Functional Consequences of Substitution of the Seven-Residue Segment LysIleArgAspGlnMetAla240 Located in the Stalk Helix S3 of the Ca^{2+}-ATPase of Sarcoplasmic Reticulum†

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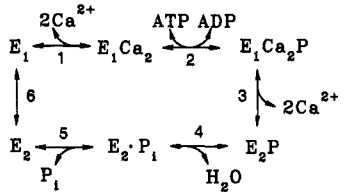
Received March 25, 1993; Revised Manuscript Received July 2, 1993●

ABSTRACT: Site-directed mutagenesis was used to substitute the seven-residue segment LysIleArgAspGlnMetAla240 located at the NH_2- terminal end of the "stalk" helix S3, near the β-strand domain, in the sarcoplasmic reticulum Ca^{2+}-ATPase of rabbit fast twitch muscle, with the corresponding Na^+,K^+-ATPase segment ArgIleAlaThrLeuAlaSer. This led to a new phenotypic variant of Ca^{2+}-ATPase. The overall turnover rates for Ca^{2+} transport and ATP hydrolysis measured at 27 and 37°C, respectively, were reduced to 30–40% of the wild-type rates. Analysis of the phosphoenzyme intermediates at 0 °C showed that the ADP-insensitive phosphoenzyme intermediate accumulated under conditions where the ADP-sensitive phosphoenzyme intermediate predominated in the wild-type Ca^{2+}-ATPase. The rate of dephosphorylation of the ADP-insensitive phosphoenzyme intermediate formed through the forward reaction with ATP, or in the "backdoor" reaction with P_i, was reduced severalfold in the mutant relative to the dephosphorylation rate measured in the wild type, but there was no significant difference between the mutant and the wild type with respect to the apparent affinity for P_i measured under equilibrium conditions. The mutant was much less susceptible to inhibition by vanadate than the wild type, under equilibrium conditions as well as during turnover with ATP and Ca^{2+}. These observations suggest that the transition state in the hydrolysis of the aspartyl phosphate bond in the ADP-insensitive phosphoenzyme intermediate was destabilized in the mutant.

The Ca^{2+}-ATPase of sarcoplasmic reticulum (SR)[1] belongs to the family of cation-transporting ATPases of "P-type", for which an acid stable phosphorylated intermediate is central to the catalytic mechanism. Another essential feature of these ATPases is their ability to switch between two different conformational states, E_1 and E_2, with reactivities to ATP/ADP and P_i/H_2O, respectively (see Scheme I). A large body of experimental evidence has supported a model for structure–function relationships in the Ca^{2+}-ATPase peptide in which the catalytic site resides in the globular cytoplasmic "headpiece" while the Ca^{2+} binding region is located in the transmembrane helical cluster (Brandl et al., 1986; Clarke et al., 1989a; Green, 1989; Green & Stokes, 1992; Inesi et al., 1990). Whereas it seems rather well documented that the largest cytoplasmic loop connecting the putative transmembrane helices M4 and M5 is involved in the binding of ATP, the functional role of the smaller cytoplasmic loop between M2 and M3 is less clear. This loop is predicted to form two "stalk" helices, S2 and S3, connected by a β-strand domain (Green, 1989; Green & Stokes, 1992). A role of the β-strand domain and the "stalk" helices in the binding of calcium ions at the high-affinity activatory sites seems to have been excluded by site-specific mutagenesis (Clarke et al., 1989b, 1990; Andersen et al., 1989; Vilsen et al., 1989), but there is evidence that the stalk contains an additional cation binding site with undefined specificity (Squier et al., 1991; Asturias & Blasie, 1991). The functional analyses of site mutants (Andersen et al., 1989; Andersen & Vilsen, 1992a; Clarke et al., 1990) and of proteolytically cleaved derivatives of Ca^{2+}-ATPase (Andersen, 1989; Imamura & Kawakita, 1989; le Maire et al., 1990) have in addition pointed to a role of the β-strand domain in the conformational switch between the E_1/E_1P and E_2/E_2P forms.

Scheme I: Minimal Reaction Cycle of Ca^{2+}-ATPase[a]



[a] E_1 and E_2 represent two distinct functional states of the dephosphoenzyme (De Meis & Vienna, 1979). E_1 is phosphorylated from ATP (following Ca^{2+} binding) but not from P_i. E_2 is phosphorylated from P_i but not from ATP. E_1P (ADP-sensitive phosphoenzyme intermediate) is able to donate the phosphoryl group back to ADP but does not react directly with water. E_2P (ADP-insensitive phosphoenzyme intermediate) is hydrolyzed by water but does not react with ADP. Vanadate binds to E_2 in competition with P_i.

