Volume 335, number 2, 261–264 FEBS 13336 December 1993

High sensitivity to site directed mutagenesis of the peptide segment connecting phosphorylation and Ca^{2+} binding domains in the Ca^{2+} transport ATPase

Ziyu Zhang, Carlota Sumbilla, David Lewis, Giuseppe Inesi*

Department of Biological Chemistry, School of Medicine, University of Maryland, Baltimore, MD 21201, USA

Received 11 October 1993

Nine residues (Leu^{321}, Lys^{329}, Asn^{330}, Val^{333}, Arg^{334}, Leu^{336}, Pro^{337}, Val^{339} and Glu^{340}), within the peptide segment intervening between the catalytic domain and the Ca^{2+} binding domain of the sarcoplasmic reticulum (SERCA 1) ATPase, were individually mutated to Ala. The mutated proteins were recovered in the microsomal fraction of COS-1 cells following transient expression, and exhibited inhibition of Ca^{2+} uptake and ATPase hydrolytic activity, while forming discernable levels of phosphorylated intermediate. Mutation of Glu^{340} to Gln (rather than to Ala) was much less effective, suggesting that the functional consequence of the mutation is related to structural perturbation, rather than loss of the acidic side chain. The high sensitivity of this peptide segment to single mutations suggests that its structural integrity is required for functional linkage of the phosphorylation and Ca^{2+} binding domains.

Ca^{2+}-ATPase mutant

1. INTRODUCTION

The primary structure of the Ca^{2+} transport ATPase of sarcoplasmic reticulum (SR) was partially obtained by direct amino acid sequencing [1]. Cloning of ATPase cDNA [2] and functional expression of full-length cDNA in COS-1 cells [3] made it possible to obtain the entire sequence and to study relationships of structure and function by site directed mutagenesis. As a result of chemical and mutational studies, it is now evident that the phosphorylation and catalytic domain resides within the extramembranous headpiece [3–6], and the Ca^{2+} binding domain is within the membrane-bound region of the enzyme [7–10]. Furthermore, mutation of a few residues located throughout the protein structure results in impairment of enzyme turnover [11,12], suggesting involvement of various protein segments in the function of the protein. On the other hand, numerous amino acid residues of the ATPase can be mutated with no significant functional consequence, as reviewed by MacLennan [13]. We have now subjected the peptide segment (Fig. 1) intervening between the phosphorylation site and the Ca^{2+} binding domain to single mutations of several amino acid residues. We found that the catalytic and transport function of the ATPase is highly sensitive to such mutations.

*Corresponding author. Fax: (1) (410) 706-8297.

2. EXPERIMENTAL

2.1. *Oligonucleotide directed mutagenesis and cDNA expression in COS-1 cells*

The chicken fast muscle SR (SERCA 1) ATPase cDNA [16] was inserted into the pUC19 plasmid for amplification, and then subcloned into the pSELECT-1 vector for site-directed mutagenesis by the 'Altered Sites' In Vitro Mutagenesis System made available by Promega (Madison, WI). Eleven unique restriction sites were introduced in the SERCA 1 cDNA by site-directed mutagenesis, retaining the original coding sequence. These unique restriction sites are spaced at approximately equal intervals, and facilitate mutagenesis by yielding cassettes of approximately three-hundred bases which can be conveniently sequenced following mutagenesis, and exchanged with the corresponding cassette of wild-type cDNA. Furthermore a c-myc tag was added to the 3′ terminal in order to monitor ATPase expression using c-myc antibodies, independent of mutations in the enzyme sequence. The mutated cDNA was finally transferred into the pCDL-SRα296 plasmid [17] for transfection of COS-1 cells by the DEAE-dextran method and transient expression under the control of the SV40 promoter. The transfection procedure was as described by Sumbilla et al. [9].

2.2. *Microsome preparation and immunodetection of expressed protein*

The procedure for microsome preparation was similar to that used by Clarke et al. [18], exactly as described by Sumbilla et al. [9]. Microsomes were stored in small aliquots at −70°C. The total microsomal protein was determined by the Lowry method [19]. The expressed ATPase was detected by blotting 7.5% Laemmli electrophoretic gels onto nitrocellulose paper, and incubating with the monoclonal antibody mAb 9E10 to the c-myc epitope [20] and, in parallel, with a monoclonal antibody to the chicken Ca-ATPase, CaF3-5C3, [14]. After incubation with secondary antibody (goat anti-mouse IgG-horseradish peroxidase-conjugated), the bound proteins were probed using an Enhanced Chemiluminescence-linked detection system (Amersham Corp.). Quantitation of immunoreactivity was obtained by densitometry, standardized with samples of the wild-type ATPase used as controls for the functional studies.

