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The Thermal Sensitivity of Sarcoplasmic Reticulum Ca²⁺-ATPase

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The thermal sensitivity of sarcoplasmic reticulum Ca²⁺-ATPase

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ABBREVIATIONS

AMP-PNP	adenyl-5'-imidodiphosphate
$C_{12}E_{9}$	polyoxyethylene-9-laurylether
DACM	N-(7-Dimethylamino-4-methyl-3-coumarinyl)-maleimide
DMSO	dimethyl sulfoxide
DOC	deoxycholate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-
	tetraacetic acid
EP	phosphoenzyme
FITC	fluoresceine isothiocyanate
GSH	glutathione
HPLC	high-performance liquid chromatography
IAA	iodoacetamide
IAEDANS	5-(2-iodoacetamidoethyl)aminonaphthalene-1-sulfonate
NCD-4	N-cyclohexyl-N'-(4-dimethylamino-1-naphthyl)carbodiimide
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyrvate
PMI	N-(3-pyrene)maleimide
SDS	sodium dodecyl sulfate
SR	sarcoplasmic reticulum
TCA	trichloroacetic acid
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonate
TFA	trifluoroacetic acid

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GENERAL INTRODUCTION

Physiological functions of sarcoplasmic reticulum

In skeletal muscle, intracellular Ca²⁺ ions play a critical role in the regulation of contraction and relaxation of muscle; When muscle cell is relaxed, concentration of Ca²⁺ is kept below 0.1 μ M. When muscle cell is excited by electric current from a nerve end, the depolarization of plasma membrane is transferred to interior of the cell *via* transverse tubules, which are vertically running against the muscle fiber, then the signal stimulates the Ca²⁺-burst from the sarcoplasmic reticulum (SR) vesicles which are surrounding myofibrils in the cell. Upon the Ca²⁺-release from SR, intracellular Ca²⁺ level increases above several μ M which suffices to trigger the muscle contraction. The entire process from excitation to contraction of muscle is called "E-C coupling". After the excitation, cytoplasmic Ca²⁺ ions are removed by SR to a level lower than 0.1 μ M, which results in the muscle relaxation (see Ref. 1 for a review).

SR vesicles can be easily isolated with retention of the full functional activities by differential centrifugation of a muscle homogenate. The isolated SR vesicles have been proven suitable for the study of the molecular mechanisms of active cation transport since this system possesses several features distinct from other transport membranes as follow; (i) A high content of the Ca²⁺-pump ATPase, (ii) a clear distinction between the inside and outside of the membrane, (iii) tightly coupled ATP hydrolysis to Ca²⁺-transport, and (iv) readily measured reaction intermediates, EP, which facilitates the kinetic analysis and characterization of the elementary steps of the reaction (see Ref. 2 for review).

Reaction scheme of Ca²⁺-ATPase

Figure 1 shows the coupling mechanism of ATP hydrolysis and Ca²⁺-transport by SR. This scheme has been proposed on the basis of a number of transient and steady state kinetic analysis (see Ref. 3 for review). In the first step of the Ca²⁺-pump reaction, Ca²⁺-ATPase forms E_1 Ca complex by the binding of 2 mol Ca²⁺ to 1 mol of enzyme. In the intact SR membrane Ca²⁺-ATPase exists in an equilibrium between two different configurations of E_1 and E_2 . In the absence of Ca²⁺, the equilibrium shifts to E_2 . When Ca²⁺ is added to the cytoplasmic side, the enzyme in E_1 form, but not in E_2 form, can sequentially bind 2 mol of Ca to form E_1 Ca complex. In the second step, E_1 Ca is reacted with ATP at the outer surface of the SR membrane to form phosphoprotein intermediate



Fig. 1 Coupling mechanism of ATP hydrolysis and Ca transport in SR. The scheme is based on the assumption of two major conformational states, E1 and E2, which are characterized by the ability to react with ATP and Pi, respectively. In the formation of E1P from E1Ca and ATP, 2 mol Ca²⁺ ions are occluded in the enzyme. After conversion of E1P to E2P, 2 mol Ca²⁺ ions are deoccluded. This is followed by dephosphorylation of E2P to E2 and Pi. For further details see text.

 (E_1P) by transferring a γ -phosphate of ATP to the specific Asp residue of the catalytic site. E₁P is highly sensitive to ADP and it can form ATP by the reversal reaction with ADP. In this step, Ca²⁺ is occluded in the enzyme and inaccessible to both of cytoplasmic and luminal sides of the SR membrane. The third step is a conversion of the Ca²⁺-ATPase from E₁P to E₂P in which Ca²⁺ ions move into luminal side. The apparent affinity of E₂P for Ca²⁺ becomes 3 to 4 order of magnitude less than that of E₁P and 2 mol of Ca²⁺ are randomly released from the enzyme into the SR lumen. In the final step, E₂P is hydrolyzed by H₂O into E₂ and inorganic phosphate, Pi at the outer surface of SR membrane and the reaction cycle is accomplished. This catalytic cycle of the Ca2+-ATPase is completely reversible. When EGTA is added to remove outer medium Ca²⁺ and, Pi and ADP are simultaneously added to the medium which contains SR vesicles that have been loaded with Ca²⁺, 2 mol of Ca²⁺ are released from the vesicles which is accompanied by the synthesis of 1 mol of ATP via the steps of E₂P formation which is followed by the conversion from E_2P to E_1P . This reverse reaction indicates that free energy required for ATP synthesis is supplied by the dissipation of Ca²⁺ gradient across the SR membrane.

Structure of Ca²⁺-ATPase

Although the kinetics has been extensively studied, the exact mechanism of this chemiosmotic energy coupling can only be obtained by detailed studies on the structure-function relationships. The importance of the structural studies and characterization of the Ca²⁺-ATPase has been increasingly recognized recently. Ca²⁺-ATPase is about 110,000 Dalton membrane spanning protein. From amino acid sequence and hydropathy profile, the second structure of Ca²⁺-ATPase is expected to be constructed by 10 transmembrane helixes and large cytoplasmic domain (Fig. 2). Analysis of the amino acid sequence (4, 5) along with extensive site-directed mutagenesis studies (6) together with the studies on the two-dimensional membrane crystals (7) have concurred in a structural model for the Ca²⁺-ATPase in which the Ca²⁺-binding region is located within a cluster of putative transmembrane α -herices, M4, M5, and M6, (8) while the ATP binding site, Lys⁵¹⁵, and phosphorylation site, Asp³⁵¹ (6) are located in the cytoplasmic domains. Therefore energy coupling between ATP hydrolysis and Ca²⁺ transport seems to require long distance communication through conformational changes. Recent studies of mutational analysis have suggested that several kinds of residues play a key role in



structure proposed by MacLennan et al (4) and Andersen, J.P. (5). Mutated residues by site-directed mutagenesis are shown by colored circles. the coupling of ATP hydrolysis to Ca^{2+} transport across the SR membrane (9). There are number of investigations by using chemical reagents and mutagenetic methods in analyzing various aspects of structure function relationship in the Ca^{2+} -ATPase protein (see Ref. 10 for review)

Functional movements of the ATPase in the SR membrane

Employing conformational probes such as spin labels and fluorescent dyes, many investigators attempted to detect a conformational changes associated with the transport of Ca^{2+} across the SR membrane (see Ref. 3 for review). However, none of these studies provide solid evidence that the ATPase molecules undergo specific movements or conformational changes that are directly coupled with the translocation of Ca^{2+} across the membrane. One of the major problems in understanding the functional movements of the enzyme molecule is to determine whether the catalytic function is performed by the Ca^{2+} -ATPase as a structural unit of a monomer or an oligomer. The oligomericity of the Ca^{2+} -ATPase in the SR membrane has been suggested, following the experimental results from a variety of techniques such as analytical centrifugation on the detergent solubilized protein, electron microscopy, fluorescence energy transfer, fluorescence anisotropy measurements as laser flash induced photodichroism (see Ref. 11 for review). However, it has not been determined whether or not the molecular interaction of the Ca^{2+} -ATPase in the SR membrane plays an essential role in the Ca^{2+} transport.

Purposes of studies on the structure function relationship of scallop Ca²⁺-ATPase

For the SR isolated from poikirothermal animal muscle, temperature has been considered to be one of a crucial factors influencing its catalytic functions. Abe *et al.* (12) were successful in preparing SR membrane from scallop (*Patinopecten yessoensis*) adductor muscle with retention of full Ca²⁺-transport activity. They found that the isolated scallop SR has characteristics in the temperature sensitivity that pronouncedly differs from those of homoiothermal animals such as a rabbit. Both activities of ATP hydrolysis and Ca²⁺ transport by scallop SR were irreversibly lost above 37°C, while almost activities of rabbit SR retained even at a temperature as high as 50°C.

The first purpose of this work is to elucidate and characterize the reaction step in the catalytic cycle of scallop Ca²⁺-ATPase upon which thermal disruption drastically affects. We found that the basic aspects of ATP hydrolysis by the scallop SR are similar for that of rabbit SR and that when the scallop enzyme exist in E_1 state it can be easily inactivated by heat, whereas the enzyme was completely protected from the heat inactivation when the enzyme was fixed in E_2 state (13). However, no protection was observed for the Ca²⁺-transport across the membrane even when the enzyme was fixed in the E_2 state. This uncoupling between the Ca²⁺ transport and the ATP hydrolysis did not lead to a rise in the Ca²⁺ permeability of SR membrane (Part I).

The second purpose of this work is to characterize the primary structure which contribute to the stabilization of the rabbit Ca^{2+} -ATPase. For this purpose we have cloned cDNA of the Ca^{2+} -ATPase (SERCA1) from scallop adductor muscle, and predicted entire amino acid sequence of this enzyme (14). Based on detailed comparison of the amino acid sequence with that of rabbit Ca^{2+} -ATPases, sequence analysis by using fluorescent SH reagent, and site-directed mutagenesis, it is suggested that Cys^{675} may binds to Cys^{349} through disulfide bond on the rabbit Ca^{2+} -ATPase, which can contribute to the thermal stabilization of the enzyme, while that lacking in the disulfide bond in the scallop Ca^{2+} -ATPase may cause the thermal destabilization of the enzyme (Part II and Part III).

The third purpose of this work is to provide direct evidence suggesting that the catalytic function of Ca^{2+} transport is performed by the Ca^{2+} -ATPase in the oligomeric form. In this study, rabbit and scallop Ca^{2+} -ATPases at various protein ratios were cross-reconstituted into proteoliposomes. When proteoliposomes containing rabbit and scallop SR at a protein ratio of 1:1 were pre-incubated at 39°C for 10 min, the Ca-transport activity was almost completely lost (15). These results indicate that molecular interaction in the SR membrane is essential for Ca^{2+} -transport (part IV).

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Part I

Protection of Scallop Sarcoplasmic Reticulum ATPase from Thermal Inactivation by Removal of Calcium from High-Affinity Binding Sites on the Enzyme

SUMMARY

Sarcoplasmic reticulum (SR) vesicles were isolated from scallop muscle by the method of Abe *et al.* (J.Biochem.112,822-827,1992) and their thermolability was examined in the presence and absence of Ca^{2+} . When SR was preincubated at 38°C in the presence of 0.1 mM Ca^{2+} , Ca^{2+} -transport activity decreased as a function of time with a half-inhibition time of about 5 min. Activities of the Ca^{2+} -dependent ATPase, phosphoenzyme (EP) formation and E_2 to E_1 transition were decreased by the heat treatment in parallel with the Ca^{2+} -transport activity. In contrast, when SR was preincubated at 38°C in the presence of 2-5 mM EGTA, all of these activities, except for the Ca^{2+} -transport and the ATPase reaction did not lead to a rise in the Ca^{2+} permeability of SR membrane. Plots of the ATPase activity or steady-state level of EP against pCa in the thermal incubation medium revealed a typical sigmoidal curve with a half-inhibition concentration and Hill number of about 0.5 μ M and 1.80, respectively. These results suggest that 2 mol of Ca^{2+} must be removed from the high-affinity Ca^{2+} binding sites on the ATPase to stabilize the Ca^{2+} -ATPase against heat inactivation.

The protection from heat inactivation disappeared if SR was preincubated at 38°C after having been solubilized with a nonionic detergent, but returned when the detergent was removed to reconstitute the SR membrane. These results suggest that the protection of ATPase from thermal inactivation in EGTA may require a membrane structure in which the ATPase molecules exist in an appropriate arrangement.

INTRODUCTION

Ca²⁺-ATPase of sarcoplasmic reticulum (SR) couples the energy derived from ATP hydrolysis with the transport of Ca²⁺ across the SR membrane against a concentration gradient (1). In the presence of Ca²⁺, ATP is hydrolyzed into ADP and Pi through two kinds of phosphorylated intermediates (E₁P and E₂P), followed by a transition of the enzyme state from E₂ to E₁ (2). Two Ca²⁺ externally bound to highaffinity binding sites of the ATPase are occluded by the enzyme when E₁P is formed, and they are released into the SR lumen when E₁P is converted into E₂P.

Much work has been done to characterize the molecular movements of ATPase which could be associated with the Ca^{2+} -transport across the SR membrane by means of variety of methods, such as chemical cross linking (3-5), fluorometric studies (6,7), saturation transfer EPR methods (8-11), and electron microscopic observations of two-dimensional crystallization of the ATPase on SR membrane (12-14), but the movements remain poorly understood.

In the present study, I investigated the effects of thermal treatment on the catalytic functions of scallop SR under various conditions. In the preceding study, Abe *et al* (15) found that scallop SR is highly sensitive to heat; both Ca²⁺-transport and ATP hydrolysis activities are rapidly lost at temperatures higher than 37°C. In the present work, I found that susceptibility of ATP hydrolysis to heat could be prevented by removing 2 mol of Ca²⁺ from the high-affinity binding sites on the enzyme or by adding unhydrolyzable nucleotide. This protection disappeared when SR membrane was destroyed by a nonionic detergent, while it was restored by removing the detergent to reconstitute the membrane. These results raise the possibility that the thermal stability of Ca²⁺-ATPase might be related to the formation of an oligomeric ATPase in the scallop SR membrane.

EXPERIMENTAL PROCEDURES

Materials --- SR was isolated from the striated portion of scallop (*Patinopecten yessoensis*) adductor muscle according to a method described previously (15). The SR sample was divided into small pieces, quickly frozen in liquid nitrogen, and stored at --80°C. Pyruvate kinase, lactate dehydrogenase, NADH, PEP were purchased from Boehringer Mannheim. [γ -³²P]ATP was purchased from Amersham, ⁴⁵CaCl₂ was from Japan RI Association, and Antipyrylazo III was from Nacalai.

Heat treatment of SR --- A small portion of the SR suspension was added at 0.5-2 mg/ml to the preincubation medium, which contained 0.1 M KCl, 10% glycerol, 5 mM MgCl₂, and 20 mM TES (pH 7.2) usually at 38°C. At the indicated times, 0.1 ml of the SR suspension was transferred into 5-20 volumes of assay medium which had been kept cool on ice. In some experiments, the preincubation medium was frozen in liquid nitrogen to stop the thermal treatment.