Here we report on the functional consequences of replacing the seven-residue segment LysIleArgAspGlnMetAla240 in the most NH_2-terminal part of the S3 helix, near the β-strand domain, with the segment ArgIleAlaThrLeuAlaSer located at the homologous position in the Na^+,K^+-ATPase [see

† This research was supported by grants from the Danish Biomembrane Research Centre, the Danish Medical Research Council, the NOVO Foundation, the Foundation for the Advancement of Medical Science, the King Chr. X Foundation, and the Nordic Insulin Foundation.

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● Abstract published in *Advance ACS Abstracts*, September 1, 1993.

[1] Abbreviations: E_1P, ADP-sensitive phosphoenzyme intermediate; E_2P, ADP-insensitive phosphoenzyme intermediate; EGTA, [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid; M1–M10, putative transmembrane helices numbered from the NH_2-terminal end of the peptide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; S1–S5, putative "stalk" helices numbered from the NH_2-terminal end of the peptide; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum.

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alignment by Green (1989)]. This chimeric mutant showed a reduced rate of dephosphorylation of the E_2P phosphoenzyme intermediate relative to the wild-type Ca^{2+}-ATPase, without a corresponding increase in the affinity for P_i. In addition, the mutant was insensitive to inhibition by vanadate. These characteristics of the mutant are consistent with a destabilization of the transition state in the hydrolysis of the aspartyl phosphate bond in E_2P.

EXPERIMENTAL PROCEDURES

Many of the methods employed in this study have been described in detail elsewhere (Maruyama & MacLennan, 1988; Clarke et al., 1989b; Andersen et al., 1989, 1992; Vilsen et al., 1989, 1991a,b). In brief, 14 nucleotides in the rabbit fast twitch muscle Ca^{2+}-ATPase cDNA were substituted by oligonucleotide-directed mutagenesis according to Kunkel (1985), using a single 36-nucleotide-long mutagenic primer. The presence of the desired nucleotide substitutions was confirmed by nucleotide sequencing according to Sanger et al. (1977), and the entire Ca^{2+}-ATPase cDNA containing the mutations was cloned into the *Eco*RI site of vector p91023(B) (Kaufman et al., 1989) for expression in COS-1 cells (Gluzman, 1981). Microsomes were prepared from transfected cells, and the expression of Ca^{2+}-ATPase protein was quantitated by a sandwich ELISA assay (Vilsen et al., 1991b). Assays for Ca^{2+} transport and Ca^{2+}-activated ATPase activity were carried out as described (Vilsen et al., 1989, 1991a; Vilsen & Andersen, 1992). The phosphoenzyme intermediates formed from ATP or P_i were examined as in Andersen et al. (1989, 1992). The kinetic studies of the disappearance of the phosphoenzyme were all conducted at 0°C. After acid precipitation, the phosphorylated microsomal protein was washed and subjected to SDS–polyacrylamide gel electrophoresis at pH 6.0 followed by autoradiography of the dried gels and quantitation by densitometric analysis using an LKB 2202 Ultroscan laser densitometer. Care was taken to ensure that the lanes of the gel contained equivalent amounts of expressed Ca^{2+}-ATPase. In some experiments, vanadate (VO_4^{3-}) was included during preincubation and/or in the reaction mixtures. To avoid contamination with decavanadate, the stock solution of vanadate was prepared by dissolving $NaVO_3$ (Merck) in excess NaOH.

All experiments were conducted at least twice, and mean values are shown in the figures, with error bars indicating the largest deviation from the mean observed in the series.

RESULTS

The chimeric mutant LysIleArgAspGlnMetAla240→ArgIleAlaThrLeuAlaSer and also the wild-type rabbit fast twitch muscle sarcoplasmic reticulum Ca^{2+}-ATPase were expressed transiently in COS-1 cells. There was no significant difference between their expression levels. Approximately 10 μg of either mutant or wild-type Ca^{2+}-ATPase protein was obtained from six 9-cm Petri dishes of cells. To analyze the effects of the amino acid replacements on the overall turnover rate and on the individual partial reactions in the Ca^{2+} transport cycle (see Scheme I), the experiments described below were carried out.

The maximum specific rates of ATP hydrolysis and Ca^{2+} transport in the mutant enzyme amounted to 30–40% of the wild-type rates. In the mutant as well as in the wild type, the ATP concentration dependence of ATP hydrolysis showed a characteristic secondary low-affinity activation (Figure 1A) consistent with a role of ATP as an allosteric modulator in addition to its role as substrate [for a review, see Andersen (1989)]. The ATP concentration dependence of phosphorylation from ATP of the mutant was identical to that of the wild type, with the $K_{0.5}$ for ATP below 1 μM at 0 °C (not shown).

