Volume 306, number 2,3, 247–250 FEBS 11316 July 1992

Mutational analysis of the role of Glu^{309} in the sarcoplasmic reticulum Ca^{2+}-ATPase of frog skeletal muscle

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Received 21 May 1992

Site-specific mutagenesis was used to analyse the role of the residue, Glu^{309}, in the function of the Ca^{2+}-ATPase of frog skeletal muscle sarcoplasmic reticulum by substitution with Ala or Lys. At pH 6.0, 100 μM Ca^{2+} was unable to prevent phosphorylation from P_i, consistent with previous observations on the Ca^{2+}-ATPase of rabbit fast twitch muscle [Clarke, D.M., Loo, T.W, Inesi, G. and MacLennan, D.H. (1989) Nature 339, 476–478]. At neutral pH, however, micromolar concentrations of Ca^{2+} were sufficient to inhibit phosphorylation of the Glu^{309}→Lys mutant from inorganic phosphate, suggesting that at least one high-affinity Ca^{2+} site was relatively intact in this mutant. The Glu^{309}→Lys mutant was unable to form a phosphoenzyme from ATP at all Ca^{2+} concentrations studied (up to 12.5 mM), whereas phosphorylation of the Glu^{309}→Ala mutant occurred at 12.5 mM Ca^{2+}, but not at Ca^{2+} concentrations in the submillimolar range. Kinetic studies demonstrated a reduced rate of dephosphorylation of the E_2P intermediate in the Glu^{309}→Lys mutant. A less pronounced stabilization of E_2P was observed with the Glu^{309}→Ala mutant, suggesting a possible role of the charge at the position of Glu^{309} in phosphoenzyme hydrolysis.

Ca^{2+}-ATPase; Sarcoplasmic reticulum; Amino acid substitution; Ca^{2+} binding; Phosphoenzyme intermediate

1. INTRODUCTION

The Ca^{2+}-ATPase of sarcoplasmic reticulum mediates the active uptake of 2 mol Ca^{2+} for each mol of ATP hydrolyzed [1]. At the beginning of the transport cycle two calcium ions are bound at high-affinity sites facing the cytoplasmic membrane surface. Following occlusion of the calcium ions in the ADP-sensitive phosphoenzyme intermediate (E_1P), protein conformational changes lead to release of the ions from lumenal low-affinity sites concurrently with the formation of an ADP-insensitive phosphoenzyme species (E_2P) [2]. To gain further insight into the translocation mechanism, it is important to identify the amino acid residues involved in Ca^{2+} binding and in the ion translocating conformational changes. Studies of the Ca^{2+}-sensitivity of the phosphorylation reaction in mutants, in which carboxylic residues of the rabbit fast twitch muscle Ca^{2+}-ATPase had been exchanged with their corresponding amides, have led to the suggestion, that 3 glutamic acid residues and 1 aspartic acid residue in the putative transmembrane segments form the core of the high-affinity Ca^{2+} binding structure [3]. Proteolytic peptides derived from the COOH-terminal half of the Ca^{2+}-ATPase have, however, been shown to bind Ca^{2+}, despite the fact that they only contained 3 of the 4 carboxylic residues proposed as Ca^{2+} ligands [4]. The last of the putative Ca^{2+} liganding carboxylic residues (Glu^{309}) is located in the 4th transmembrane segment in a region which is highly conserved among the cation transporting ATPases ('PEGLP segment'). We previously demonstrated that substitution of Pro^{312} in this segment led to an enzyme, in which the E_1P–E_2P transition was blocked ('E_1/E_1P type' mutant) [5], whereas substitution of Gly^{310} led to a very stable E_2P form ('E_2/E_2P type' mutant) [6]. Hence, the PEGLP segment is central to the Ca^{2+} translocating step in the enzyme cycle, and it seems that our understanding would be a great deal furthered by clarification of the role of Glu^{309}. In the present study we have examined the functional consequences of substituting Glu^{309} in the frog skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase (see the accompanying paper [7]) with either Ala or Lys. The latter substitution was carried out to test whether the positive charge of Lys would be able to mimick the presence of bound Ca^{2+}, as might be expected on the basis of a location in the Ca^{2+} binding pocket.