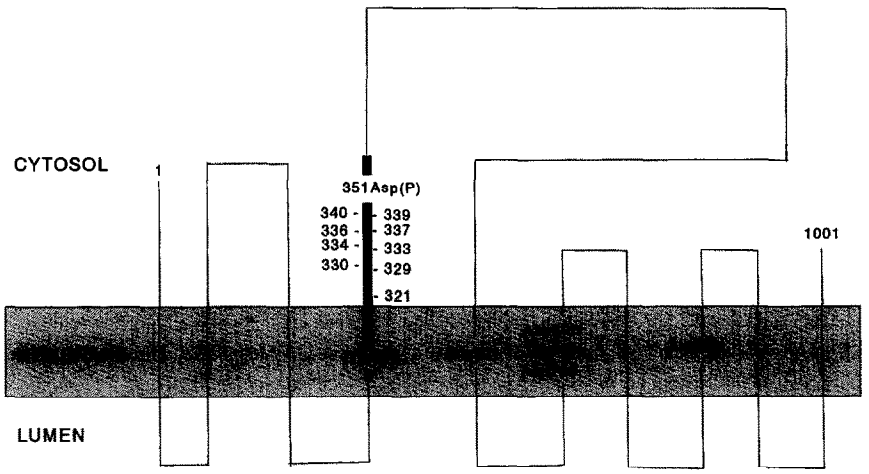

Fig. 1. Topology of the Ca-ATPase in the sarcoplasmic reticulum membrane. Large segments at the top extend into the cytosol; short segments are exposed to the lumenal side of the membrane. The model is based on that of Clarke et al. [7]. Asp^{351} undergoing phosphorylation is shown in the extramembranous region. Residues Glu^{309}, Glu^{771}, Asp^{800}, Thr^{799}, Asn^{796}, and Glu^{908}, involved in Ca^{2+} binding are shown in the membrane region. Presumably, the membrane spanning segments M4, M6, M7 and M8, are clustered to form a Ca^{2+} binding channel in the ATPase folded structure. The phosphorylation and Ca^{2+} binding domains are separated by a distance of approximately 50 Å [14]. The thick line denotes the segment (residues 297 to 359) linking the phosphorylation and Ca^{2+} binding domains, which retains a high degree of homology in several cation transport ATPases [15]. The nine residues subjected to mutation analysis in our experiments are denoted by their numbers.

2.3. *Functional studies*

The studies of Ca^{2+} transport and phosphoenzyme formation with [γ-^{32}P]ATP or [^{32}P]phosphate were carried out as described by Clarke et al. [18]. ATPase hydrolytic activity was assessed by determination of P_i production [21].

3. RESULTS AND DISCUSSION

As previously reported [3,22], the microsomal fraction of COS-1 cells transfected with cDNA encoding the wild-type Ca^{2+}-ATPase exhibits ATP dependent Ca^{2+} transport (Fig. 2), with initial rates varying between 15 and 25 nmol·mg^{-1} of microsomal protein·min^{-1}, at 25°C. As previously reported [23], we were also able to measure ATPase activity, provided that the microsomal vesicles were rendered leaky with a Ca^{2+} ionophore, and that the incubation was carried out at 37°C, to yield maximal catalytic turnover. Under these conditions, we obtained rates ranging between 70 and 102 nmol·mg^{-1} of microsomal protein·min^{-1}. These rates correspond to the difference between total and Ca^{2+}-independent ATPase activities, and therefore reflect the Ca^{2+}-dependent ATPase activity (Fig. 3).

Within the stretch of amino acids between Lys^{321} and Glu^{340}, nine amino acids were selected for single mutations to Ala. Glu was mutated to Ala and Gln. The expression level of all these mutants by the transfected COS-1 cells, as revealed by immunodetection of the c-myc epitope, was comparable or even higher than the expression of wild-type ATPase (results not shown). All mutations to Ala resulted in profound impairment of transport and catalytic functions, while the more conservative mutation of Glu^{340} to Gln produced much less interference. Examples of related measurements are shown in Figs. 2 and 3, and all the measurements are summarized in Table I. In general, Ca^{2+} transport was interfered with more strongly, resulting in inhibition as high as 90–95% in some mutants. On the other hand, the ATPase activity appeared to be inhibited to a