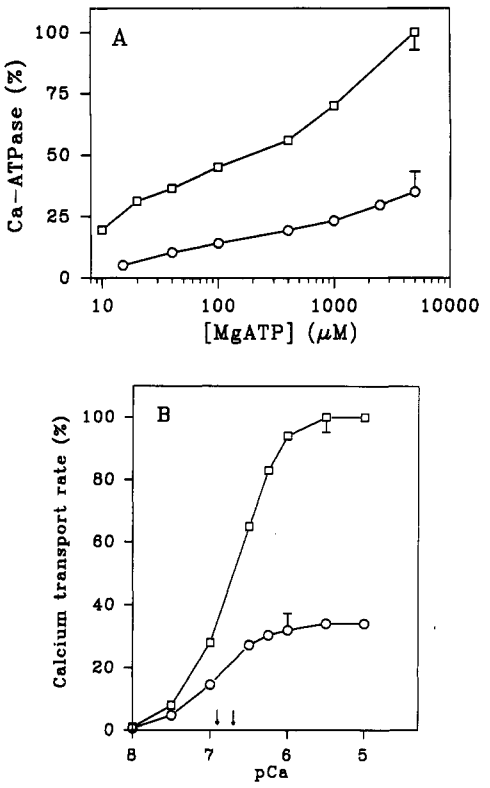

FIGURE 1: ATP and Ca^{2+} concentration dependencies of steady-state turnover of the chimeric mutant (○) and the wild-type Ca^{2+}-ATPase (□) expressed in COS-1 cells. The specific activities (calculated per milligram of expressed Ca^{2+}-ATPase protein) are shown as percentages of the values measured with the wild type at saturating Ca^{2+} and ATP concentrations [100% corresponding to a turnover rate of 20–25 μmol min^{-1} (mg of Ca^{2+}-ATPase)$^{-1}$ at 37 °C]. (A) Ca^{2+}-activated ATP hydrolysis catalyzed by the microsomes was measured spectrophotometrically at 37 °C in the presence of 50 mM TES, pH 7.5, 0.1 M K^+, 1 mM Mg^{2+}, 0.1 mM Ca^{2+}, and the indicated concentrations of MgATP, with phosphoenolpyruvate (1 mM), lactate dehydrogenase (10 units/mL), pyruvate kinase (10 units/mL), and the Ca^{2+} ionophore A23187 (2 μM) added. The data points show the values obtained after subtraction of Ca^{2+}-independent ATP hydrolysis measured in the presence of 2 mM EGTA without added Ca^{2+}. (B) Oxalate-supported uptake of $^{45}Ca^{2+}$ in the microsomes was measured by Millipore filtration after 5 min incubation at 27 °C in the presence of 5 mM MgATP, 0.1 M K^+, 5 mM oxalate, 20 mM MOPS, pH 6.8, and various concentrations of Ca^{2+} set with EGTA. The *arrows* indicate the Ca^{2+} concentrations at which the transport rates were half-maximal.

The $[Ca^{2+}]$ dependence of Ca^{2+} transport is shown in Figure 1B. It can be seen that the mutant displayed a slightly higher apparent affinity for Ca^{2+} than the wild type. A similar increase in the apparent Ca^{2+} affinity was observed in studies of the $[Ca^{2+}]$ dependence of steady-state phosphorylation from ATP Figure 2A. On the other hand, no significant difference between the apparent Ca^{2+} affinities of the mutant and the wild type was observed, when the Ca^{2+}-induced inhibition of equilibrium phosphorylation in the "backdoor" reaction with P_i was titrated (Figure 2B).

The phosphoenzyme intermediate of the mutant like that of the wild type was found to exist in two states, one being ADP-sensitive (E_1P) and the other being ADP-insensitive (E_2P). Figure 3A shows that in the mutant a significant fraction of the phosphoenzyme present at steady state was resistant to a 7-s incubation with ADP plus EGTA (the latter compound included to terminate the phosphorylation by chelation of Ca^{2+}). Moreover, almost all the mutant phos-