Abbreviations: Ca^{2+}-ATPase, Ca^{2+}-activated adenosine triphosphatase; E_1P, ADP-sensitive phosphoenzyme intermediate; E_2P, ADP-insensitive phosphoenzyme intermediate; EGTA, [ethylenebis(oxy-ethylene-nitrilo)]tetraacetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate.

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2. MATERIALS AND METHODS

Mutations were introduced into the *Kpn*I(636)–*Bam*HI(1157) restriction fragment, that had been excised from the full-length frog

Ca^{2+}-ATPase cDNA [7] and inserted into the Bluescript vector, using synthetic oligonucleotides according to the site-specific mutagenesis method of Kunkel [8]. The presence of the correct mutation was confirmed by nucleotide sequencing. Finally the mutated fragment was excised from the Bluescript vector and religated back into its original position in the full-length clone. The mutant and wild-type frog muscle Ca^{2+}-ATPase cDNAs were expressed in COS-1 cells and the isolated COS-1 cell microsomes assayed for Ca^{2+} transport and ATPase activity as described in the accompanying paper [7]. Phosphorylation from ATP or P_i was measured as previously [5,6,9,10] with modifications as described in the figure legends. 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. The autoradiograms are shown in the figures.

3. RESULTS

The functional role of Glu^{309} in the frog muscle Ca^{2+}-ATPase was analysed by substitution with Ala or Lys. Either of these mutations led to an enzyme, which was well expressed in the COS-1 cell microsomes, but which was inactive as ATPase and as Ca^{2+} transporter. To examine the Ca^{2+} binding properties of the mutants, we studied the inhibition by Ca^{2+} of phosphorylation from P_i [6]. One of the findings previously leading to the conclusion that the Glu^{309}→Gln mutant of the rabbit enzyme was deficient in Ca^{2+} binding was that in this mutant, in contrast with the wild-type enzyme, the presence of 0.1 mM Ca^{2+} did not prevent phosphorylation from P_i [3]. As seen in Fig. 1, we were able to reproduce this result at 25°C, pH 6.0. However, as the Ca^{2+} affinity of the Ca^{2+}-ATPase is known to depend strongly on pH, presumably because of competition between Ca^{2+} and H^+ [11,12], we also performed the titration of Ca^{2+} inhibition of phosphorylation from P_i at neutral pH (Fig. 2), and under these conditions even micromolar concentrations of Ca^{2+} inhibited the phosphorylation of the Glu^{309}→Lys mutant from P_i ($K_{0.5} \approx 10\ \mu M$). For the Glu^{309}→Ala mutant, the $K_{0.5}$ for Ca^{2+} inhibition of phosphorylation from P_i was higher ($\approx 100\ \mu M$), but still the data indisputably demonstrated an inhibitory effect of Ca^{2+}. This shows that each of the mutant enzymes was capable of binding at least one calcium ion.

Fig. 3 describes the results of experiments, in which we examined the Ca^{2+} dependency of phosphorylation from ATP. No significant phosphoenzyme formation was observed with the Glu^{309}→Lys mutant at the Ca^{2+} concentrations examined (up to 12.5 mM, i.e. more than 10,000-fold the concentration giving half-maximum phosphorylation from ATP in the wild-type). At submillimolar Ca^{2+} concentrations, the Glu^{309}→Ala mutant was unable to form a phosphoenzyme with ATP, as well, but at 12.5 mM Ca^{2+} the phosphorylation level detected with this mutant was almost as high as with the wild-type, suggesting that the activatory Ca^{2+} sites were still functioning, albeit with an unusually low apparent affinity.

Fig. 4 shows the results of kinetic experiments, in

Fig. 1. Ca^{2+} dependency at pH 6.0 of phosphorylation from P_i of the Glu^{309}→Ala and Glu^{309}→Lys mutants and the wild-type Ca^{2+}-ATPase. Phosphorylation of expressed Ca^{2+}-ATPase in microsomal fractions isolated from COS-1 cells transfected with mutant or wild-type frog muscle Ca^{2+}-ATPase cDNA was carried out for 10 min at 25°C in the presence of 100 μM $^{32}P_i$, 100 mM MES/Tris (pH 6.0), 5 mM $MgCl_2$, 20% (v/v) dimethylsulfoxide and 2 mM EGTA ('E') or 100 μM Ca^{2+} ('C').

which the rates of dephosphorylation of the E_2P phosphoenzyme intermediates formed with P_i in the wild-type and Glu^{309} mutants of the frog muscle Ca^{2+}-ATPase were observed after dilution in a 200-fold excess of non-radioactive P_i. While dephosphorylation was complete in the wild-type within 10 s, both mutants retained the bound phosphate for longer time, demonstrating that the covalent or the non-covalent enzyme–phosphate bonds were more stable in the mutants than in the wild-type. It can be seen that the stabilization was more pronounced with the Glu^{309}→Lys mutant than with the Glu^{309}→Ala mutant.