Measurements of activities --- Ca²⁺-transport by SR was measured in a medium containing 0.025-0.05 mg/ml SR protein, 0.1 M KCl, 5 mM MgCl₂, 5-10% glycerol, 40 μ M CaCl₂, 0.2 mM Antipyrylazo III, and 20 mM TES at pH 7.2. The reaction was started at 23°C by the addition of 0.2-1 mM ATP. Ca²⁺ uptake into SR was determined by measuring the absorption at 700 nm in the reaction mixture (16).

The permeability of the SR membrane to Ca^{2+} was measured by essentially the same method as that described previously (16) except that ${}^{45}Ca^{2+}$ loading and Ca^{2+} efflux assay were both carried out at 0 and 25°C, and that ${}^{45}Ca^{2+}$ -loaded SR vesicles were incubated in the 5 mM EGTA medium for 5 s.

ATP hydrolysis was measured under similar conditions to those of Ca²⁺transport assay except that the Ca²⁺ indicator was omitted and 2-5 μ M A23187 was added to the reaction mixture. ATP hydrolysis was measured in the presence of an ATPregenerating system (0.5 mM NADH, and 1.5 mM PEP, 0.1-1 unit of lactate dehydrogenase and 0.2-2 unit of pyruvate kinase), and the amount of ADP liberated from ATP was determined by measuring the decrease in absorbance of NADH at 340 nm.

Phosphorylation of Ca²⁺-ATPase --- SR, 0.05-0.1 mg/ml, was phosphorylated with 10-100 μ M AT³²P at 0°C. At the indicated time, 5% TCA with 2 mM ATP and 0.5 mM phosphate were added to stop the reaction. The amount of EP was determined as

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described previously (17).

 E_2 - E_1 transition was measured as a fluorescence change of tryptophan in Ca²⁺-ATPase after addition of 2 mM CaCl₂ to the SR suspension containing 2 mM EGTA. For these measurements, a Shimadzu 1000 fluorometer was used.

Reconstitution of SR membrane --- SR (2.5 mg/ml) was solubilized with 30 mg/ml of $C_{12}E_9$. The suspension was centrifuged at 540,000 \times g for 20 min to remove insoluble debris. Soybean asolectin (25 mg/ml) was added to the supernatant and the suspension was incubated at 23°C for 1 h with 0.2 g/ml of Bio-beads SM-2 to remove the detergent. The Bio-beads were removed by filtration, and the filtrate was centrifuged at 540,000 \times g for 30 min to precipitate reconstituted SR vesicles.

RESULTS

Inactivation of Ca²⁺-transport and ATP hydrolysis by heat treatment of SR in the presence of Ca²⁺ --- 2 mg/ml SR was preincubated at 38°C for various time periods in 0.1 ml of reaction medium containing 0.1 mM CaCl₂, 10% glycerol, 0.1 M KCl, 20 mM TES (pH 7.2) and 5 mM MgCl₂, then rapidly transferred into liquid nitrogen to stop the heat treatment. Ca2+-transport assay was carried out at 23°C under the conditions described in "EXPERIMENTAL PROCEDURES". The Ca2+-transport showed a slow decrease in rate immediately after initiating the preincubation. This was followed by a rapid decrease, and the activity almost disappeared within 8 min (Fig. 1). The rate of inactivation increased as the preincubation temperature increased (data not shown). In the following experiments, scallop SR was mainly preincubated at 38°C, because the catalytic activities of SR decreased at a rate slow enough to allow accurate analysis of the inactivation process. The activity of Ca²⁺-dependent ATPase decreased essentially in parallel to that of Ca²⁺-transport. These complicated time courses of heat inactivation might suggest the existence of different kinds of thermally sensitive regions on the ATPase protein. The loss of ATPase activity may be attributed to the lack of EP formation but not to EP decomposition, because the steady-state level of EP decreased at a similar rate to that of the Ca^{2+} -ATPase (Fig. 1).

Figure 2 compares the Ca²⁺ concentration dependence of EP formation after preincubation of SR for 6 min at 38 and 23°C. In both cases, plots of the EP level against pCa revealed a typical sigmoidal curve with a Kd and Hill number of about 0.2 μ M and 1.8, respectively. Therefore, the inhibition of EP formation by heat treatment of SR does not seem to be due to a decrease in the affinity of this enzyme for Ca²⁺.

Protection of ATPase from thermal inactivation in the presence of EGTA --- The Ca²⁺-ATPase of scallop SR became very stable at high temperature if EGTA was added to the preincubation mixture. Under this condition, more than 90, 70, and 80% of the activities of ATP hydrolysis, EP formation and E_2 - E_1 transition, respectively, remained at 12 min after initiation of the thermal treatment (Fig. 3). Figure 4 shows the dependence of susceptibility to thermal inactivation of ATPase and E_2 - E_1 transition on the Ca²⁺ concentration of the preincubation medium. Scallop SR was preincubated at 38°C for 10 min at different concentrations of Ca²⁺ from pCa 9 to 5. Significant

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Fig. 1. Time courses of heat inactivation of Ca²⁺ -transport, ATP hydrolysis and EP formation. SR was added at 38°C to the preincubation medium containing 0.1 mM CaCl2 to a final concentration of 2 mg/ml. At the indicated times, 0.1 ml of the SR suspension was immediately frozen in liquid nitrogen to terminate the thermal treatment. Mesurements of Ca²⁺-transport (O), ATP hydrolysis(\Box), and EP formation(Δ) were carried out at 23°C as described in "EXPERIMENTAL PROCEDURES".



Fig. 2. Effect of heat treatment of SR on the Ca²⁺ concentration dependence of EP formation. SR was preincubated at $38^{\circ}C(\bigcirc)$ or $23^{\circ}C(\square)$ for 6 min in the presence of 0.1 mM CaCl2, then centrifuged to wash the SR membrane. Phosphorylation of ATPase was initiated at $23^{\circ}C$ by adding 1 mM [γ -32P]ATP in the presence of 0.1 mM CaCl2. 5 sec later, the reaction was terminated by addition of 5% TCA, and the amount of EP was determined as described in "EXPERIMENTAL PROCEDURES".



Fig. 3. Protection of ATP hydrolysis, EP formation, and E2-E1 transition against thermal treatment of SR in EGTA. SR was preincubated at 38°C in the presence of 5 mM EGTA (open symbols) or 0.1 mM CaCl2 (closed symbols) for the indicated times. These samples were washed by centrifugation, and resuspended in the assay medium. ATP hydrolysis(\bigcirc, \bigcirc), EP formation ($\triangle, \blacktriangle$), and E1-E2 transition (\square, \blacksquare) were measured as described in "EXPERIMENTAL PROCEDURES". All the activities were presented as a percentage of those obtained with the untreated SR.

enhancement of the thermal inactivation was observed by increasing the concentration of Ca²⁺ above pCa 6, while maximal activities of both ATP hydrolysis and E_2-E_1 transition were retained even after 12 min of thermal treatment below pCa 8. The plots of the remaining activities of ATP hydrolysis and E_2-E_1 transition against pCa of the preincubation medium revealed typical sigmoidal curves with a dissociation constant and Hill number of about 0.5 μ M and 1.6-1.8, respectively. Essentially the same sigmoidal curve of Ca²⁺ dependence was observed for EP formation (data not shown). These observations indicate that removal of 2 mol of Ca²⁺ from the high-affinity sites on the Ca²⁺-ATPase or fixing the enzyme in the E_2 state might be required to make this enzyme resistant to heat inactivation.

Uncoupling of Ca²⁺-transport from ATP hydrolysis after thermal treatment of SR in EGTA --- As shown in Fig. 5, the thermal treatment of SR in the presence of EGTA unexpectedly failed to protect the Ca²⁺-transport activity. These results support the possibility that the thermally sensitive region for the Ca²⁺-transport is different from that for ATP hydrolysis. Uncoupling of the Ca2+-transport from the ATPase reaction may lead to an increase in the Ca²⁺ permeability of the SR membrane. In order to test this possibility, I examined the leakiness of the SR membrane to Ca²⁺ as shown in Fig. 6. SR vesicles were preincubated in the presence of 2 mM EGTA at 38°C for the indicated times. The treated SR vesicles were passively loaded with ⁴⁵Ca²⁺ by incubation with 10 mM $^{45}Ca^{2+}$ for 5 h at 0°C, or for 2 h at 23°C. After the Ca²⁺ concentrations inside and outside the SR membrane had reached equilibrium, a small amount of SR suspension was transferred into a solution containing 5 mM EGTA and incubated for 5 s. The amount of ⁴⁵Ca²⁺ remaining in the SR vesicles was measured by the filtration method (16). Heat treatment of SR at 38°C for up to at least 15 min did not result in any rise in the Ca²⁺ permeability of the SR membrane. Therefore the lack of Ca^{2+} -transport in the SR is due to some facts other than Ca^{2+} leakiness of the membrane.

Lepock *et al.*(18,19) observed that incubation of rabbit SR at 37°C in the presence of EGTA resulted in a rapid loss of Ca²⁺-transport with no effect on ATPase activity. With rabbit SR, however, neither ATP hydrolysis nor Ca²⁺-transport was destroyed by incubation with Ca²⁺ below 49°C. These observations are in contrast with our data for scallop SR, indicating that the mechanism of thermal inactivation in scallop SR may differ from that in rabbit SR.



Fig. 4. Ca²⁺ concentration dependence of heat sensitivity of Ca²⁺ ATPase and E2-E1 transition. SR was preincubated at 38°C for 10 min in the presence of various concentrations of Ca²⁺. Ca²⁺ ATPase (\square) and E2-E1 transition (\bigcirc) activities were measured as described in "EXPERIMENTAL PROCEDURES".



Fig. 5. Uncoupling of ATP hydrolysis from Ca²⁺ transport after thermal treatment of SR in the presence of EGTA. SR was preincubated at 38°C in the presence of 2 mM EGTA for the indicated times. ATP hydrolysis (\bigcirc) and Ca²⁺ transport (\bigcirc) were measured as described in "EXPERIMENTAL PROCEDURES".



Fig. 6. Effects of heat treatment on the Ca²⁺ permeability of SR membrane. SR was treated at 38°C for the indicated times as presented in Fig. 5, then the SR vesicles were loaded with 10 mM $^{45}Ca^{2+}$ at 0°C for 5 h (\Box) or at 25°C for 2 h(O). The SR suspension was transferred into 20 volumes of assay medium containing 5 mM EGTA. After 5 s, the reaction medium was passed through a membrane filter, and the amount of Ca²⁺ retained on the filter was determined as described in "EXPERIMENTAL PROCEDURES".

Role of membrane structure in thermal stabilization of Ca²⁺-ATPase in EGTA --- To test whether or not EGTA protection of Ca²⁺-ATPase from heat inactivation requires some membrane structure of SR, I compared the thermal lability of the ATPase solubilized in $C_{12}E_9$ and the reconstituted membrane-bound ATPase (Fig. 7). These samples were preincubated at 38°C in the presence and absence of 2 mM EGTA for various time periods. As shown in Fig. 7a, the Ca²⁺ -dependent ATPase activity of the solubilized ATPase was completely lost during the preincubation even in the presence of EGTA. When solubilized ATPase was reconstituted into the proteoliposome, EGTA protection of the enzyme from thermal lability was fully restored (Fig. 7b).

Other factors affecting thermal inactivation of SR functions --- Figure 8 shows the effect of nucleotide on the thermal inactivation of SR function. Preincubation of SR at 38°C in the presence of 1 mM AMP-PNP induced rapid inactivation of Ca²⁺- transport with complete retention of ATP hydrolytic activity. The Ca²⁺-ATPase activity shows strong resistance to the heat treatment in spite of the presence of 0.1 mM Ca²⁺. Other nucleotides (ADP and AMP) up to 10 mM showed no effect on the SR functions (data not shown). I examined the effects of other reagents such as β -mercaptoethanol, dithiothreitol, vanadate, and polyclonal antibody against the Ca²⁺-ATPase purified from scallop SR, but they failed to offer protection against heat inactivation of SR catalytic functions.



Fig. 7. a: Inactivation of the solubilized ATPase by thermal treatment in the presence and absence of Ca²⁺. SR at 2 mg/ml was solubilized with 30 mg/ml C12E9, then preincubated in the presence of 0.1 mM CaCl2 (\bigcirc) or 2 mM EGTA (\bigcirc) at 38°C for the indicated times. The SR suspension was added to 20 volumes of assay medium. ATPase activity was measured at the final Ca²⁺ concentration of 0.1 mM as described in "EXPERIMENTAL PROCEDURES". b: Restoration of thermal resistance of the Ca²⁺ ATPase in EGTA on removal of detergent from solubilized ATPase. SR membrane was reconstituted as described in "EXPERIMENTAL PROCEDURES", and the reconstituted membrane was preincubated at 38°C in the presence of 0.1 mM CaCl2 (\bigcirc) or 5 mM EGTA (\bigcirc) for the indicated times. ATPase activity of the reconstituted SR was measured as described in (a).



Fig. 8. Effects of thermal treatment of SR in the presence of AMP-PNP on the ATP hydrolysis and Ca²⁺ transport. SR (5 mg/ml) was preincubated at 38°C in the presence of 0.1 mM CaCl₂, and 1 mM AMP-PNP for the indicated time, then washed by centrifugation to remove the nucleotide. ATP hydrolysis (O) and Ca²⁺ transport (\square) were measured as presented in Fig. 5.

DISCUSSION

SR membranes isolated from cold water fish, including scallop SR, have striking characteristics of thermolability (15,20,21). As shown in Fig. 1, most of the Ca²⁺-transport and ATPase activities of scallop SR were almost completely lost within 10 min of incubation at 38°C in the presence of Ca²⁺. Based on preliminary tests, it was considered that the thermal treatment of SR directly affects the Ca²⁺-ATPase in the SR membrane, because the thermal inactivation was still observed when Ca²⁺-ATPase was purified from scallop SR by HPLC in C₁₂E₉ and reconstituted into soybean liposome (data not shown).

The data in Figs. 3 and 4 suggest that removal of 2 mol of Ca^{2+} from the highaffinity sites on the Ca^{2+} -ATPase makes the enzyme thermally resistant. One of the simplest explanations is that the enzyme becomes heat stable if it exists in the SR membrane in the E_2 form. However, this is ruled out by the fact that the Ca^{2+} -ATPase of scallop SR was easily inactivated by heat treatment at low pH without KCl, where the enzyme is believed to exist in the E_2 state (22-25). In addition, the heat treatment of SR with 1 mM AMP-PNP, which stimulates the conversion of the enzyme from the E_2 to the E_1 state (26), completely protected Ca^{2+} -ATPase from thermal lability (Fig. 8).

As shown in Fig. 7a, when SR membrane was destroyed by $C_{12}E_9$, the Ca²⁺-ATPase became thermolabile in EGTA. On the other hand, the thermal resistance of the ATPase was fully restored when the detergent was removed to reconstitute the SR membrane (Fig. 7b). These results suggest that the thermal stability of Ca²⁺-ATPase requires not only removal of Ca²⁺ from the enzyme but also correct location in the SR membrane.