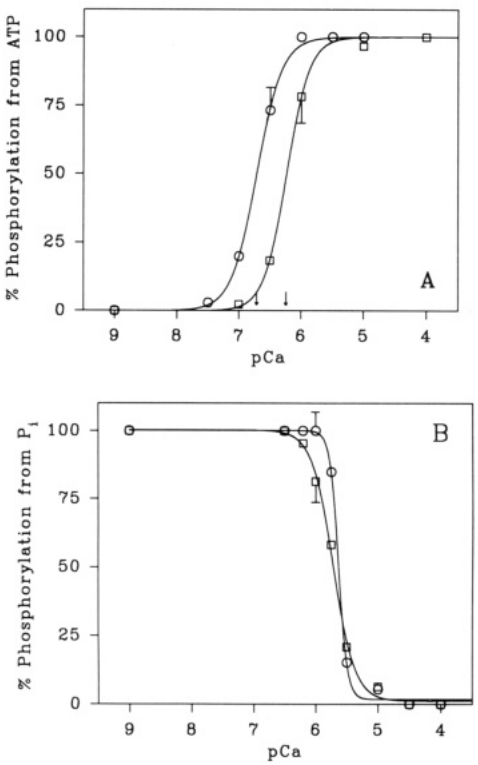

FIGURE 2: Ca^{2+} dependence of phosphorylation from ATP and P_i of the chimeric mutant (○) and the wild-type Ca^{2+}-ATPase (□) expressed in COS-1 cells. The acid-quenched ^{32}P-phosphorylated samples were subjected to SDS–polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels were quantitated by densitometry. The specific phosphorylations are shown as percentages of their maximal values. (A) Phosphorylation from ATP was carried out at 0 °C for 15 s in the presence of 50 mM MOPS buffer (pH 7.0), 80 mM K^+, 5 mM Mg^{2+}, 2 μM Ca^{2+} ionophore A23187, 2 μM [γ-^{32}P]ATP, and the indicated Ca^{2+} concentrations. The *arrows* indicate the Ca^{2+} concentrations at which the phosphorylation levels were half maximal. (B) Phosphorylation from P_i was carried out for 10 min at 25 °C in the presence of 500 μM [$^{32}P_i$], 100 mM TES/Tris buffer (pH 7.0), 10 mM $MgCl_2$, 20% (v/v) dimethyl sulfoxide, and the indicated Ca^{2+} concentrations.

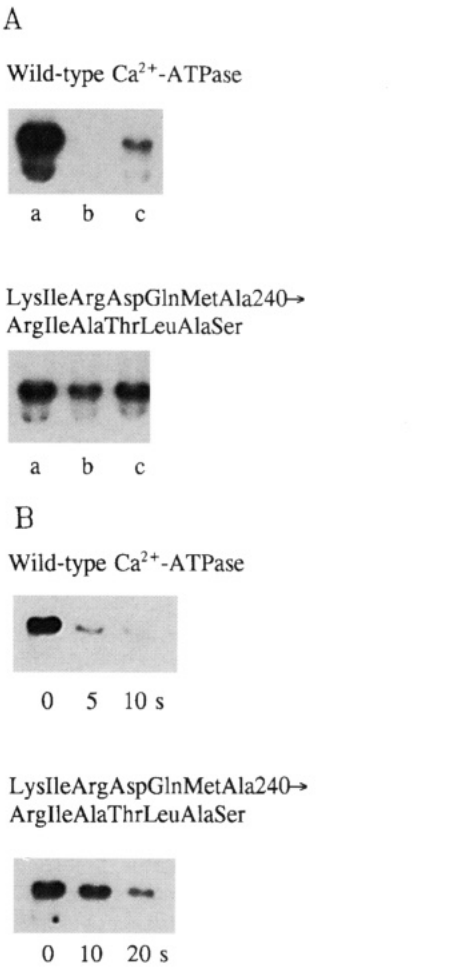

FIGURE 3: Analysis of the phosphoenzyme intermediates formed with either ATP or P_i in the chimeric mutant and in the wild-type Ca^{2+}-ATPase expressed in COS-1 cells. (A) Phosphorylation with [γ-^{32}P]ATP was carried out at 0 °C for 15 s in the presence of 50 mM MOPS buffer (pH 7.0), 80 mM K^+, 5 mM Mg^{2+}, 0.1 mM Ca^{2+}, 2 μM A23187, and 2 μM [γ-^{32}P]ATP. Following 15-s reaction with [γ-^{32}P]ATP, the sample was quenched directly (a) or incubated with 1 mM ADP and 1 mM EGTA (b) or with 1 mM EGTA without ADP (c) for 7 s prior to acid-quenching. The acid-quenched ^{32}P-phosphorylated samples were subjected to SDS–polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels are shown. The film was exposed longer for the wild type than for the mutant. (B) Phosphorylation with [$^{32}P_i$] was carried out at 25 °C for 10 min in the presence of 100 μM [$^{32}P_i$], 100 mM MES/Tris (pH 6.0), 5 mM Mg^{2+}, 2 mM EGTA, and 20% (v/v) dimethyl sulfoxide. Following cooling of the sample to 0 °C, dephosphorylation was initiated by 20-fold dilution of an aliquot into ice-cold medium containing 50 mM MOPS (pH 7.0), 80 mM K^+, 5 mM Mg^{2+}, 1 mM nonradioactive P_i, and 2 mM EGTA, without dimethyl sulfoxide, and acid quenching was performed either instantaneously ("0") or at the indicated time intervals after the dilution.

phoenzyme was stable for 7 s in the presence of EGTA alone. By contrast, in the wild-type Ca^{2+}-ATPase, all the phosphoenzyme present at steady state disappeared rapidly upon addition of ADP plus EGTA, and the decay of the wild-type phosphoenzyme was also much faster than that of the mutant in the presence of EGTA alone. In the presence of ADP, most of the dephosphorylation is presumed to occur through reversal of the phosphorylation with resulting ATP synthesis (step 2 in Scheme I), whereas in the presence of EGTA alone the dephosphorylation of E_1P occurs through conversion of E_1P to E_2P (step 3 in Scheme I) and hydrolysis of the latter intermediate (step 4). The data shown in Figure 3 may be interpreted in terms of a reduced rate of hydrolysis of E_2P in the mutant, relative to the wild-type hydrolysis rate, leading to steady-state accumulation of E_2P in the mutant. The results illustrated in Figure 3B are in line with this interpretation, since they demonstrate an unusually low rate of E_2P dephosphorylation in the mutant. This was measured following dilution in nonradioactive P_i of the E_2P phosphoenzyme formed by "backdoor" phosphorylation with [$^{32}P_i$]. The half-life of the E_2P intermediate estimated by densitometric analysis of the autoradiograms was 7–10 s for the mutant and close to 1 s for the wild type. The latter value constitutes an upper limit set by the time resolution of our manual mixing technique.

