM Tris buffer (pH 6.5), and various concentrations of "Ca2+" set with EGTA1 as previously described (Vilsen et al., 1989).

Calcium-activated ATPase activity was measured spectrophotometrically at 37°C using the NADH-coupled assay described by Mellor et al. (1980). Microsomes (0.5–2 μg of Ca2+-ATPase) were added to 2.5 ml of medium containing 20 mM TES (pH 7.5), 0.1 mM Ca2+, 1 mM Mg2+, 0.01–5 mM MgATP, 0.1 mM Ca2+, 0.15 mM NADH, 1 mM phosphonoepiperuvate, lactate dehydrogenase (approximately 30 IU), and pyruvate kinase (approximately 30 IU). To avoid back inhibition caused by an accumulation of calcium, 2 μM calcium ionophore A23187 was included, resulting in a 2–5-fold increase of the rate of ATP hydrolysis. The ATPase activity was calculated from the slopes of the linear curves showing the decrease of absorbance at 340 nm as a function of time. To calculate the activity referable to Ca2+-ATPase, the basic activity measured in the presence of 2 mM EGTA was subtracted from the total ATPase activity. For the wild-type enzyme, the basic activity amounted to about 10–20% of the total activity, depending on the ATP concentration.

Phosphoenzyme intermediates formed from ATP were analyzed at 0°C using the procedures described by Andersen et al. (1985, 1989). The concentrations of [γ-32P]ATP and free Ca2+ were varied as indicated in the text and figure legends. To test the ADP sensitivity of the phosphoenzyme, 1 mM ADP was added 5 s prior to acid quenching. In some of the experiments, phosphorylation with 2 μM [γ-32P]ATP was carried out in a medium promoting accumulation of E2P, i.e. in 100 mM TES/Tra (pH 8.5), 10 mM Mg2+, 20 μM Ca2+, 10 mM EDTA, 0.1% bovine serum albumin, 30 μM oxalate, 100 μM ATP, and 20% (v/v) dimethyl sulfoxide. Acid quenching, washing, and electrophoresis at low pH of the phosphorylated protein as well as autoradiography of the dried gels were performed as described previously (Andersen et al., 1989).

**Glutaraldehyde-induced Cross-linking—** Glutaraldehyde treatment of the microsomal preparation containing mutant or wild-type Ca2+-ATPase protein was carried out according to the procedures described by Ross and McIntosh (1987a). Microsomes (0.2–0.3 μg of Ca2+-ATPase) were incubated for 5 min at 20°C in the presence of 5 mM glutaraldehyde, 50 mM MOPS/Tris buffer (pH 6.0), 1 mM EGTA, 5 mM Mg2+, or polyvalent cations or polyvalenate at the concentrations indicated in the figure legends. The reaction was terminated by the addition of 50 mM hydrazine followed by an equal volume of solubilization medium (0.1 M Tris/HCl (pH 6.8), 4% (w/v) SDS, 8 M urea, and 4% (w/v) dithreitol). Electrophoresis in 8% (w/v) SDS-polyacrylamide gels was performed as described previously (Kaufman et al., 1989).

The monoclonal antibody A52 was used to detect the gluteraldehyde-treated Ca2+-ATPase protein. An alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Promega) was bound on top of A52, and the blot was

1 The abbreviations used are: EGTA, [ethylenebis(oxyethylene-nitrito)]etracetic acid; E, P, ADP-sensitive phosphoenzyme intermediate; E; P, ADP-insensitive phosphoenzyme intermediate; MES, 2-(N-morpholino)ethanesulfonic acid; TES, N-tris(hydroxymethyl)- 2-aminoethanesulfonic acid; TNK, ATP, 5',5'-dithiobis-(2-nitro-l-phenyl)adenosine triphosphate; SDS, sodium dodecyl sulfate, MOPS, 3-(N-morpholino)propanesulfonic acid.
visualized using 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium chloride.

To obtain polyvanadate, a monovanadate solution was treated with HCl followed by readjustment of the pH back to 8.0. The resulting yellow solution contains decavanadate, which acts as an ATP analog (Coan et al., 1986).