Based on these considerations, I propose the following mechanism for the thermal inactivation of Ca^{2+} -ATPase in scallop SR membrane. The susceptibility of Ca^{2+} -ATPase to heat inactivation may vary depending upon the arrangement of the enzyme molecules in the SR membrane. When the enzyme molecules exist in an oligomeric form, they will be heat resistant, while in a monomeric form, they are easily inactivated by heat treatment. Using fluorometric techniques (6,7), EPR spectorometric analysis (8-11), and electron microscopic observations of unstained SR membrane (12-14,27), many studies have suggested that arrangement of the ATPase molecules in the SR membrane can be varied by adjusting the concentrations of divalent cations and

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nucleotides. Stokes and Lacapere (27) demonstrated that simultaneous addition of vanadate and EGTA induced two-dimensional crystallization of ATPase on the surface membrane of rabbit SR, and that the crystal formation was prevented by Ca²⁺ at physiological concentrations. They observed that non-hydrolyzable nucleotides affected the crystal formation. These observations are consistent with our present results. Moreover, Yamasaki and co-worker (3,5) suggested that when the SR membrane is allowed to react with a cross linking reagent, N, N'-(1,4-phenylene)bismaleimide (PBM), in the presence of 1 mM AMP-PNP, the Ca²⁺-ATPase molecules predominantly form the dimer in the SR membrane. This corresponds well with the finding that the nucleotide prevented heat inactivation of the ATPase (Fig. 8).

Thus, I might be able to determine whether the Ca^{2+} -ATPase molecules exist in the scallop SR membrane in a monomeric form or an oligomeric form simply by examining the susceptibility of this enzyme to thermal inactivation.

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Part II

cDNA cloning and predicted primary structure of scallop sarcoplasmic reticulum Ca²⁺-ATPase
SUMMARY

Sarcoplasmic reticulum (SR) Ca^{2+} -ATPase of the scallop cross-striated adductor muscle was purified with deoxycholate and digested with lysyl endopeptidase for sequencing of the digested fragments. Overlapping cDNA clones of the ATPase were isolated by screening the cDNA library with a RT-PCR product, as a hybridization probe, which encodes the partial amino acid sequence of the ATPase. The predicted amino acid sequence of the ATPase contained all the partial sequences determined with the proteolytic fragments and consisted of the 993 residues with about 70% overall sequence similarity to those of the SR ATPases from rabbit fast-twitch and slow-twitch muscles. An outline of the structure of the scallop ATPase molecule is predicted to mainly consist of 10 transmembrane and 5 stalk domains with two large cytoplasmic regions as observed with the rabbit SR. The sequence relationship between scallop and other sarco/endoplasmic reticulum-type Ca^{2+} -ATPases is discussed.

INTRODUCTION

Intracellular calcium concentrations in eucaryotic cells are regulated by the plasma membrane and by intracellular organelles. In cardiac and skeletal muscle cells, the sarcoplasmic reticulum (SR) plays a primary role in regulating cytoplasmic calcium concentrations (1). The Ca²⁺-ATPase of the SR is an integral membrane protein that helps to relax the striated muscles contraction by pumping calcium from the cytoplasm into the SR by hydrolytic coupling with ATP. The transport reaction is performed with a cyclic change of E_1 (high affinity conformation of the enzyme for calcium) and E_2 (low affinity conformation of the enzyme for calcium) states of the ATPase, accompanied by the monomer-dimer transition of the ATPase molecules on the SR membrane (2). By using the SR of rabbit fast-twitch skeletal muscle, the mechanism of calcium transport in the SR has been extensively studied from physiological, biochemical, and molecular biological points of view (3, 4). Almost all such studies on the invertebrate SR have been carried out with the SR from cross-striated adductor muscle of adult scallop (5-11). Although the scallop SR shares many important properties with the rabbit SR (9), distinct differences between them have been found as follows: (i) Scallop ATPase activity was found to be much more labile to heat than that of rabbit ATPase (9). (ii) When these ATPase molecules were monomerized in the absence of the calcium ion, the scallop molecules became inactivated, while the rabbit counterparts maintained their activities (10). (iii) The dimer state of the scallop molecules was stable without vanadate or phosphate which is required for stabilization of the rabbit molecules in the dimer state (5). The relationship between stability of the activity and the organized state of the scallop ATPase is currently being investigated (10-12). For this purpose, it is necessary to know the primary structure of the scallop ATPase. Although the primary structures of several invertebrate SR Ca²⁺-ATPases have been deduced from the nucleotide sequences of their cDNAs (13-15), the structure of the scallop Ca²⁺-ATPase is not known. Here, we analyzed proteolytic fragments of the ATPase protein from the cross-striated muscle cells of adult scallop, isolated and sequenced its cDNA, and predicted its amino acid sequence. The relationship of amino acid sequences between scallop and other sarco/endoplasmic reticulum-type Ca2+-ATPases (SERCA) is discussed.

EXPERIMENTAL PROCEDURES

Sequencing of fragments of the ATPase protein --- The SR was prepared from the cross-striated adductor muscle of adult scallop (Patinopecten yessoensis) as reported previously (9). The Ca²⁺-ATPase was purified by washing the SR with sodium deoxycholate in a 2:5 ratio of sodium deoxycholate to the reticulum protein according to the methods previously used for purification of Ca²⁺-ATPase from the SR of rabbit fast-twitch skeletal muscle (16). The preparations of the SR and the purified ATPase were analyzed by SDS-PAGE by using gels containing 7.5 and 4% acrylamide as separating and stacking gels, respectively. The gel was stained with Coomassie blue. After delipidation of the purified ATPase preparation with chloroform and methanol (2: 1), the protein was reduced and carboxymethylated according to the method of Suzuki et al. (17), and then digested with lysyl endopeptidase (18). The digested products were first isolated by reverse-phase chromatography on a column of Cosmosil 5C₁₈-300 (4.6 \times 150 mm) with a linear gradient of 0-90% acetonitrile in 0.1% trifluoroacetic acid. Peptides for sequence analysis were purified further by re-chromatography on the same column with a linear gradient of the acetonitrile in 10 mM ammonium acetate (18). By the re-chromatography, 12 preparations of the purified peptide were obtained. The amino acid sequences of the peptides were determined by an automated protein sequencer (Applied BioSystems 476A).

Cloning and sequencing of cDNA --- Total RNA was extracted from the cross-striated adductor muscle of a one-year-old adult scallop by the method (19) of guanidium thiocyanate/CsCl ultracentrifugation using cesium TFA instead of CsCl. Poly(A)⁺ RNA was isolated from the total RNA by oligo(dT)-cellulose chromatography. cDNA with average length of 1.5-kb was synthesized from the isolated poly(A)⁺ RNA by using SuperscriptTM reverse transcriptase (GIBCO BRL) with random primers. A part of the cDNA was inserted into λ gt10 for construction of a cDNA library. The remaining cDNA was used for RT-PCR using a pair of degenerated primers, i.e., 5'-AA(A/G)AA(A/G)GAGTT(T/C)AC(A/T/C/G)CT(T/C/G)GA(A/G)TT-3' as an upstream primer and 5'-AT(A/T/C/G)GCCAT(A/G)TA(A/G)CG(A/G/T/C)AA(A/G) AA-3' as a downstream primer. These primers were designed based on the two conserved amino acid sequences of SERCA-type Ca²⁺-ATPases, SKEFTLEF and FFRYMAI, which are close to the ATP-binding region (19) and in one of the

transmembrane regions (19), respectively (see text for details). After 3 cycles of PCR (94°C for 1 min, 45°C for 1 min and then 72°C for 1 min), 27 cycles of PCR (94°C for 1 min, 55°C for 1 min and then 72°C for 1 min) were performed. The PCR products (about 1,000 bp) were subcloned into a phagemid vector pBluescript SK(+). The nucleotide sequences of the products were determined by the dideoxy chain-termination method using the Dye Terminator Cycle sequencing kit and an automated DNA sequencer (Applied Biosystems, model 373A18). One of the cDNA clones was found to include one of the partial amino acid sequences (see text for details) of the scallop ATPase protein. This was used as a probe for screening the cDNA library by the method of plaque hybridization. The hybridization was carried out under the standard protocol of hybridization (20), and then, the filter was washed with $0.5 \times SSC$ (0.15M NaCl, 0.015M sodium citrate) containing 0.1% SDS at 65°C. 25,000 independent plaques of the library were screened and 11 clones were obtained. By sequencing cDNA of the obtained 11 clones, the longest four clones were found to overlap with the screening probe which was mentioned above. The overlapping clones were sequenced with the automated DNA sequencer, described above. The plasmid pBluescript SK(+) was utilized for subcloning the different DNA fragments of the overlapping clones to be sequenced.

RESULTS AND DISCUSSION

In rabbit cross-striated muscle cells, two types of Ca²⁺-ATPase genes have been found to be expressed, i.e., they are fast-twitch form (SERCA1) gene and slow-twitchform (SERCA2) gene, respectively (19). The difference between the predicted amino acid sequences of these ATPases have been shown to be about 17% and be scattered over the whole sequences rather than concentrated in some region of the sequences (19). In order to obtain enough data to examine the homogeniety of the ATPase gene in crossstriated muscle cells of adult scallop, we thoroughly analyzed the amino acid sequence of the scallop Ca²⁺-ATPase (Fig. 1). The ATPase was purified with deoxycholate from the SR preparation in which the ATPase protein comprised about 40 % of the total SR proteins (Fig. 1A). The content of the ATPase protein in the purified ATPase preparation was estimated to be more than 90% by gel electrophoresis. From lysyl endopeptidase digests of the purified ATPase, 12 peptides, L1-L12, were isolated for sequencing (Fig. 1B). The determined amino acid sequences of the peptides are as follows: IRDE (L2), MFVF (L3), TCDY IRASLLVPGDIVEISV (L1). (L4), **MNFFNTGRSGLNLREQGTVCNHVIQ** (L5), GAPE (L6), NCRK (L7), ATAEAICRRIGVFGENESTEGMSFT (L8), SRLF (L9), AEIGIAMGSGTAVAK (L10), SASEMVLADDNFATIVSAVEEGRAIYNN (L11) and ISMPVILIDETLK (L12).

4 overlapping cDNA clones were obtained by screening 25,000 plaques of the cDNA library by using a RT-PCR product as a probe; the product ,which was used, included one (AEIGIAMGSGTAVAK) of the partial amino acid sequences which were mentioned above. The complete nucleotide sequence of these clones is shown in Fig. 2A. The sequence contains the longest open reading frame of 2,979 bp, which encodes a polypeptide of 993 amino acids with a calculated Mr of 109,581 and contains a 5'untranslated leader sequence of 172 nucleotides and a 3'-untranslated 732-nucleotide sequence. The putative initiator methionine was assigned, because the nucleotide methionine fit the Kozak's consensus sequence around the sequence (GCCA/GCCATGG) for translational initiation and a stop codon (TAA) was present at 5' codons upstream of the methionine. The conserved amino acid sequences (SKEFTLEF and FFRYMAI) of SERCA-type Ca²⁺-ATPases, which were used to design the RT-PCR primers, were in the sequences of 479-486 and 833-839, respectively. All of the partial amino acid sequences determined with the purified ATPase were found in

the deduced sequences of 139-154, 234-237, 365-389, 403-406, 451-475, 515-518, 612-615, 629-653, 672-675, 714-727, 728-755 and 972-984. That is, no sequence, which does not correspond to the deduced sequences, was obtained. This suggests that the cDNA encodes the scallop ATPase. In other word, one type of SERCA gene seems to be expressed in the scallop striated muscle cells. In some of vertebrates and invertebrates, SERCA genes have been found to undergo alternative splicing of their primary transcripts, i.e., In rabbit (21), human (22), rat (23) and bird (24), it is shown that the position of amino acid, 993, in their translated amino acid sequences is involved in such pre-mRNA processing. As mentioned above, the position of 993 is that of the last amino acid in scallop amino acid sequence. The scallop sequence size, 993, is smallest in a SERCA family of 20 species as ever known. In Crustacean *Artemia* (25), amino acid residue, 997, was recently shown to be in a position involving such processing. It is, therefore, interesting to know the splicing in the scallop gene.

A secondary structure predicted according to the methods of Kyte and Doolittle (26) and of Brandl *et al.* (19) shows an outline of scallop ATPase molecule which mainly consists of 10 transmembrane domains (M1-M10), 5 amphipathic α -helical regions (stalk regions) (S1-S5) continuous to the transmembrane domains, and 2 large cytoplasmic domains (Fig. 2B), as observed with the ATPase (19) from rabbit fast-twitch skeletal muscles. This supports the report of their similar three-dimensional structures determined from their electron micrographies (2, 5). Based on the similarity of the scallop sequence to that of the rabbit (19), Asp350 and Lys514, respectively, in one (amino acid residues 327-727) of the cytoplasmic domains seem to be a phosphorylation site and a fluorescein 5-isothiocyanate binding site which forms a part of the ATP-binding sites. Although the present results of the primary structure do not provide any direct information about the difference (5, 9, 10, 11) of the scallop ATPase from the rabbit ATPase with regard to the relationship between stability of the ATPase activity and the aggregation state of the ATPases.

The overall amino acid sequence of the scallop Ca^{2+} -ATPase exhibited about 70 (70.1-75.7)% similarity to those of the SERCA-type ATPases of *Schistosoma*, *Artemia*, *Drosophila*, rabbit, etc., which belong to Animalia, while it exhibited about 30 (28.8-34.3) and 50 (47.1-48.5)% similarity to those of yeasts, and *Trypanosoma* and *Plasmodium*, which belong to Fungi and Protista, respectively (Fig. 3). Sequence



Fig. 1. Isolation of lysyl endopeptidase peptides of scallop Ca²⁺-ATPase. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of the SR (a) and the purified ATPase (b). The protein band indicated by an arrow was shown in a previous paper (9) to have almost the same molecular weight as that (100kDa) of rabbit SR Ca²⁺-ATPase of the fast-twitch muscle. The protein was also shown to be Ca²⁺-ATPase protein based on the observations of calciumdependent phosphorylation of the protein with ATP, hydroxylamine-sensitivity of the phosphoprotein and time-dependent liberation of the phosphate incorporated into the protein (9). (B) First reverse-phase chromatography of the lysyl endopeptidase digest of scallop ATPase. Twelve peptides, L1-L12, were sequenced (see "Materials and Methods" for details).