Since it has been demonstrated that ADP can activate partial reversal of the $E_1P \rightarrow E_2P$ transition in the wild-type Ca^{2+}-ATPase (Froehlich & Heller, 1985), the apparent insensitivity of part of the mutant phosphoenzyme to ADP might also be explained by a block of this reversal in the mutant. This hypothesis was examined as illustrated in Figure 4. In one set of experiments, ADP with EGTA was added to phosphorylated mutant enzyme present in microsomes made leaky to Ca^{2+} with ionophore, and the dephosphorylation was followed by acid quenching at serial time intervals. Two kinetic phases of dephosphorylation could be distinguished. The fast phase presumably represents the reaction of ADP with the E_1P present at steady state, whereas the slow phase (corresponding to about 75% of the phosphoenzyme as judged from extrapolation to the ordinate intercept) might represent either the forward hydrolysis of E_2P or the combination of forward E_2P hydrolysis with $E_2P \rightarrow E_1P$ back-transition followed by reaction of E_1P with ADP. The latter possibility would imply that the

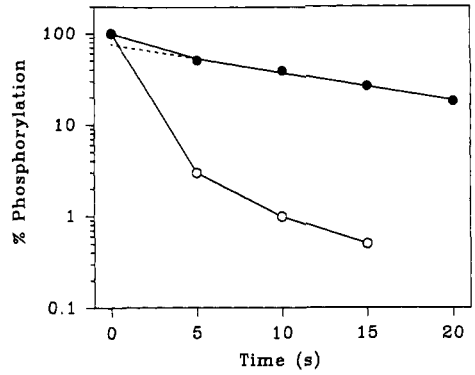

FIGURE 4: Analysis of the Ca^{2+} sensitivity of ADP-induced dephosphorylation of the chimeric mutant. Phosphorylation with [γ-^{32}P]ATP was carried out at 0 °C for 15 s in the presence of 50 mM MOPS buffer (pH 7.0), 80 mM K^+, 5 mM Mg^{2+}, 0.1 mM Ca^{2+}, 2 μM Ca^{2+} ionophore A23187, and 2 μM [γ-^{32}P]ATP. Following 15-s reaction with [γ-^{32}P]ATP, the sample was quenched directly (time point zero = 100%) or incubated with 1 mM ADP plus 1 mM EGTA (●) or with 1 mM ADP plus 1 mM $CaCl_2$ (○). Acid quenching was performed at series time intervals as indicated on the abscissa. The acid-quenched ^{32}P-phosphorylated samples were subjected to SDS–polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels were quantitated by densitometry. Note the logarithmic ordinate scale. The dashed line indicates that the slow phase extrapolates back to 75% at zero time. The slope of the slow phase corresponds to a half-life of 10 s.

$E_2P \rightarrow E_1P$ back-transition can occur even in the absence of free Ca^{2+} (which in this experiment was removed by added EGTA and ionophore). This might be possible if the Ca^{2+} needed for the back-transition (see Scheme I) remained occluded in the E_2P form for some time (Froehlich & Heller, 1985). The $E_2P \rightarrow E_1P$ transition of enzyme containing Ca^{2+} in the occluded state in E_2P would, on the other hand, be rather insensitive to added free Ca^{2+}. However, the second experiment shown in Figure 4 demonstrated that addition of Ca^{2+} with ADP led to a much more rapid disappearance of all of the phosphoenzyme. Therefore, added Ca^{2+} was able to accelerate the $E_2P \rightarrow E_1P$ transition, and there was no sign of a block of this transition in the mutant. Hence, we conclude that the most significant reason for the steady-state accumulation of E_2P in the mutant was the block of its hydrolysis to E_2 and P_i. In further support of the interpretation of the slow phase of dephosphorylation as hydrolysis of E_2P is also the finding that the rate of the slow phase was very similar to the rate of hydrolysis of E_2P formed by "backdoor" phosphorylation with P_i (compare Figure 4 with Figure 3B).