RESULTS

Expression Level and Ca\(^{2+}\) Transport—In this study we mutated 18 amino acid residues located in the region of the Ca\(^{2+}\)-ATPase, stretching from Glu\(^{646}\) to Asp\(^{731}\). The mutated carboxylic residues include all the carboxylic residues of the hinge domain as well as 2 lysines and 3 prolines. In those cases in which two carboxylic residues were juxtaposed, both of these residues were replaced simultaneously by a double mutation. With the exception of the Asp-Asp\(^{730}\) \(\rightarrow\) Ala-Ala mutant, which was expressed poorly, expression of the mutants varied between 80 and 110\% of that of the wild type, as determined by quantitative enzyme-linked immunosorbent assay. This suggests that with one exception the mutations did not affect the incorporation of the expressed protein into the membrane and its subsequent folding.

A summary of the maximum specific Ca\(^{2+}\) uptake rates of the mutants, indicated as percent of the Ca\(^{2+}\) uptake rate of the wild-type enzyme, is shown in Table I. The measurements were performed at pCa 5.5, ensuring saturation of the high affinity Ca\(^{2+}\) transport sites in the wild-type enzyme. The Glu-Glu\(^{647}\) \(\rightarrow\) Ala-Ala, Asp-Asp\(^{660}\) \(\rightarrow\) Ala-Ala, Glu\(^{660}\) \(\rightarrow\) Ala, Asp-Glu\(^{666}\) \(\rightarrow\) Ala-Ala, Glu\(^{713}\) \(\rightarrow\) Ala, and Glu\(^{733}\) \(\rightarrow\) Ala mutants all transported Ca\(^{2+}\) at rates close to or identical to that of the wild type. For the mutants Pro\(^{691}\) \(\rightarrow\) Ala, Pro\(^{709}\) \(\rightarrow\) Ala, and Lys\(^{728}\) \(\rightarrow\) Ala, we observed a decrease of the transport rate to 27, 48, and 81% of the wild type, respectively, whereas the Ca\(^{2+}\) transport activity was abolished completely for the Lys\(^{685}\) mutants and the Asp\(^{700}\) \(\rightarrow\) Ala and Asn-Asp\(^{705}\) \(\rightarrow\) Ala-Ala mutants.

For those mutants, which were able to transport Ca\(^{2+}\) at a measurable rate, the Ca\(^{2+}\) affinity was examined by titration of the Ca\(^{2+}\) dependence of Ca\(^{2+}\) transport. Examples of this are presented in Fig. 1, which shows that the Glu-Glu\(^{647}\) \(\rightarrow\) Ala-Ala, Asp-Asp\(^{660}\) \(\rightarrow\) Ala-Ala, and Glu\(^{660}\) \(\rightarrow\) Ala mutants all displayed an apparent affinity for Ca\(^{2+}\) identical to that of the wild type. This was also the case for the Asp-Asp\(^{666}\) \(\rightarrow\) Ala-Ala, Glu\(^{713}\) \(\rightarrow\) Ala, and Glu\(^{733}\) \(\rightarrow\) Ala mutants (not shown). Furthermore, none of the mutants Pro\(^{691}\) \(\rightarrow\) Ala, Pro\(^{709}\) \(\rightarrow\) Ala, and Lys\(^{728}\) \(\rightarrow\) Ala, which were found to have reduced transport activity, displayed any alteration of the Ca\(^{2+}\) affinity relative to the wild type (Fig. 1). This indicates that the high affinity Ca\(^{2+}\) binding sites were intact in these mutant enzymes and that the reduction of transport activity at pCa 5.5 was caused by a decrease of V\(_{\text{max}}\).

ATPase Activity Measurements—In this study we were able to measure the specific ATPase activities of the mutants as a result of the higher level of enzyme expressed after a slight modification of the procedure for transfection (see “Experimental Procedures”). Table I presents the maximum calcium-stimulated specific ATPase activities of the mutants, indicated as percent of the wild-type enzyme. The measurements were performed at pCa 5.5, 5 mM MgATP, 27 °C. Calcium transport was measured at pCa 5.5, 2 mM MgATP, 27 °C. The rate of ATP hydrolysis in the wild-type Ca\(^{2+}\)-ATPase in COS-1 cell microsomes displayed a secondary rise in the millimolar ATP concentration range, identical to that observed for the Ca\(^{2+}\)-ATPase in sarcoplasmic reticulum. The biphasic ATP concentration dependence is consistent with a role for ATP as an allosteric modulator, in addition to its role as a substrate (for a review see Andersen, 1989). For the two proline mutants a biphasic ATP concentration dependence of ATPase activity was likewise observed, and the ATPase activity was reduced to almost the same fraction of the wild-type activity throughout the ATP concentration range. Thus, the mutants retained the regulatory site for modulation of turnover by ATP.