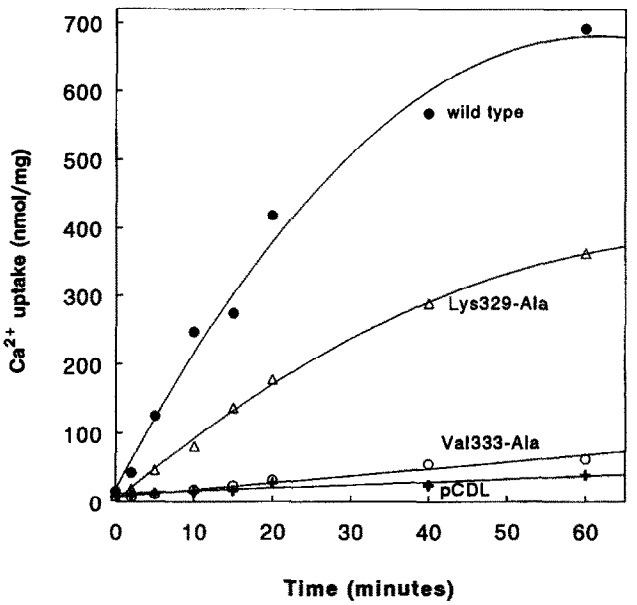

Fig. 2. ATP-dependent Ca^{2+} transport sustained by microsomes obtained from COS-1 cells transfected with wild-type Ca^{2+}ATPase cDNA (●–●), cDNA containing the single mutation of Lys^{329} → Ala (△–△) or Val^{333} → Ala (○–○), and pCDL vector only with no cDNA insert (+–+). Ca^{2+} transport was obtained at 25°C in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM $^{45}CaCl_2$, 5 mM potassium oxalate, 5–10 μg microsomal protein per ml, and 3 mM ATP. Fractional samples were filtered serially through 0.45-μm Millipore filters. The filters were washed with 3 mM $LaCl_3$, 10 mM MOPS, pH 7.0, and processed for scintillation counting. The experimental data were corrected for the levels of specific protein expression.

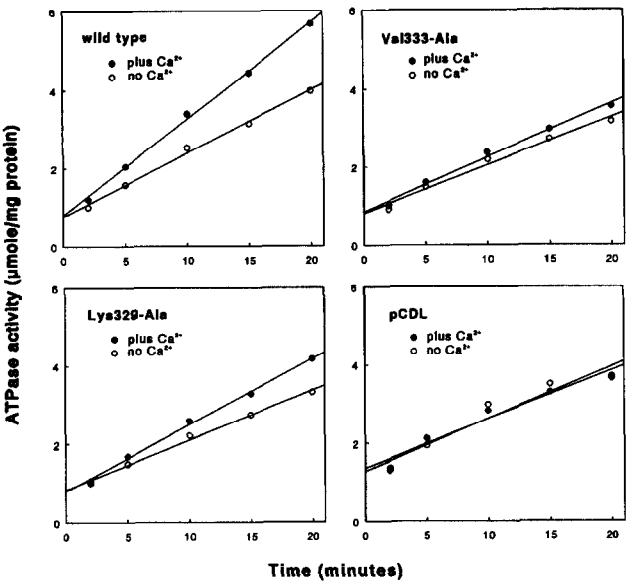

Fig. 3. ATPase activity of microsomes obtained from COS-1 cells transfected with wild-type Ca^{2+}-ATPase cDNA, cDNA containing the single mutations $Lys^{329} \rightarrow Ala$ or $Val^{333} \rightarrow Ala$ and pCDL vector only with no cDNA insert. ATP hydrolysis was obtained at 37°C in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 5 mM sodium azide, 30 μg microsomal protein per ml, 5 μM ionophore A23187, and 3 mM ATP in the absence (2 mM EGTA, ○–○) or presence of 10 μM free Ca^{2+} (●–●). Following the addition of ATP, serial samples were taken for determination of P_i. The Ca^{2+}-dependent ATPase activities were corrected for the levels of specific protein expression.

slightly lesser degree (Table I). It should be pointed out that the ATPase was tested under conditions enhancing its activity (i.e. in the presence of the Ca^{2+} ionophore A23187, and at 37°C), which are not strictly comparable to the conditions required for Ca^{2+} transport.

As opposed to mutational interference with catalytic and transport activities, we found that the levels of phosphorylated intermediate formed upon addition of ATP were still evident in the mutated ATPase. Turnover of the intermediate, however, appeared to be slower (Fig. 4), consistent with the observed inhibition of catalytic activity. It is apparent then that the main functional impairment produced by our mutations is an interference with the ATPase cycle. Furthermore, at least in some of the mutants, an uncoupling effect may be also produced, as the rates of net Ca^{2+} uptake are reduced more than the Ca^{2+}-dependent ATPase activity. These effects are likely due to structural perturbations produced by mutation of the involved residues to the less space filling alanine. The slight effect of mutating Glu^{340} to Gln, as compared to the larger effect of mutating the same residue to Ala (Table I), shows clearly that it is not the acidic function of Glu^{340} that is required for function, but rather the rigidity conferred to the neighbouring structure by the native residue.