Figure 5 shows the P_i concentration dependence of equilibrium phosphorylation from P_i. The measurements were carried out either in the absence (A) or in the presence (B) of the organic solvent dimethyl sulfoxide. The latter compound increases the apparent affinity of the Ca^{2+}-ATPase for P_i, possibly due to an effect on the structure of water (de Meis et al., 1980). Hence, using the maximal phosphorylation levels determined in the presence of dimethyl sulfoxide as reference, the affinity for P_i could be estimated even under low-affinity conditions in the absence of dimethyl sulfoxide. It can be seen from Figure 5 that the mutant and the wild type displayed similar if not identical apparent affinities for P_i under varying ionic conditions and in the presence and absence of dimethyl sulfoxide. It may thus be concluded that the decrease in the "off" rate for the phosphoryl group in the mutant, demonstrated by the results of the kinetic experiments described above, must have been accompanied by a similar reduction of the "on" rate. This suggests that the transition state in the hydrolysis of the E_2P phosphoenzyme intermediate was less stable in the mutant than in the wild-type Ca^{2+}-ATPase. Further evidence in support of this hypothesis was obtained in studies of the

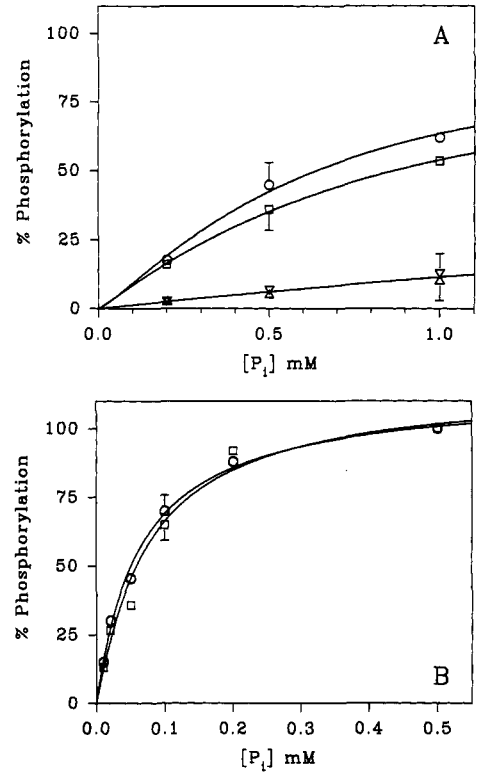

FIGURE 5: P_i concentration dependence of phosphorylation from P_i of the chimeric mutant (○, △) and the wild-type Ca^{2+}-ATPase (□, ▽) expressed in COS-1 cells. (A) Phosphorylation with [$^{32}P_i$] was carried out at 25 °C for 10 min in the presence of the indicated concentrations of [$^{32}P_i$] and in the absence of dimethyl sulfoxide. The buffer contained 100 mM MES/Tris (pH 6.0), 10 mM Mg^{2+}, and 2 mM EGTA (○, □) or 50 mM MOPS (pH 7.0), 5 mM Mg^{2+}, 80 mM K^+, and 2 mM EGTA (△, ▽). (B) Phosphorylation with [$^{32}P_i$] was carried out at 25 °C for 10 min in the presence of the indicated concentrations of [$^{32}P_i$] and 20% (v/v) dimethyl sulfoxide, 100 mM TES/Tris (pH 7.0), 5 mM Mg^{2+}, and 2 mM EGTA. Quantitation was obtained by densitometric analysis of the autoradiograms of the dried gels. In both panel A and panel B the specific phosphorylations are shown as percentages of the maximal values measured at 1 mM P_i in the presence of dimethyl sulfoxide.

interaction of the mutant enzyme with the transition state analog vanadate.

In the experiments illustrated in Figure 6A, wild-type and mutant enzymes were preincubated with various concentrations of vanadate in the absence of Ca^{2+} to allow the binding of the analog before measurement of equilibrium phosphorylation in the presence of a fully saturating concentration of [$^{32}P_i$]. As vanadate and inorganic phosphate bind at the same site in the E_2 state, and as the dissociation of vanadate is rather slow (Andersen & Møller, 1985), the titration of vanadate-induced inhibition of phosphorylation from P_i provided an indirect measure of the affinity of the enzyme for vanadate. It is seen in Figure 6A that the concentration of vanadate required to prevent phosphorylation of the mutant was more than 10-fold higher than that required with the wild-type Ca^{2+}-ATPase.

In the experiments illustrated in Figure 6B, vanadate was added directly during the turnover of the enzyme with ATP and Ca^{2+}, and again the results demonstrated a much weaker binding of vanadate to the mutant compared with the binding to the wild type. Under these conditions, vanadate presumably interacted with an enzyme form produced in the course of the catalytic cycle (probably also E_2, see Scheme I).