Phosphorylation from ATP and P\(_{\text{i}}\) — A phosphorylated intermediate of the wild-type Ca\(^{2+}\)-ATPase can be formed either from ATP or from P\(_{\text{i}}\). The reaction with ATP requires the binding of two Ca\(^{2+}\) ions at high affinity sites for initiation of the transfer of the terminal phosphate group of ATP to the

### Table I

| Mutation           | Conservation* | Specific activity | Specific phosphorylation |
|--------------------|---------------|------------------|--------------------------|
| Glu-Glu\(^{647}\)→Ala-Ala | (++)          | 93               | >90                      | >90                      |
| Asp-Asp\(^{660}\)→Ala-Ala | (+)           | 100              | >90                      | >90                      |
| Pro\(^{661}\)→Ala  | (+)           | 27               | 70                       | 70                       |
| Lys\(^{684}\)→Ala  | (+)           | 0                | 0                        | 0                        |
| Pro\(^{709}\)→Ala  | (+)           | 48               | >90                      | >90                      |
| Asn-Asp\(^{705}\)→Ala-Ala | (+)           | 105             | 92                       | >90                      |
| Glu\(^{728}\)→Ala  | (+)           | 81               | 87                       | >90                      |
| Glu\(^{733}\)→Ala  | (+)           | 106             | 91                       | >90                      |

* indicates full conservation of the residues among Ca\(^{2+}\)-ATPase, Na\(^{+}\), K\(^{+}\)-ATPase, and H\(^{+}\)-ATPase (Serrano, 1988) whereas (+) indicates conservation among two of these ATPases with allowance for a conservative substitution in the third. Calcium transport was measured at pCa 5.5, 5 mM MgATP, 27 °C. Calcium-activated ATP hydrolysis was measured at pCa 4.0, 5 mM MgATP, 37 °C. Phosphorylation from ATP was measured at pH 7.0 in the presence of 100 μM Ca\(^{2+}\) and 2 μM [γ\(^{-32}\)P]ATP. This ATP concentration is slightly lower than that required to provide 100% saturation of the wild-type enzyme. In those cases in which no phosphorylation was observed an increase of the ATP concentration to 60 μM was without any effect on the result. Phosphorylation from P\(_{\text{i}}\) was measured at 500 μM [γ\(^{-32}\)P]P\(_{\text{i}}\) in the absence of Ca\(^{2+}\). In those cases in which no phosphorylation was observed an increase in the P\(_{\text{i}}\) concentration to 2 mM was without any effect on the result. This result was also obtained when incubation with [γ\(^{-32}\)P]ATP was performed at pH 6.0. Expression was <10% of wild type.

To examine whether the reduced ATPase activity observed for the mutants Pro\(^{661}\)→Ala and Pro\(^{709}\)→Ala could be ascribed to a defect in the binding of ATP to a low affinity regulatory site we measured the ATP concentration dependence of calcium-activated ATP hydrolysis (Fig. 2). The rate of ATP hydrolysis in the wild-type Ca\(^{2+}\)-ATPase in COS-1 cell microsomes displayed a secondary rise in the millimolar ATP concentration range, identical to that observed for the Ca\(^{2+}\)-ATPase in sarcoplasmic reticulum. The biphasic ATP concentration dependence is consistent with a role for ATP as an allosteric modulator, in addition to its role as a substrate (for a review see Andersen, 1989). For the two proline mutants a biphasic ATP concentration dependence of ATPase activity was likewise observed, and the ATPase activity was reduced to almost the same fraction of the wild-type activity throughout the ATP concentration range. Thus, the mutants retained the regulatory site for modulation of turnover by ATP.

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enzyme. Thus, assay of phosphorylation from ATP serves to characterize the intactness of the Ca²⁺-binding sites as well as the nucleotide binding site and the phosphorylation site. The intactness of the phosphorylation site can, in addition, be characterized by assay of phosphorylation of the enzyme with P_i. This reaction occurs even when the nucleotide part of the catalytic site is blocked and requires the absence of Ca²⁺.