Figure2-A -151-121 -91 CGAACACCAAGGAAAATCAAGCTAGAAATTGTATGTTTCTGGCTTGAATTTTCGTTTCCTTTCACCAATATTTCATTTTGGGA -61 -31 -1 TATATTTGGGGTCAAGGGTCCATTCACAGGATAATTTTACATGAAGTGAGGGTGATTTTTCGTGAAATTTATACTTAAACATTCGGTGCA 30 60 90 M E Y A H T K S C E E V L E Y F N V E T D G L S E E Q V K T 120 150 180 AATACAGAGAAATACGGTCCGAATGAACTACCGACAGAAGAGGGTAAACCACTATGGGAACTGATCCTAGAACAGTTCGACGATCTATTA N T E K Y G P N E L P <u>T E E G K</u> Ρ <u><u>w</u> Š1</u> <u>E L I L E Q F D D L L</u> L 240 210 GTGAAGATTCTGTTGCTAGCAGCTATAATCTCATTTGTATTAGCTTGGTTTGAGGAGAGTGAAGAACAAGTGACAGCCTTTGTAGAGCCA <u>V K I L L L A A I I S F V L A W F E E S E E Q V T A F Y E P</u> 300 330 360 TTTGTAATTCTGACGATTTTAATATGTAACGCCGTTGTAGGAGTATGGCAGGAAAAAAATGCAGAGGATGCTATTGAAGCGCTGAAAGAG FVILTILICNAVVGVWQERN<u>AEDAIE</u> M2 Α Е 390 420 450 TATGAACCAGAAATCGCAAAAGTGGTGCGTAAGGGACAAAGAGGAGTACAGAAGATCCGCGCCAGTTTGTTGGTCCCTGGAGACATTGTC <u>Y E P E I A K V V R</u> K G Q R G V Q K [<u>I</u> R G D v А 480 510 540 <u>GAGATCTCCGT</u>TGGAGACAAAATTCCCAGCAGATATTCGTATCCTCCACAATTTATTCCACAACACTTAGGATAGACCAGTCCATTCTTACA <u>SV</u>GDKIPADIRILHIYSTTLRIDQSIL т 570 600 630 GGAGAGAGTGTGAGCGTGATCAAACACACCGATCCCATCCCCGACCCTCGGGCTGTCAACCAGGACAAGAAAAACATCCTCTTGGA G E S V S V I K H T D P I P D P R A V N Q D K K N I L F S G 660 690 720 ACAAACATCTCAGCAGGAAAAATGCAAGGGAATCGCTTTCGGAACCGGCCTGAATACTGCTATTGGCAAGATCCGAGACGAGATGATGGAG TNISAGKCKGIAFGTGLNTAIGK<mark>IRDE</mark>MM Ε 750 780 810 ACAGAGACCGAGAAGACACCTCTTCAACAGAAACTGGACGAGTTCGGAACTCAACTTTCTAAGGTTATCACTATCATCTGTATCTGCGTA <u>E K T P L Q Q K L D E F G T Q L S K V I T I I C I C V</u> M3 т 840 870 900 TGGGCCATCAACATCGGTCATTTCAACGATCCCGCCCACGGAGGATCATGGATGAAGGGCGCCATCTACTACTTCAAGATTGCTGTGGCT WAINIGHFNDPAHGGSWMKGAIYYFKIAVA M4 930 960 990 CTGGCTGTGGCTGCCATTCCTGAGGGTCTGCCTGCTGTCATCACAACCTGTCTGGCTCTCGGTACCAGAAGGATGGCTAAGAAGAATGCC L <u>LAVAAI</u>PEGL<u>PAVITTC</u> <u>L G T R R M A K K N A</u> A 1050 1020 1080 ATTGTCAGGAGTCTGCCGTCCGTTGAGACCCTTGGCTGTACCTCAGTCATCTGCTCTGATAAGACTGGCACACTGACCACCAGATG I V R S L P S V E T L G C T S V I C S D K T G T L T T N O M 1110 1140 1170 TCTGTTTGCAAGATGTTTGTGTTTTAACAAAGTTGAGGGTGCTGACATTCAAACCCAGCAGTTCGAGATCACCGGCTCTACTTATGCCCCA C K K F V F N K V E G A D I Q T Q Q F E I T G S T Y A P 1200 1230 1260 GAGGGAGATGTTTACTTGGGTGGTAAGAAGGTGAAG<u>ACATGCGACTA</u>CGATGGTCTAGAGGAGATGGCCACCATCTGTGCGATGTGCAAT E G D V Y L G G K K V K <u>T C D Y</u> D G L E E M A T I C A M C N 1290 1320 1350 GACTCCAGCGTAGATTACAATGATACCAAGGGAGTGTATGAGAAGGTTGGTGAGGGCCACAGAGACTGCCCTGACTGTTCTGTGTGAGAAG D S S V D Y N D T K G V Y E K V G E A T E T A L T V L C E K 1380 1410 1440 <u>GLNLREQG</u>T N V <u>CNHVIQ</u>QMW<u>S</u>K R S 1470 1500 1530 1560 1590 1620 MFAKGA P <u>E</u>GLLDRCTHVRVGKDKVPMSPA 1650 1680 1710 AAGAACGAAATCTTGAAATACACCAAGGCCTATGGAACTGGACGTGATACGCTGCGTTGTCTTGCCCTGGCCACCATTGATGCTCCTCCA K N E I L K Y T K A Y G T G R D T L R C L A L A T I D A P P

Figure2-A (Continued)

.