The buffer conditions used for the studies with vanadate described in Figsure 6A,B were widely different, not only with respect to the presence of ATP and P_i but also with respect to pH and the presence (Figure 6B) or absence (Figure

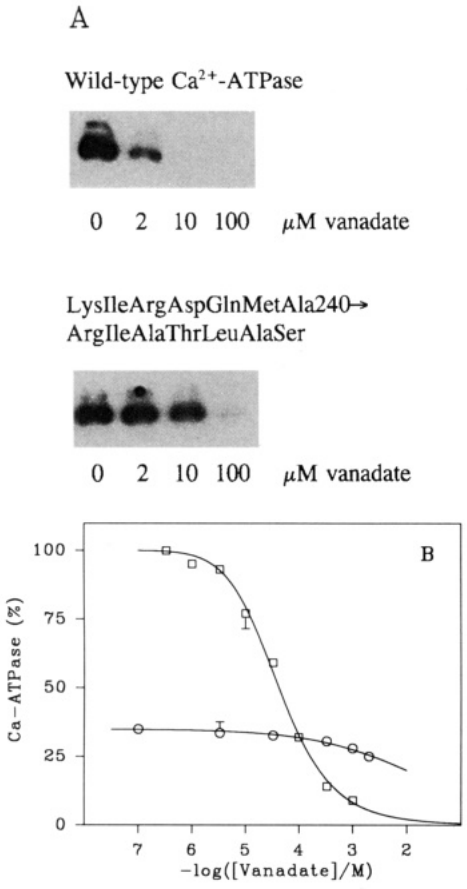

FIGURE 6: Titration of the inhibition by vanadate of the phosphorylation by P_i and the Ca^{2+}-activated ATPase activity in the chimeric mutant and in the wild-type Ca^{2+}-ATPase expressed in COS-1 cells. (A) Phosphorylation with [$^{32}P_i$] was carried out at 25 °C for 10 min in the presence of 0.5 mM [$^{32}P_i$], 100 mM TES/Tris (pH 7.0), 5 mM Mg^{2+}, 2 mM EGTA, 20% (v/v) dimethyl sulfoxide, and the indicated concentrations of vanadate. Prior to the addition of [$^{32}P_i$], the enzyme had been preincubated with the indicated concentrations of vanadate in the same buffer solution at 25 °C for 30 min. The acid-quenched ^{32}P-phosphorylated samples were subjected to SDS–polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels are shown. The film was exposed longer for the wild type than for the mutant. (B) The Ca^{2+}-activated ATPase activity was measured as described for Figure 1A, at 37 °C in the presence of 50 mM TES, pH 7.5, 0.1 M K^+, 1 mM Mg^{2+}, 0.1 mM Ca^{2+}, and 5 mM MgATP. Vanadate was added to the microsomes after the addition of MgATP, and the oxidation of NADH was recorded continuously. The activities shown were determined at steady state after the effect of vanadate had been fully expressed. (○) Chimeric mutant; (□) wild type.

6A) of K^+ (activates E_2P dephosphorylation). It may therefore be concluded that the effect of the amino acid replacements on vanadate binding does not depend on these ionic conditions.

DISCUSSION

The substitution of a seven-residue segment in the predicted "stalk" helix S3 of the Ca^{2+}-ATPase, with the peptide located at the homologous position in the Na^+,K^+-ATPase, led to relative mild functional changes which allowed measurement of turnover rates for Ca^{2+} transport and ATP hydrolysis at 27 and 37 °C and of the properties of the phosphoenzyme intermediates at 0 °C. The major findings were the following:

(1) In the mutant, the overall rates of ATP hydrolysis and Ca^{2+} transport were reduced to 30–40% of the wild-type rates.

(2) In the mutant, about 75% E_2P accumulated during ATP hydrolysis, under conditions in which only E_1P was detected in the wild-type Ca^{2+}-ATPase.

(3) Kinetic studies demonstrated a reduced rate of dephosphorylation of E_2P that explained the accumulation of E_2P in the mutant.

(4) The apparent affinity of the mutant enzyme for P_i measured by titration of equilibrium phosphorylation was similar to that of the wild-type Ca^{2+}-ATPase, indicating that the decrease in the dephosphorylation rate must have been accompanied by a decrease in the rate of phosphorylation of E_2 with P_i.

(5) The mutant was much less susceptible than the wild type to inhibition by vanadate. As vanadate is considered an analog of the pentacoordinated transition state of the phosphoryl group (Cantley et al., 1978), this finding suggests that the binding affinity of the mutant enzyme for the phosphoryl group in its transition state was reduced.

These observations can be explained according to the concept that the catalytic rate (in this case the rate of E_2P dephosphorylation) depends directly on the ability of the enzyme to bind the activated complex (transition state) tightly (Pauling, 1946; Fersht, 1974; Jencks, 1975). Our results are comparable to those obtained with other catalytic systems, in which mutations affecting the stability of the transition state have led to a reduced rate of catalysis with relatively little concomitant change in the binding affinities for either substrates or products [see, for instance, Leatherbarrow et al. (1985)].