The mutants that transported Ca²⁺ were all found to be phosphorylated from ATP in the presence of 100 μM Ca²⁺ and from P_i in the absence of Ca²⁺ (Table I). Except for the Pro⁶⁸→Ala mutant, all the transport-active mutants retained the same level of maximum phosphorylation as the wild type. In the Pro⁶⁸→Ala mutant the maximum phosphorylation level was slightly lower than that of the wild type (Table I). The apparent affinities of the transport-active mutants observed by variation of the concentrations of ATP and P_i were the same as those of the wild-type enzyme. No phosphorylated intermediates were formed from ATP in the absence of Ca²⁺ or from P_i in the presence of Ca²⁺ (not shown).

The transport-defective mutants Lys⁶⁶⁴→Ala, Asp⁷⁰³→Ala, and Asn-Asp⁷⁰⁷→Ala-Ala were unable to form a phosphorylated intermediate by reaction with ATP or P_i, irrespective of the presence or absence of Ca²⁺. These mutants were, therefore, defective either in the noncovalent binding of ATP and P_i or in the subsequent catalytic transfer of the phosphate group to Asp⁷⁰¹.

The Effect of Charge on the Phosphorylation of Lys⁶⁶⁴ Mutants—To examine if the positive charge of Lys⁶⁶⁴ is essential in the phosphorylation reaction we replaced Lys⁶⁶⁴ with the other positively charged residues arginine and histidine, the latter being charged only at low pH. In addition we substituted glutamine for Lys⁶⁶⁴ to remove the positive charge while retaining the approximate bulk of the side chain and some of the polarity.

Fig. 3A shows that the Lys⁶⁶⁴→Arg mutant, in contrast to the Lys⁶⁶⁴→Gln and Lys⁶⁶⁴→His mutants, was able to phosphorylate with ATP to a level identical to that of the wild type, in the presence of 2 μM ATP and 100 μM Ca²⁺. When the free Ca²⁺ concentrations were varied between 0.1 and 100 μM and the ATP concentrations were varied between 0.1 and 10 μM the Lys⁶⁶⁴→Arg mutant displayed similar apparent affinities for Ca²⁺ and ATP as the wild-type enzyme (Fig. 4, A and B). These results demonstrate that the Lys⁶⁶⁴→Arg mutant retained the high affinity binding sites for ATP and Ca²⁺ as well as the ability to catalyze the transfer of the γ-phosphate of ATP to the enzyme. Similar titration experiments with the Lys⁶⁶⁴→His and Lys⁶⁶⁴→Gln mutants indicated that these mutants were unable to phosphorylate, even at an ATP concentration as high as 60 μM (not shown).

Since the standard ATP phosphorylation assay was carried out at pH 7.0 and the histidine residue was expected to be positively charged only at lower pH, we examined the ability of the Lys⁶⁶⁴→His mutant to react with ATP at pH 6.0. Even at this low pH and at an ATP concentration as high as 60 μM the Lys⁶⁶⁴→His mutant did not form a phosphorylated intermediate (not shown).

When phosphorylation was examined with inorganic phosphate as substrate, none of the Lys⁶⁶⁴→mutants, including the Lys⁶⁶⁴→Arg mutant, formed a phosphoenzyme intermediate at P_i concentrations up to 2 mM in the presence of dimethyl sulfoxide and in the absence as well as in the presence of Ca²⁺ (Fig. 3B). The P_i concentration providing half-saturation of the wild-type enzyme under similar conditions is about 20-fold lower (Andersen et al., 1989).

Fig. 3B. Phosphorylation of the Lys⁶⁶⁴ mutants expressed in COS-1 cells. A, phosphorylation from ATP was carried out for 15 s at 0 °C in 20 mM MOPS buffer (pH 7.0), 80 mM K⁺, 5 mM Mg²⁺, 2 μM [γ-³²P]ATP in the presence of 0.1 mM Ca²⁺ (C) or 1 mM EGTA (E). B, phosphorylation from P_i was carried out for 2 min at 20 °C in 50 mM MES buffer (pH 6.0), 5 mM Mg²⁺, 20% (v/v) dimethyl sulfoxide, 0.5 mM ³²P, in the presence of 0.1 mM Ca²⁺ (C) or 2 mM EGTA (E). The acid-quinched samples containing an equivalent amount of expressed ATnPase were subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0, and radioactivity was detected by autoradiography. The amino acids substituted for Lys⁶⁶⁴ are indicated above the lanes (Lys indicates the wild type).
Interconversion of the Phosphoenzyme Intermediates of the \( \text{Lys}^{684} \rightarrow \text{Arg} \) Mutant—The finding that the \( \text{Lys}^{684} \rightarrow \text{Arg} \) mutant formed a phosphorylated intermediate from ATP, but not from \( \text{P}_{\text{i}} \), combined with the inability of this mutant to transport \( \text{Ca}^{2+} \) led us to conduct a series of experiments under conditions similar to those described previously (Andersen et al., 1989) to examine the partial reactions after phosphorylation from ATP, i.e. the conversion of the ADP-sensitive phosphorylase intermediate (EIP) to the ADP-insensitive phosphorylase intermediate (E2P) and the subsequent hydrolysis of E2P with liberation of \( \text{P}_{\text{i}} \). (Andersen et al., 1985).