CGCCGCGAGGACATGGACCTGG			
RREDMDLE	D S R K F I Q	YETNMTFV	GGAGTCGTAGGAATGTTGGAC G V V G M L D
	1830	1860	1890
P P R M E V F D	SIK <u>NCRK</u>	GCTGGAATCCGTGTCATTGTCATC A G I R V I V I	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
	1920	1950	1980
GCTGAGGCTATCTGTCGTAGGA A E A I C R R I	TCGGTGTGTGTCGGCGAAAACGAG GVFGENE	TCTACAGAAGGCATGTCCTTCACT	GGCCGCGAGTTTGATGACCTG G R E F D D L
	2010	2040	. 2070
TCACATGAAGAGCAGCGATTGG S H E E O R L A	CGGTAACAAAGAGTCGTCTGTTT V T K S R L F	CCCCGTGTAGAGCCTGCTCACAAG A R V E P A H K	AGTAAGATCGTAGAGTACCTG S K I V E Y L
-	2100	2120	2160
CAAGGAGAAGGAGAAATCTCGG	CCATGACAGGAGATGGAGTAAAC	GACGCCCCTGCTCTGAAGAAAGCC	GAAATTGGTATCGCCATGGGA
QGEGEISA	MTGDGVN	DAPALKKA	EIGIAMG
	2190	2220	2250
TCTGGAACTGCTGTGGCCAAGT	CTGCCTCTGAGATGGTGTTGGCI	CATGACAACTTCGCAACAATTGTG D D N F A T I V	TCCGCTGTGGAAGAGGGCCGC S A V E E G R
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(2037፩ምር/ዋል(ንልልር ነልልር ነልጣር ነልል ልር	2280 מסשירימיינימיינימיינימיינימיינימיינימיינימ	2310 TCCAACATTGGAGAGGGTCGTATGT	2340 ATCTTCTTGACTGCTGCTCTT
A I Y N N M K Q	FIRYLIS	S N I G E V V C	IFLTAAL
<u></u>	2370	2400 M	5 2430
GGCATCCCTGAGGCTCTCATCC	CCGTACAGCTTCTGTGGGTGAAC	TTGGTAACTGATGGTCTTCCAGCC	ACTGCCCTCGGCTTCAACCCC
GIPEALIP	VQLLWVN	LVTDGLPA	TALGFNP
	2460	2490	2520
CCAGATATGGACATCATGAAGA	AACCTCCCAGGAATGCAAAGGA	AGGTCTCATCACCGGCTGGTTGTTC G I. I T G W I. F	TTCAGATATATGGCTATTGGA
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ႺႺႶͲႦႶႺႥͲႺႺႦႥႺͲႺႶႺႦႶͲႺ	2550 mmccmccmccccccccccccccc	2580 IATGGTCTATGACAAAGGGCCCCAA	2610 CTCAACTACTACCAGCTGACC
G Y V G C A T V	GAAAW W F	MVYDKGPQ	LNYYQLT
M /			
	2640	2670	2700
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q	2640 CAAGATGAGCGTTTCCTCGGTGTC DERFLGV	2670 CGACTGCAAGGTTTTTGACCACCCT D C K V F D H P	2700 GCACCTATGACAATGGCTCTG A.P.M.T.M.A.L
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q	2640 CAAGATGAGCGTTTCCTCGGTGTC DERFLGV	2670 CGACTGCAAGGTTTTTGACCACCCT D C K V F D H <u>P</u> 2760	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA	2640 CAAGATGAGCGTTTCCTCGGTGTC DERFLGV 2730 ATGCTTAACGCTCTCAACAGCTTC	2670 CGACTGCAAGGTTTTTGACCACCCT D C K V F D H <u>P</u> 2760 STCTGAGAACCAGTCCCTGCTGGTG	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 GATGCCCCCTTGGTGTAACAAG
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCGTCATTGAAA S V L V V I E M	2640 CAAGATGAGCGTTTCCTCGGTGTC DERFLGV 2730 ATGCTTAACGCTCTCAACAGCTTC LLNALNSL	2670 CGACTGCAAGGTTTTTGACCACCCT D C K V F D H <u>P</u> 2760 FTCTGAGAACCAGTCCCTGCTGGTG S E N Q S L L V	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 GATGCCCCCTTGGTGTAACAAG M P P W C N K
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCGTCATTGAAA S V L V V I E M M8	2640 CAAGATGAGCGTTTCCTCGGTGTC D E R F L G V 2730 ATGCTTAACGCTCTCAACAGCTTC L N A L N S L 2820	2670 CGACTGCAAGGTTTTTGACCACCCT D C K V F D H <u>P</u> 2760 STCTGAGAACCAGTCCCTGCTGGTG S E N Q S L L V 2850	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 ATGCCCCCTTGGTGTAACAAG M P P W C N K 2880
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC	2640 AAGATGAGCGTTTCCTCGGTGTC D E R F L G V 2730 ATGCTTAACGCTCTCAACAGCTTC L N A L N S L 2820 CTGTCTATGGGACTCCACTTTTGC S M C L H F C	2670 CGACTGCAAGGTTTTTGACCACCCT D C K V F D H <u>P</u> 2760 STCTGAGAACCAGTCCCTGCTGGTG S E N Q S L L V 2850 CATCCTCTACATTGACGTCATGTCCC L L V L D V M S	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 ATGCCCCCTTGGTGTAACAAG M P P W C N K 2880 ACCATCTTCCAAATCACGCCT
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A J M9	2640 CAAGATGAGCGTTTCCTCGGTGTC D E R F L G V 2730 ATGCTTAACGCTCTCAACAGCTTC L N A L N S L 2820 CTGTCTATGGGACTCCACTTTTGC S M G L H F C	$\begin{array}{c} 2670\\ \hline \\ \textbf{CGACTGCAAGGTTTTTGACCACCCT}\\ \textbf{D} \textbf{C} \textbf{K} \textbf{V} \textbf{F} \textbf{D} \textbf{H} \underline{P} \\ 2760\\ \hline \\ \textbf{STCTGAGAACCAGTCCCTGCTGGTG}\\ \textbf{S} \textbf{E} \textbf{N} \textbf{Q} \textbf{S} \textbf{L} \textbf{L} \textbf{V} \\ 2850\\ \hline \\ \textbf{CATCCTCTACATTGACGTCATGTCG}\\ \hline \underline{\textbf{I}} \underline{\textbf{L}} \underline{\textbf{Y}} \textbf{I} \textbf{D} \textbf{V} \textbf{M} \textbf{S} \end{array}$	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 GATGCCCCCTTGGTGTAACAAG M P P W C N K 2880 GACCATCTTCCAAATCACGCCT T I F Q I T P
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A I M9	2640 CAAGATGAGCGTTTCCTCGGTGTC D E R F L G V 2730 MTGCTTAACGCTCTCAACAGCTTC L N A L N S L 2820 CTGTCTATGGGACTCCACTTTTGC S M G L H F C 2910 CCCGTACTCAAGATCTCAATGCC	2670 CGACTGCAAGGTTTTTGACCACCCT D C K V F D H <u>P.</u> 2760 STCTGAGAACCAGTCCCTGCTGGTG S E N Q S L L V 2850 CATCCTCTACATTGACGTCATGTCG <u>I L Y</u> I D V M S 2940 FGTGATACTTATAGACGAAACATTG	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 ATGCCCCCTTGGTGTAACAAG M P P W C N K 2880 ACCATCTTCCAAATCACGCCT T I F Q I T P 2970 BAGTTCTGTGCTCGCAAATTC
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A J M9 CTTGGATTTGAAGAATGGTTCG L G F E E W F A	2640 CAAGATGAGCGTTTCCTCGGTGTC D E R F L G V 2730 ATGCTTAACGCTCTCAACAGCTTC L N A L N S L 2820 CTGTCTATGGGACTCCACTTTGC S M G L H F C. 2910 CCGTACTCAAGATCTCAATGCC V L K <u>I S M P</u>	$\begin{array}{c} 2670\\ \hline \\ \textbf{CGACTGCAAGGTTTTTGACCACCCT}\\ \textbf{D} \ \textbf{C} \ \textbf{K} \ \textbf{V} \ \textbf{F} \ \textbf{D} \ \textbf{H} \ \underline{P} \\ \hline \\ 2760\\ \hline \\ \textbf{STCTGAGAACCAGTCCCTGCTGGTG}\\ \textbf{S} \ \textbf{E} \ \textbf{N} \ \textbf{Q} \ \textbf{S} \ \textbf{L} \ \textbf{L} \ \textbf{V} \\ \hline \\ \begin{array}{c} 2850\\ \hline \\ \textbf{CATCCTCTACATTGACGTCATGTCG}\\ \hline \\ \textbf{I} \ \textbf{L} \ \textbf{Y} \ \textbf{I} \ \textbf{D} \ \textbf{V} \ \textbf{M} \ \textbf{S} \\ \hline \\ \begin{array}{c} 2940\\ \hline \\ \textbf{FGTGATACTTATAGACGAAACATTG}\\ \hline \\ \textbf{V} \ \textbf{I} \ \textbf{L} \ \textbf{I} \ \textbf{D} \ \textbf{E} \ \textbf{T} \ \textbf{L} \end{array}$	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 GATGCCCCCTTGGTGTAACAAG M P P W C N K 2880 GACCATCTTCCAAATCACGCCT T I F Q I T P 2970 GAAGTTCTGTGCTCGCAAATTC K F C A R K F
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A J M9 CTTGGATTTGAAGAATGGTTCC L G F E E W F A M10	2640 $AAGATGAGCGTTTCCTCGGTGTCOCGGTGTCOCGGTGTCAACGCTTCAACGCTCTCAACAGCTTCAACGCTTCAACAGCTTCOCGCTCTCAACAGCTTCOCGCTACTGGGACTCCAACTTTGGGACTCCAACGCCCCACTTTGGGACTCCAACGCCCCACTCCAACGCCCCACTCCAACGCCCCACTCCAACGCCCCACTCCAACGCCCCACTCCAACGCCCCCACTCCAACGCCCCCACTCCAACGCCCCCACTCCAACGCCCCCACTCCAACGCCCCCACTCCAACGCCCCCACTCCAACGCCCCCACTCCAACGCCCCCACTCCAACGCCCCCCCC$	$\begin{array}{c} 2670\\ \hline \\ \textbf{CGACTGCAAGGTTTTTGACCACCCT}\\ \textbf{D} \textbf{C} \textbf{K} \textbf{V} \textbf{F} \textbf{D} \textbf{H} \underline{P}_{\dots}\\ 2760\\ \hline \\ \textbf{STCTGAGAACCAGTCCCTGCTGGTG}\\ \textbf{S} \textbf{E} \textbf{N} \textbf{Q} \textbf{S} \textbf{L} \textbf{L} \textbf{V}\\ \hline \\ 2850\\ \hline \\ \textbf{CATCCTCTACATTGACGTCATGTCG}\\ \hline \underline{\textbf{I}} \underline{\textbf{L}} \underline{\textbf{Y}} \textbf{I} \textbf{D} \textbf{V} \textbf{M} \textbf{S}\\ \hline \\ \begin{array}{c} 2940\\ \hline \\ \textbf{FGTGATACTTATAGACGAAACATTG}\\ \hline \hline \textbf{V} \textbf{I} \textbf{L} \textbf{D} \textbf{E} \textbf{T} \textbf{L}\\ \end{array}$	$\begin{array}{c} 2700\\ \hline \\ \mathbf{GCACCTATGACAATGGCTCTG}\\ \underline{A} & \underline{P} & \underline{M} & \underline{T} & \underline{M} & \underline{A} & \underline{L} \\ \hline \\ & & & & & & & & & & \\ & & & & & &$
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A I M9 CTTGGATTTGAAGAATGGTTCG L G F E E W F A M10 ACTGACGCGTAAATATTTCGAQ	$\begin{array}{c} 2640\\ \hline \\ \textbf{AAGATGAGCGTTTCCTCGGTGTC}\\ \textbf{D} & \textbf{E} & \textbf{F} & \textbf{L} & \textbf{G} & \textbf{V}\\ \hline \\ 2730\\ \hline \\ \textbf{ATGCTTAACGCTCTCAACAGCTTC}\\ \hline \\ \textbf{L} & \textbf{N} & \textbf{A} & \textbf{L} & \textbf{N} & \textbf{S} & \textbf{L}\\ \hline \\ 2820\\ \hline \\ \textbf{CTGTCTATGGGACTCCACTTTTGC}\\ \hline \\ \textbf{C} & \textbf{S} & \textbf{M} & \textbf{G} & \textbf{L} & \textbf{H} & \textbf{F} & \textbf{C}.\\ \hline \\ 2910\\ \hline \\ \textbf{SCCGTACTCAAGATCTCAATGCCT}\\ \hline \\ \textbf{V} & \textbf{L} & \textbf{K} & \boxed{\textbf{I} & \textbf{S} & \textbf{M} & \textbf{P}}\\ \hline \\ 3000\\ \hline \\ CACATTATACAAGACACAGACAAGACAACA$	$\begin{array}{c} 2670\\ \hline \\ \textbf{CGACTGCAAGGTTTTTGACCACCCT}\\ \textbf{D} \ \textbf{C} \ \textbf{K} \ \textbf{V} \ \textbf{F} \ \textbf{D} \ \textbf{H} \ \underline{P}_{\dots}\\ 2760\\ \hline \\ \textbf{STCTGAGAACCAGTCCCTGCTGGTG}\\ \textbf{S} \ \textbf{E} \ \textbf{N} \ \textbf{Q} \ \textbf{S} \ \textbf{L} \ \textbf{L} \ \textbf{V}\\ \hline \\ \textbf{2850}\\ \hline \\ \textbf{CATCCTCTACATTGACGTCATGTCG}\\ \hline \underline{\textbf{I}} \ \underline{\textbf{L}} \ \underline{\textbf{Y}} \ \textbf{I} \ \textbf{D} \ \textbf{V} \ \textbf{M} \ \textbf{S}\\ \hline \\ \textbf{2940}\\ \hline \\ \textbf{FGTGATACTTATAGACGAAACATTG}\\ \hline \\ \hline \textbf{V} \ \textbf{L} \ \textbf{L} \ \textbf{D} \ \textbf{E} \ \textbf{T} \ \textbf{L}\\ \hline \\ \textbf{3030}\\ \hline \\ GGACAAGTTGTGAGGTCCAGTGAAGCACAGTGAAGCACAGTGAAGCAAGAACATTGAGGTCAGTGAAGCAAGTGAAGCAAGTGAAGCAAGTGAAGCAAGTGAAGCAAGTGAAGCAAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAAGCAAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAAGGTCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAAGGTCAGTGAAGCAAGTGAAGGTCAAGTGAAGGTCCAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGGTCCAGTGAAGCAAGTGAAGTGAAGGTCCAGTGAAGCAAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAAGACAAGTGAAGGTCCAGTGAAGGTCCAGTGAAGGTCCAGTGAAGCAAGTGAAGTGAGGTCCAGTGAAGCAAGTGAAGAACAATGACAAGTGAAGTGAAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAGAACAATGACAAGTGAGGTCCAGTGAAGGTCAGTGAAGGTCAGTGAAGGTCAGTGAAGTGTGAGGTCCAGTGAAGCAAGTGTGAGGTCCAGTGAAGTGAGGTCCAGTGAAGCAAGTGAGGTCAGTGAAGGTCAGTGAGGTCAGTGAAGGTCAGTGAAGTGAGGTCAGTGAAGGTCAGTGAAGTGTGAGGTCCAGTGAAGGTCAGTGAGGTCAGTGAAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGAGTGAGAGTGAGGTCAGTGAGAGTGAGGTCAGTGAGAGTGAGAGTGAGAGTGAGGTCAGTGAGGTCAGTGAGAGTGAGGTCAGTGAGTG$	$\begin{array}{c} 2700\\ \hline \\ \mathbf{GCACCTATGACAATGGCTCTG}\\ \underline{A} & \underline{P} & \underline{M} & \underline{T} & \underline{M} & \underline{A} & \underline{L} \\ \hline \\ 2790\\ \hline \\ \mathbf{ATGCCCCCTTGGTGTAACAAG}\\ \underline{M} & \underline{P} & \underline{P} & \underline{W} & \underline{C} & \underline{N} & \underline{K} \\ \hline \\ \mathbf{ATGCCCCCTTGGTGTAACAAG}\\ \underline{M} & \underline{P} & \underline{W} & \underline{C} & \underline{N} & \underline{K} \\ \hline \\ \mathbf{ACCATCTTCCAAATCACGCCT}\\ \underline{T} & \underline{I} & \underline{F} & \underline{Q} & \underline{I} & \underline{T} & \underline{P} \\ \hline \\ \underline{AAGGTCTGTGCTCGCAAATC}\\ \underline{K} & \underline{F} & \underline{C} & \underline{A} & \underline{R} & \underline{K} & \underline{F} \\ \hline \\ \mathbf{SAAGGCAGCCATTTCGTACAAC \\ \hline \end{array}$
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A I M9 CTTGGATTTGAAGAATGGTTCC L G F E E W F A M10 ACTGACGCGTAAATATTTCGAC T D A *	$\begin{array}{c} 2640\\ \textbf{AAGATGAGCGTTTCCTCGGTGTC}\\ \textbf{D} & \textbf{E} & \textbf{R} & \textbf{F} & \textbf{L} & \textbf{G} & \textbf{V}\\ 2730\\ \textbf{MTGCTTAACGCTCTCAACAGCTTC}\\ \textbf{L} & \textbf{N} & \textbf{A} & \textbf{L} & \textbf{N} & \textbf{S} & \textbf{L}\\ 2820\\ \textbf{TGTCTATGGGACTCCACTTTTGC}\\ \textbf{S} & \textbf{M} & \textbf{G} & \textbf{L} & \textbf{H} & \textbf{F} & \textbf{C}\\ 2910\\ \textbf{CCCGTACTCAAGATCTCAATGCC'}\\ \textbf{V} & \textbf{L} & \textbf{K} & \textbf{I} & \textbf{S} & \textbf{M} \\ \textbf{G} & \textbf{L} & \textbf{M} & \textbf{F} \\ 12\\ 3000\\ CACATTATACAAGACACAGACAAACA$	$\begin{array}{c} 2670\\ \text{CGACTGCAAGGTTTTTGACCACCCT}\\ \text{D} \ \text{C} \ \text{K} \ \text{V} \ \text{F} \ \text{D} \ \text{H} \ \underline{P}_{\dots}\\ 2760\\ \text{STCTGAGAACCAGTCCCTGCTGGTGGTG\\ \text{S} \ \text{E} \ \text{N} \ \text{Q} \ \text{S} \ \text{L} \ \text{L} \ \text{V}\\ 2850\\ \text{CATCCTCTACATTGACGTCATGTCG}\\ \underline{I}_{\dots} \ \underline{L}_{\dots} \ \underline{Y}_{\dots} \ \text{I} \ \text{D} \ \text{V} \ \text{M} \ \text{S}\\ 2940\\ \underline{rgtGATACTTATAGACGAAACATTG}\\ \underline{V \ \text{I} \ \text{L} \ \text{I} \ \text{D} \ \text{E} \ \text{T} \ \text{L}\\ 3030\\ SGACAAGTTGTGAGGTCCAGTGAAGCAAGTGAAGCAAGTGAAGCAAACATGAGAAACATGAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGCAAGTGTGAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGTGAGGTCCAGTGAAGTGAGGTCCAGTGAAGTGAGGTCCAGTGAAGTGAGGTCCAGTGAAGCAAGTGAGGTCCAGTGAAGTGAGGTCCAGTGAGGTCCAGTGAGGTCAGTGAGGTCCAGTGAGGTCAGTGAGGTCCAGTGAGGTCCAGTGAGGTCCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCCAGTGAGGTCAGTGAGGTCCAGTGAGGTCAGTGAGGTCCAGTGAGGTCAGTGAGGTCCAGTGAGGTCAGTGAGGTCCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCCAGTGAGAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTGAGGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTGAGGTGAGGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTGAGGTGAGGTCAGTGAGGTGAGGTCAGTGAGGTGAGGTCAGTGAGGTCAGTGAGGTCAGTGAGGTGAGGTGAGGTCAGGTGAGGTGAGGTCAGGTGAGGTCAGGTGAGGTGAGGTGAGGTGGGTCAGGTGGAGGTGGAGGTGGAGGTGGAGGTGGAGGTGGGGGG$	$\begin{array}{c} 2700\\ \hline \\ \mathbf{GCACCTATGACAATGGCTCTG}\\ \underline{A} & \underline{P} & \underline{M} & \underline{T} & \underline{M} & \underline{A} & \underline{L} \\ \hline \\ 2790\\ \hline \\ \mathbf{ATGCCCCCCTTGGTGTAACAAG}\\ \underline{M} & \underline{P} & \underline{W} & \underline{C} & \underline{N} & \underline{K} \\ \hline \\ \mathbf{ATGCCATCTTCCAAATCACGCCT}\\ \underline{T} & \underline{I} & \underline{F} & \underline{Q} & \underline{I} & \underline{T} & \underline{P} \\ \hline \\ \mathbf{ACCATCTTGTGCTCGCAAATTC}\\ \hline \\ \underline{C} & \underline{A} & \underline{C} & \underline{K} & \underline{F} \\ \hline \\ \hline \\ \mathbf{AAGGTCTGTGCTCGTACAAC} \\ \hline \end{array}$
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A J M9 CTTGGATTTGAAGAATGGTTCG L G F E E W F A M10 ACTGACGCGTAAATATTTCGAC T D A *	$\begin{array}{c} 2640\\ \hline \\ \textbf{AAGATGAGCGTTTCCTCGGTGTC}\\ \textbf{P} \textbf{D} \textbf{E} \textbf{R} \textbf{F} \textbf{L} \textbf{G} \textbf{V}\\ \hline \\ 2730\\ \hline \\ \textbf{TGCTTAACGCTCTCAACAGCTTC}\\ \hline \textbf{L} \textbf{N} \textbf{A} \textbf{L} \textbf{N} \textbf{S} \textbf{L}\\ \hline \\ 2820\\ \hline \\ \textbf{CTGTCTATGGGACTCCACTTTTGC}\\ \hline \\ \textbf{S} \textbf{M} \textbf{G} \textbf{L} \textbf{H} \textbf{F} \textbf{C}.\\ \hline \\ 2910\\ \hline \\ \textbf{CCGTACTCAAGATCTCAATGCCT}\\ \hline \\ \textbf{V} \textbf{L} \textbf{K} \textbf{\overline{I} \textbf{S} \textbf{M} \textbf{P}\\ \hline \\ \\ 3000\\ \hline \\ CACATTATACAAGACACAGACAAACAACAACAACAACAACAACAAACAAAAAA$	$\begin{array}{c} 2670\\ \hline \\ \textbf{CGACTGCAAGGTTTTTGACCACCCT}\\ \textbf{D} \textbf{C} \textbf{K} \textbf{V} \textbf{F} \textbf{D} \textbf{H} \underline{P} \\ 2760\\ \hline \\ \textbf{STCTGAGAACCAGTCCCTGCTGGTG}\\ \textbf{S} \textbf{E} \textbf{N} \textbf{Q} \textbf{S} \textbf{L} \textbf{L} \textbf{V} \\ \hline \\ 2850\\ \hline \\ \textbf{CATCCTCTACATTGACGTCATGTCG}\\ \hline \\ \textbf{L} \textbf{L} \textbf{Y} \textbf{I} \textbf{D} \textbf{V} \textbf{M} \textbf{S} \\ \hline \\ \begin{array}{c} 2940\\ \hline \\ \textbf{FGTGATACTTATAGACGAAACATTG}\\ \hline \\ \hline \textbf{V} \textbf{I} \textbf{L} \textbf{D} \textbf{E} \textbf{T} \textbf{L} \\ \hline \\ 3030\\ \hline \\ \textbf{SGACAAGTTGTGAGGTCCAGTGAAGC} \\ \hline \\ 3120 \\ \end{array}$	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 GATGCCCCCTTGGTGTAACAAG M P P W C N K 2880 GACCATCTTCCAAATCACGCCT T I F Q I T P 2970 GAAGTTCTGTGCTCGCAAATTC K F C A R K F 3060 GAAGGCAGCCATTTCGTACAAC
CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A I M9 CTTGGATTTGAAGAATGGTTCG L G F E E W F A M10 ACTGACGCGTAAATATTTCGAC T D A *	$\begin{array}{c} 2640\\ \hline \\ \textbf{AAGATGAGCGTTTCCTCGGTGTC}\\ \textbf{D} & \textbf{E} & \textbf{F} & \textbf{L} & \textbf{G} & \textbf{V}\\ \hline \\ 2730\\ \hline \\ \textbf{ATGCTTAACGCTCTCAACAGCTTC}\\ \hline \\ \textbf{L} & \textbf{N} & \textbf{A} & \textbf{L} & \textbf{N} & \textbf{S} & \textbf{L}\\ \hline \\ 2820\\ \hline \\ \textbf{CTGTCTATGGGACTCCACTTTTGC}\\ \hline \\ \textbf{C} & \textbf{C} & \textbf{C} & \textbf{M} & \textbf{G} & \textbf{L} & \textbf{H} & \textbf{F} & \textbf{C}.\\ \hline \\ 2910\\ \hline \\ \textbf{CCGTACTCAAGATCTCAATGCCT}\\ \hline \\ \textbf{V} & \textbf{L} & \textbf{K} & \textbf{I} & \textbf{S} & \textbf{M} & \textbf{P}\\ \hline \\ 3000\\ \hline \\ CACATTATACAAGACACAGACAACAACAACAACAACAACAACAACAAC$	$\begin{array}{c} 2670\\ \hline \\ \hline$	$\begin{array}{c} 2700\\ \hline \\ \mathbf{GCACCTATGACAATGGCTCTG}\\ \underline{A} & \underline{P} & \underline{M} & \underline{T} & \underline{M} & \underline{A} & \underline{L} \\ \hline \\ & & & & & & & & & & & & & & \\ & & & & & & & & & & & & & \\ \hline \\ \mathbf{ATGCCCCCCTTGGTGTGTGTAACAAG}\\ \underline{M} & \underline{P} & \underline{P} & \underline{W} & \underline{C} & \underline{N} & \underline{K} \\ \hline \\ & & & & & & & & & & \\ \hline \\ \mathbf{ATGCCATCTTCCAAATCACGCCT}\\ \underline{T} & \underline{I} & \underline{F} & \underline{Q} & \underline{I} & \underline{T} & \underline{P} \\ \hline \\ & & & & & & & & & \\ \hline \\ \mathbf{CACCATCTTGTGCTCGCAAATCC}\\ \underline{K} & \underline{F} & \underline{C} & \underline{A} & \underline{R} & \underline{K} & \underline{F} \\ \hline \\ \hline \\ & & & & & & \\ \hline \\ \mathbf{AAGGTCTGTGATCTGTGCTCGTACAAC} \\ \hline \\ & & & & & \\ \hline \\ \mathbf{SAGGCAGCCATTTCGTACAAC} \\ \hline \\ & & & & & \\ \hline \\ \mathbf{S150} \\ \hline \\ \mathbf{TTAGTGATTTTAACCAATCAGT} \\ \hline \\ & & & & & \\ \hline \\ & & & & & \\ \hline \end{array}$
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CATCACTCACAGTGTCTGGCTC H H S Q C L A Q TCCGTCCTGGTCGTCGTCATTGAAA S V L V V I E M M8 TGGCTGTTAGGAGCCATGGCTC W L L G A M A I M9 CTTGGATTTGAAGAATGGTTCG L G F E E W F A M10 ACTGACGCGTAAATATTTCGAC T D A *	2640 CAAGATGAGCGTTTCCTCGGTGTC D E R F L G V 2730 ATGCTTAACGCTCTCAACAGCTTC L N A L N S L 2820 CTGTCTATGGGACTCCACTTTGC S M G L H F C. 2910 CCCGTACTCAAGATCTCAATGCC V L K I S M P L12 3000 CACATTATACAAGACACAGACAAC 3090 AGAAACATCGGTAAGGGAAGGACC 3180 CCCTTCATCTCAGTGGAATACAAC 3270 ATGTTATATATATTAGATAGAAGC 3360 AATTTTACTCCTTACCCAAAATTC 3450	$\begin{array}{c} 2670\\ \hline \\ \textbf{CGACTGCAAGGTTTTTGACCACCCT}\\ \textbf{D} \ \textbf{C} \ \textbf{K} \ \textbf{V} \ \textbf{F} \ \textbf{D} \ \textbf{H} \ \textbf{P}_{\dots}\\ 2760\\ \hline \\ \textbf{STCTGAGAACCAGTCCCTGCTGGTGGTG\\ \textbf{S} \ \textbf{E} \ \textbf{N} \ \textbf{Q} \ \textbf{S} \ \textbf{L} \ \textbf{L} \ \textbf{V}\\ 2850\\ \hline \\ \textbf{CATCCTCTACATTGACGTCATGTCG}\\ \hline \textbf{I} \ \textbf{L} \ \textbf{Y} \ \textbf{I} \ \textbf{D} \ \textbf{V} \ \textbf{M} \ \textbf{S}\\ \hline \\ 2940\\ \hline \hline \\ \textbf{FGTGATACTTATAGACGAAACATTG}\\ \hline \\ \textbf{V} \ \textbf{I} \ \textbf{L} \ \textbf{I} \ \textbf{D} \ \textbf{E} \ \textbf{T} \ \textbf{L}\\ \hline \\ \textbf{3030}\\ \hline \\ \textbf{GGACAAGTTGTGAGGTCCAGTGAAGGTCCAGTGAAG\\ \hline \\ \textbf{3120}\\ \hline \\ \textbf{GCTGGTGATCCCCGACTCGTGTGAAAT\\ \hline \\ \textbf{3210}\\ \hline \\ \textbf{ACAGTTGACGATGATGACGATGCCTTATAT\\ \hline \\ \textbf{3300}\\ \hline \\ \textbf{FTTCAGCGTCTACCAACCCAGGAAACATTGAGAAATGACGAAATTCAAAAT\\ \hline \\ \textbf{3390}\\ \hline \\ \textbf{CTCCAGGAAATTCAAAAT\\ \hline \\ \textbf{3480}\\ \hline \end{array}$	2700 GCACCTATGACAATGGCTCTG A P M T M A L 2790 ATGCCCCCTTGGTGTAACAAG M P P W C N K 2880 ACCATCTTCCAAATCACGCCT T I F Q I T P 2970 AAGTTCTGTGCTCGCAAATTC K F C A R K F 3060 AAGGCAGCCATTTCGTACAAC 3150 TAGTGATTTTAACCAATCAGT 3240 CGGTCGTGTGTGTACTAGTTCCTT 3330 CACATAAGAAGCTCATGTGTG 3420 TAGGTGAATTATCAATCTTCAA
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CCTTCAATGATAAACAGGGGGG