It was previously pointed out that the peptide segment (thick line Fig. 1, between residues 297 and 359) intervening between catalytic and Ca^{2+} binding domains retains a high degree of homology in cation transport ATPases (Table I), indicating that structural integrity of this domain is prominently required for function [15]. It has been previously demonstrated [28,29] that mutations of this segment near the Ca^{2+} binding domain (M4 transmembrane segment) interfere with function. We now find that mutations throughout the longer portion of this segment, spanning the region between the membrane surface and the phosphorylation site, also interfere with function. We suggest that while the entire tertiary structure of the protein participates in the process of energy transduction [30], the segment intervening between the phosphorylation and Ca^{2+} binding domains may function as a 'rigid pin', sensing perturbations in the two domains and transmitting them to the neighbouring protein structure.

Acknowledgements: The authors are grateful to Dr. Mary Kirtley for helping in the analysis of sequence homologies. This work was supported by the National Institutes of Health and American Heart Association.

Table I

Functional consequences of single mutations within the peptide segment connecting catalytic and Ca^{2+} binding domains of the SERCA1 ATPase

Amino acid substitution	Relative Ca^{2+} transport	Relative ATPase activity	Amino acid homologies of wild-type Ca^{2+}-ATPase with other ATPases
Wild-type	1.00	1.00	
$Leu^{321} \rightarrow Ala$	0.32	0.58	a, b, c
$Lys^{329} \rightarrow Ala$	0.26	0.61	a, b, c, d
$Asn^{330} \rightarrow Ala$	0.34	0.56	a, b, c
$Val^{333} \rightarrow Ala$	0.04	0.20	a, b, c, d
$Arg^{334} \rightarrow Ala$	0.06	0.22	a, b(K), c(K), d(Q)
$Leu^{336} \rightarrow Ala$	0.04	0.17	a, b, c, d
$Pro^{337} \rightarrow Ala$	0.30	0.31	a
$Val^{339} \rightarrow Ala$	0.35	0.33	a, b, c
$Glu^{340} \rightarrow Ala$	0.10	0.11	a, b, c, d
$Glu^{340} \rightarrow Gln$	0.67	0.78	a, b, c, d

The levels of functional activities are corrected for the level of expressed ATPase in the microsomal protein. The functional values reported for each mutant are averages of five or six measurements performed with three microsomal preparations obtained from COS-1 cells subjected to transfection in separate sessions. Homologies of amino acid in the wild-type fast muscle Ca-ATPase, SERCA1 [16], relate to: a = slow muscle Ca-ATPase, SERCA2 [24]; b = sheep Na,K-ATPase [25]; c = pig H,K-ATPase [26]; d = yeast H-ATPase [27].

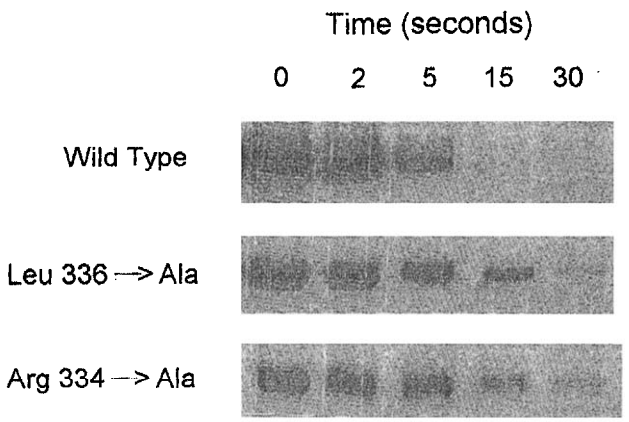

Fig. 4. Decay of radioactive phosphoenzyme following a chase with non-radioactive ATP in microsomes obtained from COS-1 cells transfected with wild-type Ca^{2+}-ATPase cDNA, cDNA containing the single mutations of $Leu^{336} \rightarrow Ala$ or $Arg^{334} \rightarrow Ala$. Phosphorylation was obtained by incubating 70 μg of microsomal protein in ice for 10 s in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM $CaCl_2$, and 2 μM [γ-^{32}P]ATP (specific activity 1×10^7 disintegration·nmol^{-1}), in a total volume of 0.5 ml. Decay of the radioactive phosphoenzyme was initiated by the addition of 0.12 mM non-radioactive ATP. Samples were collected before addition of cold ATP and at serial times following initiation of decay. Quenching was obtained with 0.4 M PCA and 2 mM P_i, and the samples were processed for electrophoresis and autoradiography.

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