The phosphoenzyme formed at pH 7.0 in the presence of \( \text{K}^{+} \) was ADP-sensitive in the \( \text{Lys}^{684} \rightarrow \text{Arg} \) mutant as well as in the wild-type enzyme (Fig. 5, lanes b). When EGTA was added without ADP the dephosphorylation from the ADP-sensitive E2P form proceeded at a much lower rate in the \( \text{Lys}^{684} \rightarrow \text{Arg} \) mutant than in the wild-type enzyme (Fig. 5, lanes c and d), suggesting that either the E2P-E2P interconversion or the E2P decomposition was defective in the mutant.

In Fig. 6, the phosphorylation was performed at pH 8.35 in the absence of \( \text{K}^{+} \). In the wild-type enzyme these conditions favor accumulation of E2P in steady state, as a result of a low rate of E2P hydrolysis. In contrast to the wild-type, the \( \text{Lys}^{684} \rightarrow \text{Arg} \) mutant did not accumulate E2P after a 15-s reaction in this medium, as demonstrated by the complete dephosphorylation obtained in the presence of ADP (Fig. 6, lane b). A relatively large amount of ADP-insensitive phosphoenzyme appeared, however, when the conversion of the ADP-sensitive phosphoenzyme intermediate to the ADP-insensitive form was allowed to proceed for an additional 45 s in the presence of EGTA (lane d). Thus, the \( \text{Lys}^{684} \rightarrow \text{Arg} \) mutant seemed to be able to form E2P in the forward running reaction mode of the enzyme but at a greatly reduced rate.

Since the \( \text{Lys}^{684} \rightarrow \text{Arg} \) mutant enzyme was unable to form E2P in the backward reaction with \( \text{P}_{\text{i}} \), it was of interest to determine whether catalysis of E2P dephosphorylation might be defective in the mutant. Fig. 7 shows that the transfer of the mutant E2P intermediate, formed in the forward reaction in the absence of \( \text{K}^{+} \) at neutral pH resulted in a rapid dephosphorylation similar to that observed for the wild-type E2P. The residual radioactivity observed in lanes b and c corresponding to the mutant must represent ADP-sensitive phosphoenzyme, since it could be removed by inclusion of ADP in the dilution buffer (lanes e and f). On the basis of this experiment it may be surmised that the slow decay of the mutant E2P form, observed at neutral pH in the presence of \( \text{K}^{+} \), in Fig. 5, proceeded through the conversion of E2P to E2P and the subsequent (fast) hydrolysis of E2P, rather than through an independent pathway.

The Effect of Nucleotides on Intramolecular Cross-linking—The finding that some of the \( \text{Lys}^{684} \) mutants and the mutants Asp\(^{703} \rightarrow \) Ala and Asn-Asp\(^{707} \rightarrow \) Ala-Ala were unable to form a phosphorylated intermediate even from ATP raised the question whether the nucleotide binding site was defective in these mutants. Since the noncovalent binding of nucleotides could not be measured directly under equilibrium conditions because of the limited quantity of enzyme expressed in the COS-1 cells, we took advantage of the finding by Ross and McIntosh (1987a, 1987b) that nucleotide binding protects the \( \text{Ca}^{2+} \)-ATPase from intramolecular cross-linking induced by...
glutaraldehyde. We found that the cross-linked Ca\(^{2+}\)-ATPase retained the ability to react with the specific monoclonal antibody A52. Therefore, the cross-linked species, having a lower mobility than the native enzyme on SDS gels, was conveniently detected on immunoblots. As the small amount retained the ability to react with the specific monoclonal antibody A52, the gels were free of any background representing other Ca\(^{2+}\)-ATPase species than the one under study. Fig. 8 shows examples of immunoblots of glutaraldehyde-treated COS-1 cell microsomes containing wild-type Ca\(^{2+}\)-ATPase or mutants which were unable to phosphorylate from ATP. With the wild-type Ca\(^{2+}\)-ATPase an enzyme product was formed which migrated on SDS-polyacrylamide gel electrophoresis with an apparent molecular weight of 125,000 (lane 2, upper band), similar to the intramolecularly cross-linked species originally described by Ross and McIntosh (1987a, 1987b) for the Ca\(^{2+}\)-ATPase in sarcoplasmic reticulum vesicles. The amounts of cross-linked enzyme formed with the Lys\(^{684}\) → Ala mutant (Fig. 8b), with the Asp\(^{703}\) → Ala mutant (Fig. 9), and with the Lys\(^{684}\) → Arg mutant (not shown) were the same as observed with the wild-type enzyme.