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Figure2-B (Continued)



Fig. 2. Predicted primary and secondary structures of the scallop Ca²⁺-ATPase. (A) Nucleotide and deduced amino-acid sequence of the Ca²⁺-ATPase. Nucleotides are numbered with '1' being A of the ATG starting codon. The translation stop codon is denoted by an asterisk. Amino acids are numbered below the nucleotide sequence. The amino acid sequences which were used to design the primers for RT-PCR are marked in broken lines. The sequences (L1-L12) of the lysyl endopeptidase peptides of the ATPase are boxed. Solid lines and dotted lines represent the _-helical stalk regions (S1-S5) and the transmembrane segments (M1-M10), respectively. (B) Scheme of the predicted ATPase topography with 10 transmembrane domains (M1-M10), 5 stalk regions (S1-S5) and two large cytoplasmic domains. The numbers above and below the membrane indicate position of the amino acid residues at each end of the transmembrane helices. Asp350 and Lys514 are a phosphorylation site and a fluorescein 5-isothiocyanate binding site, respectively.

similarity between the two yeasts, which belong to the same phylum of Ascomycota, was about 40%, while the similarity between Trypanosoma and Plasmodium, which belong to the different phylums of Mastigophora and Apicomplexa, was about 30%. These results suggest the existence of differences in the phylogenetic classification among the ATPases of these kingdoms and in the diversity of the ATPases within each kingdom. As to the ATPases in Animalia, the following relations among them could be recognized: The ATPases in Chordata are in three branches of SERCA1, 2 and 3 in a group, as shown by Nolan et al., (27) and Song and Fambrough (28). The ATPases of scallop, Artemia and Drosophila are in two branches of Mollusca and Arthropoda in a group. Schistosoma is in a branch of common origin for these Animalia ATPases. It is interesting to know an evolutionary relation of amino acid sequences between the SERCA-type ATPases and the plasma membrane type Ca²⁺-ATPases (PMCA) which exhibit the differences in calmodulin-regulation of the ATPase activity (29) and in a ratio of calcium transported to ATP hydrolyzed (30,31). At present time, however, it is difficult to discuss such relation, because that the variety of the species, of which PMCA-type ATPases have been sequenced, is poor (4 for Chordata and one for Fungi).

The nucleotide sequence reported in this paper has been submitted to the DDBJ Data Bank with accession number AB002112.



Fig. 3. Schematic representation of the relationship of overall amino acid sequences between scallop ATPase and other SERCA-type ATPases. The amino acid sequence of scallop ATPase, which was deduced from the nucleotide sequence of its cDNA, was analyzed for similarity to those from the following organisms, by using the programm GENE-TYX-Mace with UPGMA (unweighted pair-group method with arithmetic mean) and arranged in the form of a ladder to illustrate common origins: fission yeast (Ref.32), budding yeast (Ref.33), *Trypanosoma brucei* (Ref.34), *Plasmodium falciparum* (Ref.35), *Schistosoma mansoni* (Ref.13), *Crustacean Artemia* (Ref. 14), *Drosophila melanogaster* (Ref. 15), frog -1 (Ref. 36), chicken -1 (Ref. 37), rabbit -1 (Ref.19), cat -2 (Ref. 38) human -2 (Ref. 39), rat -2 (Ref.40), rat -3 (Ref.41) and Human-3 (Ref.42). The appended numbers, 1, 2 and 3, represent the fast-twitch skeletal muscle cell form (SERCA1), the slow twitch skeletal/cardiac form (SERCA2), and the non-muscle form (SERCA3), respectively.

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Part III

Change in the thermal sensitivity of sarcoplasmic reticulum Ca²⁺-ATPase by chemical modifications and mutagenesis of specific Cys residues

SUMMARY

In the proceeding study I have cloned cDNA of the sarcoplasmic reticulum (SR) Ca^{2+} -ATPase (SERCA) from scallop adductor muscle, and predicted entire amino acid sequence of this enzyme (1). In comparison of the amino acid sequence with that of rabbit SR Ca^{2+} -ATPases, I have further explored the role of Cys residues on thermal stability of this enzyme. When rabbit SR was incubated above 36°C in the presence of 6.4 mM glutathione, Ca^{2+} -transport activity was lost within 5 min, while in the absence of glutathione, full activity was retained up to 45°C. This is in contrast that Ca^{2+} pump activity of scallop SR was lost above 36°C irrespective of the presence or absence of . These results raise a possibility that the pronounced difference in thermal stability between rabbit and scallop SR is caused by the formation of a disulfide bond among Cys residues on their Ca^{2+} -ATPase.

I have attempted to identify the Cys residues linking through a disulfide bond by examining accessibility of SH-groups to N-(7-Dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM). Sequence analysis of the DACM-labelled peptides isolated from rabbit Ca²⁺-ATPase revealed that almost all of the cytoplasmically exposed Cys residues were readily accessible to DACM except for Cys12, Cys377, Cys349, Cys674, and Cys675. By contrast Cys349 in the Scallop Ca²⁺-ATPase was found to be easily modified by DACM.

Site-directed mutagenesis was performed on Cys12, Cys377, Cys674, and Cys675 in the rabbit Ca²⁺-ATPase to respective amino acids at the same position of sequence in the scallop Ca²⁺-ATPase and the effects of mutations are compared for heat sensitivity of the mutants expressed in Sf21 cells. It was demonstrated that the activity of phosphoenzyme (EP) formation was pronouncedly inhibited when Cys675 \rightarrow Leu mutant was incubated at 41°C for 10 min. While other mutants, Cys12 \rightarrow Val, C377 \rightarrow Ile, C674 \rightarrow Arg, as well as the wild type of rabbit Ca²⁺-ATPase, were virtually unaffected by the heat treatment. Single substitution of Cys349 of the rabbit Ca²⁺-ATPase with Ala resulted in destabilization of the enzyme by the thermal treatment. These results suggest that on the rabbit Ca²⁺-ATPase, Cys675 may bind to Cys349 through disulfid bond which can contribute to the thermal stabilization of the enzyme. while that lacking in the disulfide bond in the scallop Ca²⁺-ATPase may cause the thermal destabilization of the enzyme.

INTRODUCTION

The active transport of Ca^{2+} into SR is mediated by an enzyme Ca^{2+} -ATPase which catalyzes the hydrolysis of ATP. In the reaction cycle, γ -phosphate from ATP is transferred to a specific Asp residue to form a phosphorylated intermediate (EP). Its formation and decomposition strictly couple to the Ca^{2+} transport across the membrane (2, 3). Although considerable information has been obtained regarding kinetic function of the enzyme, the structural basis for coupling of ATP hydrolysis to Ca^{2+} translocation across the membrane remains unsolved as major issues. Recent protein chemical and molecular biology techniques have been providing important information about structural features of the Ca^{2+} -ATPase.

In the proceeding paper I have studied the effects of temperature on the catalytic functions of the scallop SR (1, 4-6). The specific activity of the scallop Ca^{2+} -ATPase was lost after several minutes incubation of SR at temperatures as high as 39°C. whereas the activity of the rabbit Ca^{2+} -ATPase decreased only marginally with almost 80% of the activity retained even after the incubation above 50°C. In order to provide answers to the question what structural difference between rabbit and scallop Ca^{2+} -ATPases is related to the pronounced difference in the temperature sensitivity, we have cloned cDNA of the scallop Ca^{2+} -ATPase and predicted its primary structure (1). The detailed comparison of the amino acid sequence with that of rabbit SR Ca^{2+} -ATPases revealed interesting differences in the distribution of Cys residues on the cytoplasmically exposed region of these enzymes.

In the present study, I have identified and characterized the Cys residues on the rabbit Ca^{2+} -ATPase which could contribute to the structural stabilization of the enzyme. It was demonstrated that mutation of Cys675 to Leu or Cys349 to Ala led to expression of Ca^{2+} -pump activity which was sharply decreased by incubation at temperatures similar to those required for the inactivation of scallop Ca^{2+} -ATPase.

EXPERIMENTAL PROCEDURES

Materials --- Scallop SR was isolated from the striated portion of scallop (*Patinopecten yessoensis*) adductor muscle according to the method described previously (4). Rabbit SR was isolated from the skeletal muscle of rabbit dorsal and hind leg as described previously (5). These SR preparations were divided into small pieces, quickly frozen using liquid nitrogen, and stored at -80°C. $[\gamma$ -³²P]ATP was purchased from Amersham, Antipyrylazo III was from Nacalai, glutathione and DACM were from Wako. Other Chemicals were analytical grade.

Modification of Ca²⁺-ATPase with DACM --- SR vesicles at 2-2.5 mg/ml protein were exposed to 0.2-0.3 mM DACM for 30 min at 23°C in the standard medium which contained 0.1 M NaCl, 20 mM TES (pH7.2), 5 mM MgCl₂, 5% glycerol. At the end of the incubation period, the mixture was taken into 25 ml of the standard solution. SR was washed twice by centrifugation in the same solution. The amount of probe incorporated was determined spectrophotometrically using molar extinction co-efficients of 19,800 at 398 nm (7).

Purification of DACM labeled peptides --- The Ca²⁺-ATPase was purified according to Meissner *et al.* (8) by solubilizing SR with DOC to remove extrinsic proteins. The purified Ca²⁺-ATPase at 10 mg/ml protein was allowed to react with 0.1 mg/ml thermolysin at 52°C for 90 min as described previously (8). Separation and purification of DACM labeled peptides from the thermolysin digest was carried out by using HPLC with ODS-120T reversed-phase column essentially as described by our previous work (9). The final purified DACM-labeled peptides were subjected to Edman degradation on a model LF3600 protein sequencer (Applied BioSystems), and the amino acid sequences were determined.