We have not been able to quantitate the difference between the activation energies of E_2P dephosphorylation in the mutant and wild-type Ca^{2+}-ATPase enzymes by detailed studies of the temperature dependence of the rate constants, since the use of rapid quench techniques as required for temperatures above 0 °C demands pure enzyme in much larger quantities than can presently be obtained from the cell culture. It is nevertheless clear from comparison of the data that we have collected at 0 °C with the steady-state measurements at higher temperatures that the temperature dependence of the E_2P dephosphorylation must be steeper in the mutant than in the wild-type Ca^{2+}-ATPase, in accordance with a higher activation energy in the mutant. Hence, in the wild-type Ca^{2+}-ATPase, the dephosphorylation of E_2P constitutes the major rate-determining step of the cycle at 27–37 °C under our conditions of measurement of steady-state turnover at millimolar ATP concentration (Champeil et al., 1986), but the E_1P-E_2P transition is the most temperature sensitive step of the cycle and becomes rate limiting at 0 °C. In the mutant, the turnover rate was as high as 30–40% of the wild-type rate at 27–37 °C, but the rate of dephosphorylation of the E_2P intermediate was at least 7–10 fold lower than that of the wild type at 0 °C and slow enough to be rate-limiting for the cycle in the mutant, as judged from the accumulation of 75% E_2P at steady state (Figure 4). In the wild type and in previously characterized Ca^{2+}-ATPase mutants with overall turnover rates comparable to that of the presently studied chimeric mutant at 27–37 °C, the rate of E_2P dephosphorylation was not slow enough at 0 °C to be revealed by our manual mixing technique (Andersen et al., 1992; Vilsen et al., 1991a,b).

The alternative possibility for interpretation of the reduced vanadate sensitivity of the chimeric mutant would be a displacement of the $E_1 \rightarrow E_2$ equilibrium in favor of E_1, the enzyme form that does not bind vanadate. Indeed, there is some indication that such a change in the equilibrium might have been induced by the amino acid substitutions, since the apparent Ca^{2+} affinity was slightly increased in the mutant relative to the wild type. This increase in Ca^{2+} affinity could, however, only be observed when the enzyme was turning over with ATP, and not by titration of Ca^{2+} inhibition of

phosphorylation from P_i under equilibrium conditions (Figure 2B). Therefore, it appears to be a kinetic effect caused by the reduction of the dephosphorylation rate with resulting accumulation of the phosphoenzyme at Ca^{2+} concentrations lower than those usually required.

Mutants with amino acid replacements in the β-strand domain of the closely related yeast plasma membrane H^+-ATPase were previously shown to turn over slowly and to be insensitive to vanadate (Ghislain et al., 1987; Portillo & Serrano, 1988, 1989). In light of the present findings, it is tempting to speculate that transition-state destabilization might be involved, and it would be interesting to know whether the H^+-ATPase mutants accumulate E_2P and display normal apparent affinity for P_i like the chimera described here.

The present findings with the chimeric mutant may also be compared with the properties of the previously characterized Ca^{2+}-ATPase mutants Glu309→Gln, Gly310→Val or Pro, and Glu771→Gln with amino acid replacements in the transmembrane domain (Andersen et al., 1992; Andersen & Vilsen, 1992b). Like the chimeric mutant, these point mutants showed a reduced rate of dephosphorylation of the E_2P intermediate, but there was a corresponding increase in the apparent affinity for P_i and a decrease in the affinity for Ca^{2+} displayed in equilibrium phosphorylation measurements, and the steady-state turnover rate was less than 2% that of the wild type at 27–37 °C, unlike the situation with the chimera. For these mutants, the reason for the reduced dephosphorylation rate must have been a stabilization of the E_2P state (possibly a stabilization of the noncovalent interaction between the phosphoryl group and the protein in E_2 conformation), rather than the destabilization of the phosphoryl group transition state described here for the chimera.

The present findings raise the question whether the replaced residues LysIleArgAspGlnMetAla240 might be located close to or in the catalytic cleft in the E_2P state. This would not be incompatible with the participation of the β-strand domain in the $E_1P \rightarrow E_2P$ transition proposed previously (Andersen et al., 1989). It is possible that the $E_1P \rightarrow E_2P$ conformational change comprises a movement of some of the replaced residues in S3, bringing them nearer to the aspartyl phosphate. It is noteworthy in this connection that the binding of vanadate, as well as phosphorylation under conditions in which E_2P accumulates, has been shown to protect against tryptic cleavage at the "T_2-site" located between Arg198 and Ala199 only 35 residues away from the peptide segment studied here (Dux & Martonosi, 1983; Andersen, 1989). In light of the present findings, this protection may be considered a consequence of direct steric hindrance to trypsin access caused by the presence of the ligand in the phosphate site in E_2 state.

ACKNOWLEDGMENT

We express our gratitude to Dr. D. H. MacLennan, in whose laboratory our mutagenesis work with Ca^{2+}-ATPase was initiated. We also thank Jytte Jørgensen, Karin Kracht, and Janne Petersen for their expert and invaluable technical assistance, Dr. N. M. Green for continued discussion of ATPase structure and mechanism, and Dr. R. J. Kaufman, Genetics Institute, Boston, for the gift of the expression vector p91023(B).

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