A lower amount of cross-linked enzyme was observed with the Asn-Asp\(^{707}\) mutant (Fig. 8e), however, and with the mutants Lys\(^{684}\) → His and Lys\(^{684}\) → Gln, no cross-linking was observed at all (Fig. 8, c and d). As also shown in Fig. 8 (lanes 3, 4, and 5), addition of increasing concentrations of ADP gradually inhibited formation of the cross-linked product. The mutants Lys\(^{684}\) → Ala, Asp\(^{703}\) → Ala, and Asn-Asp\(^{707}\) → Ala-Ala all displayed the same apparent affinity for ADP as the wild-type enzyme even though the rate of cross-link formation in the absence of any nucleotide was reduced in the Asn-Asp\(^{707}\) → Ala-Ala mutant as described above. When ATP was added the same effect was observed as with ADP, but since part of the ATP is hydrolyzed during the incubation with glutaraldehyde because of the presence of calcium-independent ATPases in the micromolar preparation, we did not attempt to determine the affinity for ATP.

In addition to ADP and ATP, the non- or slowly hydrolyzable ATP analog TNP-ATP and polyvanadate solutions containing polyvanadate were able to prevent glutaraldehyde from inducing cross-link formation, as shown for the Asp\(^{703}\) → Ala mutant in Fig. 9. For this mutant as well as for the Lys\(^{684}\) → Ala and Asn-Asp\(^{707}\) → Ala-Ala mutants we found that the concentration dependence of inhibition of intramolecular cross-linking by TNP-ATP and vanadate was similar to those of the wild-type enzyme (not shown). With TNP-ATP, the protection of cross-link formation was saturated in the micromolar concentration range (Fig. 9), as expected from the high affinity of the enzyme for this ATP analog (Suzuki et al., 1990). The inhibition of cross-link formation observed with the vanadate solution can be ascribed to the fraction of vanadate present as the polyanionic decavanadate and is in accordance with the ability of decavanadate to act as an ATP analog binding at the nucleotide site (Coan et al., 1986; Ross and McIntosh, 1987b). We did not observe any effect on cross-linking of monovanadate solutions free from polyvanadate (not shown).

**FIG. 8. Effect of ADP on glutaraldehyde-induced cross-linking of wild-type Ca\(^{2+}\)-ATPase and Lys\(^{684}\) → Ala, histidine, glutamine, and Asn-Asp\(^{707}\) → Ala-Ala mutants expressed in COS-1 cells.** Microsomes containing Ca\(^{2+}\)-ATPase corresponding to the wild-type (a), the Lys\(^{684}\) → Ala mutant (b), the Lys\(^{680}\) → His mutant (c), the Lys\(^{684}\) → Gln mutant (d), or the Asn-Asp\(^{707}\) → Ala-Ala mutant (e) were incubated for 5 min with and without glutaraldehyde and ADP. The samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting, using the highly specific monoclonal antibody A52 to visualize the Ca\(^{2+}\)-ATPase protein. Lane 1, control experiment without glutaraldehyde. Lane 2, glutaraldehyde (5 mM) added. Lane 3, glutaraldehyde (5 mM) and ADP (10 μM) added. Lane 4, glutaraldehyde (5 mM) and ADP (100 μM) added. Lane 5, glutaraldehyde (5 mM) and ADP (1 mM) added.