Measurements of Activities --- Ca²⁺-transport by SR was measured in a reaction medium containing 0.025 -0.05 mg/ml SR protein, 0.1 M KCl, 5 mM MgCl₂, 5 mM oxalate, 5% glycerol, 50 μ M CaCl₂, 0.2 mM Antipyrylazo III, and 20 mM TES at pH 7.2. The reaction was started at 23°C by addition of 100 μ M ATP. Ca²⁺ uptake into SR was determined by monitoring the absorption at 700 nm in the reaction mixture (6). Phosphorylation of SR was measured in 20 μ l of reaction mixture which contained 0.5 mg/ml protein of microsome membrane, 0.1 M KCl, 5 mM MgCl₂, 5% glycerol, and 50 mM TES at pH 7.2. The reaction was initiated at 23°C by the addition of 1 mM [γ -

³²P]ATP and quenched after 10 sec with an ice cold solution containing 5% TCA,10 mM ATP and 0.1 M Pi. The amount of $[\gamma^{-32}P]$ phosphoprotein formed was determined as described elsewhere (10).

Construction and expression of mutant cDNAs --- Full-length rabbit SERCA1a gene and monoclonal antibody A52 were provided from MacLennan, D.H. Full-length scallop Ca²⁺-ATPase gene (Scal-SERCA) was described previously (1). Each genes were subcloned into pBluescript SK+. Site-directed mutagenesis was used to insert a Spe I site at the initiator methionine codon of SERCA1a and a Hin dIII site immediately after the stop codon. In the same way, a Bam HI site was inserted into the scallop Ca²⁺-ATPase gene (Scal-SERCA) before first ATG. Mutant genes of SERCA1a and Scal-SERCA were constructed used PCR with LA-Taq (TAKARA). Mutants and mutagenic primers are as follows; C12V; CACAGAGGAAGTTCTGGCCTATTTTGG. C377I; GACGGAGACTTCATTTCGCTGAACGAG, C674R; CTGCCGCCGCGCCC-GCTGCTTCGCGCG, C675L; CGCCGCGCCTGCCTCTTCGCGCGCGTG. The PCR reaction was carried under the standard condition suggested by TAKARA. After PCR reaction, template DNA was digested by restriction enzyme Dpn I which digests only methylated DNA and then PCR products were separated by agarose gel electrophoresis and purified by Prep-A-Gene DNA purification systems (BIO-RAD). Then self-ligated mutant cDNAs were transfected in E.coli DH5a competent cells. The presence of the desired mutations and the absence of unexpected ones were verified by DNA sequencing. 4 desired mutation, 1683C → T, 2202C → G, 2253G → C and 2367C → T, were available in SERCA1a gene used in this study, but these mutations do not change the native amino acid.

Recombinant baculoviruses which contain wild type or mutant genes of Ca²⁺-ATPase were constructed by Bac-to-Bac baculovirus expression systems (GIBCO). Preparation of ER fraction from Sf21 cells was as follows; 60 hour passed from virus infection, Sf21 cells were harvested and homogenized by polytron homogenizer after washing with PBS twice. Microsomal fraction was purified by step-wise centrifugation by the method described previously (5) and finally suspended in 0.1 M KCl, 5 mM Trismaleate, and 10% glycerol and stocked at -80°C. An anti-peptidase was not used through preparation.

RESULTS

We have demonstrated a striking difference in temperature sensitivity between the catalytic functions of rabbit and scallop SR (1, 4-6). As illustrated in Fig. 1, incubation of scallop SR for 5 min at temperature at 38°C suffices to produce complete inactivation of ATP hydrolysis, whereas rabbit SR ATPase is not inhibited by the incubation at temperatures up to at least 50°C. The difference can be attributed to a difference in the amino acid sequences of these enzymes. In the proceeding study I have cloned cDNA of SR Ca²⁺-ATPase from scallop adductor muscle, and predicted entire amino acid sequence of this enzyme (1). Based on the detailed comparison between the entire amino acid sequences of the rabbit and scallop Ca²⁺-ATPases, it was revealed that there are several candidate amino acids which could contribute to the stabilization of the enzyme structure. One of the most striking difference relates to the distribution of Cys residues on the cytoplasmically exposed domain in the Ca²⁺-ATPase molecule. In the experiment shown in Fig. 2, I studied the effects of glutathione on the rabbit and scallop Ca²⁺-ATPases, as expressed by Ca²⁺-pump activity. When rabbit SR was incubated above 36°C in the presence of 6.4 mM glutathione, Ca²⁺-transport activity was lost within 5 min, while in the absence of glutathione, full activity was retained up to 50°C (Fig. 2A, open and closed circles). Fig. 2B shows the time courses of decrease in Ca²⁺pump activity of rabbit SR by the incubation at 43°C in the presence of different concentration of glutathione. The half time of inactivation is 10 min for 3.2 mM, 5 min for 6.4 mM and 30 sec for 10 mM. Ca²⁺-pump activity of rabbit SR was decreased in the presence of DTT at high temperature in fashion similar to that observed with glutathione, although half concentration of DTT for heat inactivation is much higher than that for glutathione (Data not shown). These results suggest that disulfide bonds of the Ca²⁺-ATPase are involved in thermal stabilization of the structure which is essential to the enzymatic function. On the other hand, Ca²⁺-pump activity of scallop SR was lost after 5 min incubation above 35°C irrespective of the presence or absence of glutathione (Fig. 2A, open and closed triangles). It may be concluded from these observations that the pronounced difference in thermal stability between rabbit and scallop SR is presumably caused by formation of a disulfide bond among Cys residues on the enzyme. This conclusion seems to be in accord with experimental observations demonstrated in Fig. 3. We compared between reactivities of SH groups with DTNB in the rabbit and



Fig. 1. Temperature sensitivities of the Ca²⁺-ATPase activities in rabbit and scallop SR. 2 mg/ml protein of rabbit (\bigcirc) or scallop (\square) SR was incubated at indicated temperatures in the medium containing 0.1 mM CaCl2, 0.1 M KCl, 20 mM TES (pH 7.2), 10% glycerol, 5 mM MgCl2. After 5 min incubation, 0.1 ml of SR suspension was transferred into 20 volumes of assay medium which had been kept cool on ice to stop the thermal treatment. ATP hydrolysis was measured in a medium which contained 0.1 mg/ml SR, 40 μ M CaCl2, 2 μ M A23187, 0.1 M KCl, 20 mM TES (pH 7.2), 5% glycerol, 5 mM MgCl2, and ATP regenerating system (0.5 mM NADH, 1.5 mM Phosphoenolpyruvate, 1 unit of lactate dehydrogenase, 1 unit of pyruvate kinase). Reaction was initiated by the addition of 0.2 mM ATP at 23°C, and the amount of ADP liberated from ATP was determined by monitoring the decrease in absorbance of NADH at 340 nm.



Fig. 2. A: Temperature sensitivity of Ca²⁺-transport activity in the presence and absence of gluthation. 3.5 mg/ml protein of rabbit (O, Φ) and scallop (Δ, \blacktriangle) SR were incubated with (O, Δ) or without (Φ, \blacktriangle) 6.4 mM GSH at the indicated temperatures in the solution containing 0.1 MKCl, 10 mM Tris-maleate (pH 6.5), and 10% glycerol. After 5 min incubation was terminated by cooling the medium in ice. Ca²⁺-transport activity remaining after the incubation was monitored at 23°C as described in "EXPERIMENTAL PROCEDURE"

B: Time courses of thermal inactivation of rabbit SR Ca²⁺-transport at various concentrations of GSH. 3.5 mg/ml protein of rabbit SR was incubated with 0 (\Box), 4.8 (Δ), and 6.4 (O) mM GSH at 42°C under the conditions described above. Ca²⁺-transport activities remaining after various incubation times were measured as in "EXPERIMENTAL PROCEDURE"

scallop Ca²⁺-ATPase. For the case of rabbit SR, the number of SH group modified with DTNB increased very slowly. It reached a steady level of 9 -10 mol per mol of ATPase after 30 min of incubation, while it increased to about 22 mol immediately after the addition of 0.2% SDS. For the case of scallop SR, the number of reacted SH increased to 12 mol per mol of ATPase within 5 min. It reached a maximum level of as high as 23 mol upon the addition of SDS. Since rabbit and scallop Ca²⁺-ATPases share a very high sequence homology in transmembrane segments but not in cytoplasmically exposed regions (1), candidate Cys residues which contribute to formation of disulfide bond can be presumably located in the latter regions. In an attempt to determine the Cys residues which are linked through disulfide bond, intact rabbit SR vesicles were allowed to react with DACM at a concentration enough to label the accessible SH groups under similar conditions to that used for glutathione or DTNB modification.

For purification of Ca²⁺-ATPase, the SR vesicles were washed by centrifugation and solubilized with 0.5% DOC and processed as described in "EXPERIMENTAL PROCEDURES". After the purification step, about 30% of the original SR protein was recovered as Ca²⁺-ATPase protein. As estimated from the absorption at 398 nm, as high as 11 mol of DACM was incorporated per mole of ATPase. This value is in agreement with observations using DTNB as in Fig.3. The DACM-labeled Ca²⁺-ATPase of rabbit SR was then extensively digested with thermolysine for 90 min at 52°C. More than 75% of the amount of DACM were recovered in the supernatant when thermolysine digest was centrifuged to removed insoluble fractions. These results indicate that SH groups in the extra cellular portion of the Ca²⁺-ATPase were mainly reacted with DACM under the conditions used.

In the experiment shown in Fig. 4, DACM-labeled peptides were applied to an ODS-120T column and eluted with 10-50% CH₃CN gradient. By repeating the chromatography under various CH₃CN concentration gradients in the presence or absence of salt, more than 15 fluorescent peaks have been obtained as homogeneous peptides. These peptide were subjected to Edman degradation on a protein sequencer and the position of Cys residues on the primary structure of Ca²⁺-ATPase was estimated (Table I).

Comparison of these motifs with the primary structure of the rabbit Ca^{2+} -ATPase revealed that Cys residues at positions of 344* 364* 417* 420* 471* 498* 525* 561* 614* 636* and 670 were readily modified with DACM (* represents common residue to



Fig. 3. Comparison of the DTNB accessibilities between rabbit and scallop SR vesicles. 0.34 mg/ml rabbit SR vesicles (O, \bullet) and 0.19 mg/ml scallop SR vesicles (Δ, \blacktriangle) were allowed to react at 23°C with 0.2 mM DTNB in the solution containing 0.1 M NaCl, 50 mM TES (pH 7.0), and 5% glycerol. At the indicated times, 0.5% SDS was added to determine total amount of DTNB reacted with SR. Sulfhydryl content was calculated from the absorbance at 412 nm using molar extinction coefficients of 14,100 for the SR suspension(\bullet, \blacktriangle), and 13,000 for the suspension in the presence of SDS (O, Δ). These coefficients were obtained by referring to the calibration curve for the absorption which was obtained with 2-mercaptoethanol of known concentration. In addition, purity of the Ca²⁺-ATPase in rabbit and scallop SR assumed to be 60 and 65%, respectively.



Fig. 4. Elution profile of thermolytic digest of the rabbit SR Ca²⁺-ATPase on reversed-phase HPLC. Rabbit SR vesicles were allowed to react with DACM, and the Ca²⁺-ATPase was extracted with DOC and digested with thermolysin as described in "EXPERIMENTAL PROCEDURES". The digest was applied to an ODS-120T column and eluted with a 10-50%CH3CN gradient in 0.1% trifluoro-acetic acid. The absorption of the eluted peptides (black solid line) was monitored at 214 nm and DACM fluorescence in the fraction (red solid line) was monitored for excitation and emission at 398 and 465nmn, respectively. The fluorescent fraction was collected, evaporated and redissolved in 10% CH3CN and subjected to further purification step.

Peak	DACM (nmol)	Protein (nmol AA)	AA/DACM	Sequence
3b	0.26	1.9	7-8	S K V E R 471C*
				F V K G A 525C*
3c	0.38	2.3	6 - 7	E Q R E A 670C*
4	3.2	22.0	7-8	K V E R 471C*
5b	0.8	13.4	17	E Q R E A 670C*
				L S K V I 471C*
5d	1.4	4.9	3 - 5	
5c	1.6	6.7	4 - 5	V Y 498C*
5e	2.5	10.0	4 - 5	V R X L P 344C*
ба	1.7	5.2	4 - 5	
6с	2.7	21.0	7-8	V Y 498C*
7a	0.66	57.0	86	N A 471C*
7b	0.56	3.8	7-8	
8	0.5	23.0	45	T G R D T 561C*
9a	4.5	23.0	4 - 5	L V E L 417C*, 420C*
				L S K V 471C*
9b-1	2.5	19.0	7-8	E Q R E A 670C*
				A I 636C*
				VIKEW 561C*
9b-2	1.8	28.5	16	A I A I 636C*
				F V K G 525C*
10	3.0	11.0	3 - 4	F V K G A P- 525C*
11a	2.1	15.2	7 - 8	LTTN 364C*
11b	1.2	9.7	8 - 9	V M G S 614C*

[Table-I] Separation and purification of SR-DACM-peptides from rabbit Ca²⁺-ATPase

*represents common residue to both Ca²⁺-ATPases of rabbit and scallop SR

both Ca^{2+} -ATPases), while Cys residues at positions of 12, 349^{*+}, 377, 674, and 675 seemed to be inaccessible to the reagent irrespective of their existence on the cytoplasmically exposed regions of the enzyme (Fig. 5). Therefore the five inaccessible Cys residues are considered to be candidate amino acids which could contribute to formation of disulfide bonds. Accessibility of Cys residues on the scallop Ca^{2+} -ATPase were also tested under similar conditions to those for rabbit Ca^{2+} -ATPase, and it was found that Cys349 was readily reacted to DACM. This is in contrary to the result obtained with rabbit Ca^{2+} -ATPase.

In order to determine which Cys residues are cross linked through disulfide bond, site-directed mutagenesis was performed on the Cys residues at positions of 12, 377, 674, or 675 in rabbit Ca²⁺-ATPase to the respective amino acid, Val, Ile, Arg, or Leu each of which locates at the same position of sequence in scallop one, and Cys349, which is common to that of scallop Ca²⁺-ATPase, was substituted with Ala. Recombinant baculovirus that contain wild type or mutant genes of Ca²⁺-ATPase were transfected to Sf21 cells. To avoid thermal inactivation of expressed Ca²⁺-ATPase, the infected cells were cultured below 27°C. After 60 hours, cells were harvested and the microsomal fraction was obtained by differential centrifugation of homogenized cell as described in "EXPERIMENTAL PROCEDURES".

Figure 6 shows SDS-PAGE and immunoblotting of the microsomes isolated from the mutant cells which were infected by wild type, C12V, C377I, C674R, and C675L baculovirus. All of these mutant ATPases are expressed in the Sf21 cells as well as that of wild type. Functional consequences of the site-directed mutation were summarized in Table II. The activity of EP formation in the presence of Ca²⁺ significantly varied from one preparation to another presumably depending upon the amount of SERCA1 expressed in the cell. In the control experiment using notransfected cell the phosphorylation activity was negligibly low.