4. DISCUSSION

The present communication presents 3 novel observations regarding the functional role of Glu^{309} in the sarcoplasmic reticulum Ca^{2+}-ATPase. First, Ca^{2+} inhibited the phosphorylation from P_i at neutral pH. In the Glu^{309}→lys mutant the inhibition occurred with a close to normal apparent affinity for Ca^{2+}, suggesting that at least one calcium binding site was intact. Second, Ca^{2+} present in millimolar concentration was able to activate phosphorylation from ATP in the Glu^{309}→Ala mutant. Hence, the presence of the negative charge of Glu^{309} is

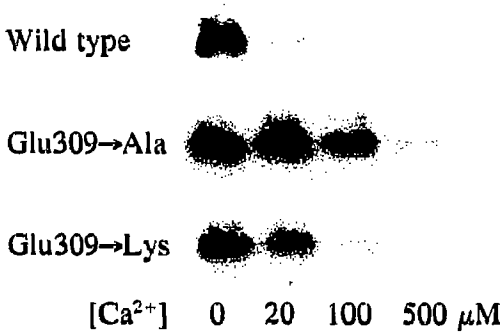

Fig. 2. Ca^{2+} dependency at pH 7.0 of phosphorylation from P_i of the Glu^{309}→Ala and Glu^{309}→Lys mutants and the wild-type Ca^{2+}-ATPase. Phosphorylation with P_i was carried out at the indicated Ca^{2+} concentrations, under conditions as described for Fig. 1 except that the buffer contained 100 mM TES/Tris (pH 7.0) instead of MES. '0' indicates the presence of 2 mM EGTA and absence of Ca^{2+}.

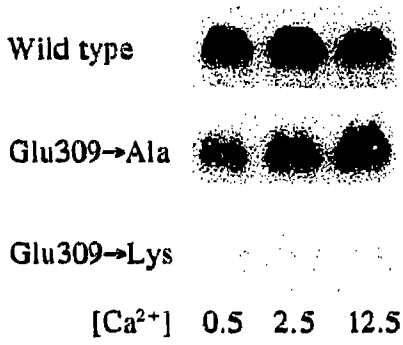

Fig. 3. Calcium dependency of phosphorylation from ATP of the Glu^{309}→Ala and Glu^{309}→Lys mutants and the wild-type Ca^{2+}-ATPase. Phosphorylation of expressed Ca^{2+}-ATPase in microsomal fractions isolated from COS-1 cells transfected with mutant or wild-type frog muscle Ca^{2+}-ATPase cDNA was 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 [γ-^{32}P]ATP, and the indicated Ca^{2+} concentrations.

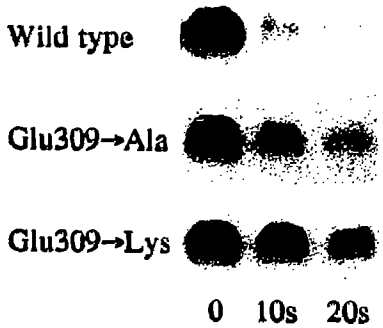

Fig. 4. Dephosphorylation of the E_2P phosphoenzyme intermediate formed with P_i in the Glu^{309}→Ala and Glu^{309}→Lys mutants and the wild-type Ca^{2+}-ATPase. Wild-type and mutant Ca^{2+}-ATPases were phosphorylated by 100 μM $^{32}P_i$ at 25°C for 10 min in the presence of 100 mM TES/Tris, pH 7.0, 5 mM Mg^{2+}, 2 mM EGTA, 20% (v/v) dimethylsulfoxide. Following cooling of the sample to 0°C, dephosphorylation was initiated by 20-fold dilution of an aliquot into an ice-cold medium containing 50 mM MOPS, pH 7.0, 80 mM K^+, 5 mM Mg^{2+}, 1 mM non-radioactive P_i, and 2 mM EGTA, and acid quenching was performed 10 s and 20 s after the dilution.

not required to confer the calcium sites the ability to transmit the signal to the catalytic site that activates the phosphoryl transfer reaction. Third, we observed a reduced rate of dephosphorylation of the E_2P intermediate in the mutants, suggesting a possible role of the charge of Glu^{309} in phosphoenzyme hydrolysis.