**FIG. 9. Effect of nucleotides and polyvanadate on glutaraldehyde-induced cross-linking of the Asp\(^{703}\) → Ala mutant Ca\(^{2+}\)-ATPase.** Incubation with glutaraldehyde was performed in the absence of ATP analogs (lane 1) or in the presence of 10 μM TNP-ATP (lane 2), 1 mM ADP (lane 3), or 1 mM vanadate, HCl treated to increase the content of polyvanadate (lane 4).

**DISCUSSION**

In this study we have focused our attention on the functional consequences of alterations to the highly conserved residue, Lys\(^{684}\), located at the beginning of the hinge domain. Moreover, we have introduced mutations to all the carboxyl residues throughout the hinge domain as well as to the conserved proline residues Pro\(^{606}\) and Pro\(^{706}\) and the lysine residue Lys\(^{685}\).

Replacement of Lys\(^{684}\) with either the other positively charged residue, arginine, or with histidine, glutamine, or alanine produced mutants that were unable to transport Ca\(^{2+}\) and hydrolyze ATP (Table 1). Hence, Lys\(^{684}\) is essential to the pump function. For each of the mutants Lys\(^{684}\) → His, Lys\(^{684}\) → Gln, and Lys\(^{684}\) → Ala the loss of overall function was associated with the inability to form a phosphorylated intermediate from ATP or P\(_i\). By contrast, the Ca\(^{2+}\)-activated phosphorylation from ATP was retained with normal apparent affinities for Ca\(^{2+}\) and ATP when the lysine was replaced by the other positively charged and structurally similar residue, arginine. On the basis of work with adenylate kinase and other nucleotide triphosphate using enzymes (Reinstein et al., 1990; Schlichting et al., 1990) it may be suggested that the positive charge takes part in the stabilization of a transition state in the γ-phosphoryl transfer reaction by making contact with the β- and γ-phosphate oxygens.

Histidine was unable to replace Lys\(^{684}\) even when phosphorylation from ATP was performed at pH 6.0. It is possible that the imidazole group remained unprotonated at pH 6.0 depending on the local environment or that the positive charge was positioned inappropriately for the catalysis of phosphoryl transfer. Alternatively, the bulky imidazole group introduced changes in the tertiary structure, disturbing the phosphorylation reaction. The latter possibility is consistent with the inability of the Lys\(^{684}\) → His mutant to undergo intramolecular cross-linking of domains at the active site (Fig. 8).

We found that the ADP-sensitive phosphoenzyme intermediate formed from ATP in the Lys\(^{684}\) → Arg mutant, decayed at a low rate relative to that of the wild-type enzyme in the presence of potassium at neutral pH. Moreover, con-
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Itions that led to immediate accumulation of the ADP-insensitive phosphoenzyme intermediate in the wild-type did not result in a detectable level of E2P in the Lys864→Arg mutant after 15 s. When the reaction was allowed to proceed for a longer time, however, significant amounts of ADP-insensitive phosphoenzyme appeared in the mutant (Fig. 6). This ADP-insensitive phosphoenzyme intermediate was able to undergo fast dephosphorylation in the presence of potassium at neutral pH and was thus similar to the E2P intermediate of the wild-type enzyme (Fig. 7). Taken together these data demonstrate that the Lys864→Arg mutant was able to form ATP in the forward running reaction mode of the enzyme although at a reduced rate. The absence of measurable Ca2+ transport and ATPase activity in this mutant is accounted for by the reduced rate of phosphoenzyme interconversion.

Surprisingly, no phosphorylation of E2 was observed in the backward reaction with inorganic phosphate as substrate, even at a P_i concentration of 2 mM in the presence of dimethyl sulfoxide. This finding suggests that the binding of P_i is very weak in the Lys864→Arg mutant. A possible interpretation of our observations is a preferential destabilization of the noncovalent phosphate-protein interaction in the E2P form of the Lys864→Arg mutant. This could arise if the bulky guanidinium group of arginine prevents an interdomain movement closing the active site cleft in relation to the E2P to E2P transition (Ross and McIntosh, 1987b). In a previous study, mutation of Gly524 in the β-strand domain to valine and arginine was likewise found to reduce the apparent affinity for P_i as well as the E2P→E2P interconversion rate (Andersen et al., 1989).