The high apparent affinity for Ca^{2+} observed with the Glu^{309}→Lys mutant in Ca^{2+} titration of phosphorylation from P_i contrasts with the inability of 12.5 mM Ca^{2+} to activate phosphorylation from ATP in this mutant. The results of the two phosphorylation assays may, however, be reconciled by assuming that one of the two Ca^{2+}-binding sites was relatively intact in the Glu^{309}→Lys mutant, whereas the positive charge of Lys prevented the binding of Ca^{2+} at the other site. The binding of Ca^{2+} at the intact site would be sufficient to prevent phosphorylation from P_i [13], while activation of phosphorylation from ATP requires the binding of two calcium ions [14]. Hence, Glu^{309} might provide ligands for the binding of one of the two calcium ions, but not for the binding of the other. The finding, that the Glu^{309}→Lys mutant displayed a higher Ca^{2+} affinity than the Glu^{309}→Ala mutant in the titration of Ca^{2+} inhibition of phosphorylation from P_i, may be explained according to the structural model, in which the binding process involves a 'jaw-closing' movement of the walls of a common binding pocket for the two ions [15,16], if it is assumed that the positively charged side chain of Lys mimicked the presence of one of the two calcium ions to such an extent that the closure of the binding pocket about the other calcium ion was aided in this mutant, consistent with the well-known positive cooperative interaction between the two Ca^{2+} sites. It must, however, also be presumed that the lysine was an inadequate substitute for Ca^{2+} with respect to the activation of phosphorylation from ATP.

The changes in the Ca^{2+} binding properties induced by the alterations to Glu^{309} were accompanied by a reduction of the rate of breakdown of the phosphoenzyme formed from P_i. A similar block of E_2P dephosphorylation has previously been described for mutants of the rabbit fast twitch muscle Ca^{2+}-ATPase, in which the juxtaposed Gly^{310} had been replaced with either Pro or Val, and we suggested that a displacement of the conformation equilibrium of the enzyme had occurred as a result of these amino acid substitutions [6]. The present observation that the replacement of Glu^{309} with Lys had a more pronounced effect on the rate of dephosphorylation than the replacement with Ala, despite the fact that one of the Ca^{2+} sites displayed a close to normal affinity in the Glu^{309}→Lys mutant, suggests that the charge at the position of the side chain of Glu^{309} may be of direct importance in the regulation of phosphoenzyme hydrolysis. This would be consistent with a model, in which the alterations to the charge, caused by the binding and dissociation of Ca^{2+} near Glu^{309}, control the dephosphorylation in the normal enzyme, explaining why E_2PCa^{2+} and $E_2P(Ca^{2+})_2$ are 'dead-end' complexes that do not undergo hydrolysis of the acyl phosphate bond until the calcium ions have dissociated to the lumenal side [17]. An alternative possibility is that the function of Glu^{309} in phosphoenzyme hydrolysis is associated with a role in the countertransport of H^+ [18–20]. By analogy with the role of K^+ in the catalytic mechanism of the Na^+, K^+-ATPase [21] the hydrolysis of E_2P of the Ca^{2+}-ATPase might require triggering by H^+ binding in exchange with Ca^{2+} at the lumenal transport site(s). A reduced H^+ affinity induced by the alteration to Glu^{309} might, therefore, result in inhibition of the dephosphorylation. Even if Glu^{309} is not directly involved in the binding of H^+, the positive charge of the Lys side chain might interfere with H^+ binding by electrostatic repulsion, consistent with the

more pronounced inhibition of phosphoenzyme hydrolysis observed with the Glu^{309}→Lys mutant relative to that of the Glu^{309}→Ala mutant.

Acknowledgements: We would like to thank Jytte Jørgensen, Karin Kracht, and Janne Petersen for their expert and invaluable technical assistance. This research was supported by grants from the Danish Biomembrane Research Centre, the Danish Medical Research Council, the NOVO Foundation, and the Nordic Insulin Foundation (Denmark).

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