The 9 acidic residues Glu-Glu^647, Asp-Asp^707, Glu^669, Asp-Glu^690, Glu^718, and Glu^729 could be replaced by alanines without affecting the maximum Ca2+ transport rates or the apparent affinities of the Ca2+-ATPase for Ca2+ or ATP (Table 1). Hence, our data exclude these oxygen donating residues as being essential to Ca2+ and ATP binding or to other partial reactions in the pump cycle.

On the other hand, the mutations Asp^707→Ala and Asn-Asp^707→Ala-Ala led to complete loss of Ca2+ transport and ATPase activity. This was associated with the inability of these mutants to be phosphorylated from ATP or P_i, suggesting that the disruption of transport function occurred either in the noncovalent binding of ATP and P_i, or in the catalysis of phosphoryl transfer to the protein. In this way the Asp^707→Ala and Asn-Asp^707→Ala mutants resembled the Lys864→glutamine, histidine, and alanine mutants.

To examine the intactness of the nucleotide binding site we took advantage of the finding by Ross and McIntosh (1987a, 1987b) that occupation of the nucleotide site prevents intramolecular cross-linking induced by glutaraldehyde. These authors suggested that the cross-link is formed between 2 lysines located in the phosphorylation and nucleotide binding domains, respectively. Apparently the mutated Lys864 is not one of the lysines participating in cross-linking since the cross-link was detected with the Lys864→Arg and the Lys864→Ala mutants. We found that TNP-ATP, ADP, and polyvanadate inhibited cross-link formation in the Lys864→Ala, Asp^707→Ala, and Asn-Asp^707→Ala-Ala mutant enzymes with the same apparent affinities as in the wild-type enzyme, indicating that the nucleotide binding site was intact in these mutants.

In a recent study replacement of Asp^707 by either asparagine or glutamic acid was shown to result in mutant enzymes that, in contrast to the Asp^707→Ala mutant, were able to phosphorylate with either ATP or P_i (Clarke et al., 1990c). Therefore it seems that the phosphorylation reaction requires the presence of the carbonyl oxygen but not the negative charge of the carboxylate side chain at position 703. Previously, Asp^707 was changed to glutamate and asparagine (Clarke et al., 1990c), with functional consequences similar to those observed for the double mutation Asn-Asp^707→Ala-Ala in the present work. We found that the rate of glutaraldehyde-induced cross-link formation was reduced in the Asn-Asp^707→Ala-Ala mutant, suggesting that the domain interactions governing cross-linking may have been disturbed. Nevertheless, the nucleotide binding site appeared intact, as judged from the prevention of cross-linking by ADP. This result is difficult to reconcile with a role of the Asn-Asp^707 motif in binding the adenine ring of ATP (Serrano, 1989).

In the present study we have for the first time been able to measure the ATPase activity in the COS-1 cell microsomes. The rate of calcium-activated ATP hydrolysis of the wild-type enzyme expressed in the COS-1 cells agreed well with that measured with the Ca2+-ATPase isolated from fast skeletal muscle (Fig. 2), and the relative decrease of ATPase activity in the mutants was consistent with the decrease of Ca2+ transport rates. For the two proline mutants Pro310→Ala and Pro310→Ala, the calcium transport and Ca2+-ATPase activities were reduced to around 30 and 50% of the wild-type activities, respectively. These mutants displayed normal apparent affinities for ATP and Ca2+ in the phosphorylation reaction. Moreover, we were able to demonstrate a biphasic ATP concentration dependence of ATP hydrolysis, similar to that observed with the wild-type enzyme. The reduction of the overall turnover rate in the proline mutants can, therefore, not be accounted for by a disruption of the catalytic or regulatory ATP sites or of the Ca2+ sites. One or both of these highly conserved prolines may play a role in the conformational changes associated with phosphoryl transfer or E2P→E2P transition. From secondary structure predictions it seems likely that Pro303 is located at the beginning of a loop structure containing the crucial Lys864 residue. The X-Pro peptide bond may serve as a conformational switch by introducing structural flexibility as proposed previously for a proline residue (Pro^310) in one of the putative transmembrane helices (Vilsen et al., 1989).

In conclusion, the present work does not support a central role of the hinge domain in nucleotide binding. On the basis of our findings it seems more likely that some of the conserved residues of the hinge take part in the catalytic events in the phosphorylation site by interacting noncovalently with phosphate. This is consistent with the original proposal that the hinge domain interacts in a hinge bending mode with the phosphorylation domain (Green et al., 1986) and suggests that the hinge domain may constitute an integral part of the phosphorylation site.

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