In the experiments in Table II, thermostabilities of the mutants were examined by monitoring the phosphorylation activity remaining after 5 min incubation at 43°C. It was found that single substitution of Cys residue at the position of 675 in the rabbit Ca^{2+} -ATPase with Leu residue which locates at the same position as that of the scallop ATPase resulted in reduction of the phosphorylating activity to about 30% of original one, while other mutants as well as wild type ATPase maintained the activities at almost the same level after the incubation. As illustrated in Fig. 7, incubation of C675L mutant



Fig. 5. Schematic representation of the Cys residue positions on the cytoplasmically exposed regions of rabbit and scallop Ca²⁺-ATPases.



Fig. 6. SDS-PAGE and immunoblotting of recombinant Ca²⁺-ATPases expressed in Sf21 cell. Sf21 microsomes from wild type, C12V, C337I, C674R, C675L baculovirus-infected cells as well as rabbit SR were subjected to SDS-PAGE and immunoblotting. Panel A shows a Coomassie Blue-stained gel, and panel B shows a corresponding immunoblot developed with SERCA1a monoclonal antibody. Arrow indicates the position of Ca²⁺-ATPase with a molecular weight of 115,000. Long bars also indicate positions of molecular weight markers.

[Table II] Effect of various mutations on the EP formation activity of SERCA-1 enzyme expressed in Sf21 cells.

	Amount of EP (pmol/mg)		
Sample	No treatment	Heat treated	Remained activity (%)
Wild Type	6.33	-	-
	5.86	-	-
	16.29 (2)	14.16	87
	17.09	12.66	74
	43.72	38.49	88
	47.16	38.81	82
Control	2.51	1.55	-
	0.93 (2)	1.34	
C12V	11.67	12.60	108
	22.80	21.58	95
	24.24	21.53	89
C377I	18.27	12.54	69
	15.45	10.80	70
	29.52 (2)	29.18	99
C674R	13.82	15.05	107
	18.03	17.77	99
	7.23(2)	7.71	107
	31.64 (3)	40.21	112
C675L	5.76	1.88	32
	8.01	1.10	60
	15.84 (2)	9.55	32

Number in () represents number of experiments. The amount of EP was measured as described in "EXPERIMENTAL PROCEDURES". Control means ER fraction from no transfected cells.



Fig. 7. Effects of single substitution of Cys675 to Leu on the thermal stability of the Ca²⁺-ATPase. Microsomes from C675L (\Box) mutant cells as well as that of wild type (O) were incubated for 10 min at indicated temperatures in the presence of 0.1 mM CaCl2. Phosphorylation of the microsomes by AT³²P was measured as described in "EXPERIMENTAL PROCEDURES".

for 5 min at 42°C lowered EP formation activity by 30%, whereas more than 80% of the activity of wild type ATPase remained even after the incubation at 50°C. From these results, it can be concluded that Cys675 presumably binds with another Cys through disulfide bond and contribute to structural stability of the rabbit Ca²⁺-ATPase.

To obtain further support for this conclusion I determined the effect of single substitution of Cys349 with Ala on the thermostability of rabbit Ca²⁺-ATPase. Autoradiogram in Fig.8 shows that EP formation activity of the C349A mutant ATPase decreased with increase in the temperature in the preincubation medium, and no activity was seen when the mutant was incubated for 10 min at temperatures higher than 40°C. Our conclusion to be drawn from these results combined with those of Table II and Fig. 7 is that Cys residues 675 and 349 may form a disulfide bond which can contribute to the thermal stabilization of the rabbit Ca²⁺-ATPase, while that lacking in the disulfide bond in the scallop Ca²⁺-ATPase may cause the thermal destabilization of the enzyme.



Fig. 8. Autoradiogram of the phosphorylated Cys349 - Ala mutant ATPase by AT³²P after incubation at various temperature. Microsomes containing C349A mutant ATPase were incubated for 10 min at indicated temperatures in the presence of 0.1 mM CaCl₂. Ca²⁺-ATPase was phosphorylated in the standard medium with 0.1 mM AT³²P at 0 °C and quenched after 15 sec by 5% TCA containing 5 mM Pi. Incorporation of ³²P was determined by separating the proteins in acidic NaDodSo4/polyacrylamide gels for autoradiography essentially according to Maruyama and MacLennan (25).

DISCUSSION

It is generally accepted that disulfide bond plays a key role in stabilizing enzyme structure. The number of related reports is increasing in the cation transport ATPase such as F_1 -ATPase of mitochondria (11, 12), Na/K-pump ATPase and Ca²⁺-pump ATPase of SR. For the Na/K-pump ATPase, reduction of disulfide bond (13, 14) or mutation (15) of Cys residues have been shown to result in a loss of ATPase activity. Further evidence was provided that structural disruption of the extra cellular portion of β -subunit drastically affects catalytic activity of α -subunit of Na/K-ATPase (16, 17). It has been shown that the SR Ca²⁺-ATPase contains 2-3 disulfide bonds (18-20), and that the ATPase is inhibited by the treatment with β -mercaptoethanol (20, 21), indicating that disulfide bonds can be involved in stabilization of the structure essential to the enzymatic function. Recently evidence has been presented that reduction of disulfide bonds on the Ca²⁺-ATPase by DTT in the presence of nucleotide and Ca²⁺ diminish its catalytic function (22), although no evidence has been obtained for identification of Cys residues which are linking through disulfide bridge.

In this report, we have further explored the role of Cys residues on thermal stability of the Ca²⁺-ATPase in comparison between primary sequences of scallop and rabbit Ca²⁺-ATPase. The detailed comparison revealed a pronounced difference between the distribution of Cys residues on the cytoplasmically exposed region of rabbit and scallop Ca²⁺-ATPases; Cys 12, 377, 670, 674, and 675 in rabbit Ca²⁺-ATPase are replaced with Val, Ile, Val, Arg, and Leu, respectively, in scallop Ca²⁺-ATPase. Cys349 of rabbit Ca²⁺-ATPase is inaccessible to DACM, whereas Cys349 of scallop ATPase is readily reacted with the reagent under the same condition described above. In addition, Thr9, Ala219, Gly405, and Val449 of rabbit ATPase are replaced with Cys residues in scallop one (1). If we assume that the Cys residues linking through disulfide bond can contribute to thermal stabilization of the Ca²⁺-ATPase, at least one of these Cys residues must be located on the rabbit ATPase, but not on scallop one. Furthermore, these residues should be inaccessible to SH reagents even they are exposed to outer medium. Among these candidates, Cys674 has already been reported to be accessible to IAF (23) and IAEDANS or IAA (24) under physiological conditions.

More definitive evidence has come from mutagenesis studies, in which single substitution of Cys349 to Ala or Cys675 to Leu were shown to drastically diminish
catalytic activity of the rabbit Ca^{2+} -ATPase when the enzyme was incubated above 40°C (Figs. 7 and 8). As a reasonable explanation for the lower sensitivity of the C675L mutant to thermal treatment, we postulate that substitution of any residues other than Cys675 may be also required for complete heat inactivation of the enzyme. These results suggested that Cys349 and Cys675 involve in the thermal stabilization of the structure essential for the enzymatic functions. However, it remains to be elucidated whether Cys349 and Cys675 are actually linking through disulfide bond on the rabbit Ca^{2+} -ATPase to contribute to thermal stabilization of the enzyme, or the local changes of environment around these residues might in some manner cause a pronounced increase in thermal stability of the enzyme. Experiments are presently under way to determine these possibilities.

In Fig.9, we propose a simplest model to explain a role of Cys residues 349 and 675 in thermal stabilization of rabbit Ca²⁺-ATPase. According to the mobile pore model (26), a portion of the ATPase molecule could be mobile, permitting to act as a carrier through which Ca²⁺ ions can pass. In our model, α -helixes of Stalk-4 and at the top of Stalk-5, whose transmembrane segments M4 and M5 form a passage of Ca²⁺ ions together with M6, are connected through disulfide bond between Cys349 and Cys675. This linkage presumably serve as a stopper which can restrict to the minimal motion for the Ca²⁺-passage. It is possible that since the motional restriction by the stopper is lacking in the scallop Ca²⁺-ATPase, this enzyme is readily destructed by thermal treatment. This possibility may be more critical in E1 state of the enzyme where binding of Ca²⁺ to the enzyme causes a conformational change allowing the Ca²⁺-entrance to open, than E2 state where the entrance is closed. This speculation is in good agreement with our previous studies (4) suggesting that removal of Ca²⁺ from the high affinity sites can protect the enzyme from thermal inactivation.



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Part IV

Temperature Sensitivity of Proteoliposomes Reconstituted from A Mixture of Scallop and Rabbit Sarcoplasmic Reticulum Ca²⁺-ATPases

SUMMARY

I reconstituted proteoliposomes by mixing scallop sarcoplasmic reticulum, SR with the rabbit one at different protein weight ratios, and investigated the effects of temperature on their Ca²⁺-transport activity. When proteoliposomes containing scallop and rabbit SR at a protein ratio of 1:1 were pre-incubated in the presence of Ca²⁺ at 39°C for 10 min, the Ca²⁺-transport activity was almost completely lost, whereas the activity of proteoliposomes containing rabbit SR alone decreased only slightly. Essentially the same results were obtained for proteoliposomes reconstituted with Ca2+-ATPases partially purified from scallop and rabbit SR. The susceptibility of the reconstituted proteoliposomes to heat inactivation increased with increase of the protein weight ratio of the scallop to rabbit SR, with the maximum being approached at a ratio higher than 1. When the scallop SR was thermally treated before reconstitution, the resulting vesicles showed Ca^{2+} -transport activity as high as that of control vesicles reconstituted from rabbit SR alone. The former vesicles were not inactivated by further treatment at high temperature. In contrast, when scallop SR was heated in EGTA before, followed by reconstitution of vesicles with rabbit SR, their Ca²⁺-transport activity was strongly inhibited by subsequent treatment at high temperature in the presence of Ca²⁺. These results can be easily explained if I assume that Ca²⁺-transport of the reconstituted vesicles can be catalyzed through dimeric interaction between the scallop and rabbit Ca²⁺-ATPases in the membrane. Pre-incubation of these vesicles at 39°C for 10 min in the presence of Ca²⁺ may destroy the dimeric interaction due to denaturation of scallop Ca²⁺-ATPase.

INTRODUCTION

The Ca²⁺-ATPase bound to SR membrane is responsible for the active transport of Ca²⁺ across the membrane. The coupling mechanism of ATP hydrolysis with Ca²⁺transport has been investigated extensively (1, 2). However, the movements of the ATPase molecules during the transport cycle have remained unknown. A number of investigators have suggested that Ca²⁺-ATPase exists in the SR membrane in an oligomeric form (3-6), but the physiological role of the molecular interaction has been poorly understood.

In the preceding study, I investigated heat inactivation of scallop Ca^{2+} -ATPase under a variety of conditions, and concluded that the thermal lability of the catalytic function may closely correlated with the arrangement of the ATPase molecules in the SR membrane (7). As an extension of our previous studies on the ATPase-ATPase interaction, I reconstituted proteoliposomes by mixing the Ca²⁺-ATPase of heat-labile scallop SR and heat-resistant rabbit SR at various protein weight ratios, and investigated the effect of temperature on their catalytic function. If there was no interaction between the two ATPases in the membrane, I could expect that the Ca²⁺-transport activity of the reconstituted vesicle would additively change depending on their respective heat sensitivities. However, incubation of the vesicles at 39°C for 10 min almost completely eliminated their Ca²⁺-transport activity, whereas heat treatment of the control vesicles reconstituted with rabbit SR alone only slightly decreased it.

These results suggest that the Ca^{2+} -ATPases of scallop and rabbit SR form an oligomer in the reconstituted membrane and that this molecular interaction is essential for Ca^{2+} -transport.

EXPERIMENTAL PROCEDURES

Materials --- Scallop SR was isolated from the striated portion of scallop adductor muscle as described previously (7, 8). Rabbit SR was prepared from rabbit dorsal and leg muscles as described previously (9). These SR were suspended in medium containing 500 mM KCl, 10% glycerol, and 20 mM TES (pH 7.3), and divided into small pieces. They were quickly frozen in liquid nitrogen, and stored at -80°C. The Ca²⁺-ATPases were partially purified from scallop and rabbit SR according to the method described by Meissner *et al* (10).

Chemical reagents --- $C_{12}E_9$ was purchased from Nikko Chemicals. Bio-beads SM-2 were obtained from Bio Rad Laboratories and repeatedly washed with methanol and water. Soybean asolectin was purchased from Sigma, A23187 from Calbiochem-Behring, and Antipyrylazo III from Nacalai. All other chemicals were of reagent grade.

Reconstitution of proteoliposomes --- Proteoliposomes were prepared by the method of Ando and Yamamoto (11) with the following modifications. SR was added to the 1 ml of reconstitution medium containing 5 mM MgCl₂, 0.1 M oxalate, 0.1 M KCl, 5-10% glycerol, and 0.1 M TES (pH 6.8) to a final concentration of 1-3 mg/ml protein. When proteoliposomes were reconstituted from a mixture of rabbit and scallop SR at indicated weight ratios, protein concentration of rabbit SR was kept at 1 mg/ml, and that of scallop SR was varied from 0 to 2 mg/ml. SR was solubilized by addition of 45 mg/ml C₁₂E₉. After several minutes, 40 mg/ml soybean asolectin solubilized in 45 mg/ml C₁₂E₉ was added to the medium. The SR mixture was further incubated at 20°C for 15-20 min under continuous supply of N_2 gas, then centrifuged to remove insoluble materials. Next, Bio-beads SM-2, 0.2-0.4 g dry weight, were added to the supernatant, and the mixture was vigorously stirred for 60-90 min under N₂ gas. The Bio-beads were removed by passing the mixture through a cotton layer, and the filtrate was centrifuged as described above to remove debris. The supernatant was again incubated with 0.2 g/ml of Bio-beads for 45 min, then centrifuged at 530,000 \times g for 30 min to collect the proteoliposomes. The pellet was suspended in 0.2-0.3 ml of the reconstitution medium from which oxalate had been omitted. The final sample was quickly frozen in liquid nitrogen and stored at -20°C.

Heat treatment of SR --- SR suspension (0.1 ml) was transferred into incubation medium containing 5 mM MgCl₂, 0.1 M KCl, 5% glycerol, and 50 mM TES

(pH 6.8) which had been warmed in a glass test tube at the indicated temperature. At the indicated time, heat treatment was stopped by transferring the mixture into ice cold assay medium.

Measurement of Ca²⁺-transport --- Ca²⁺-transport was measured at 23°C in assay medium containing SR or reconstituted vesicles at 0.02-0.1 mg/ml protein, 20-50 μ M CaCl₂, and 0.2 mM Antipyrylazo III, as the Ca²⁺ indicator. The reaction was initiated by addition of 0.2 mM ATP. The change in the Ca²⁺ concentration in the reaction mixture was continuously monitored by measuring the absorption of the Ca²⁺ indicator at 700 nm.

 Ca^{2+} efflux from the reconstituted vesicles was measured under conditions similar to those for the Ca²⁺-transport except for the absence of oxalate. The reaction was started by the addition of 5 μ M A23187 to the reaction mixture.

RESULTS

Sarcoplasmic reticulum isolated from cold water fish muscles including scallop adductor muscle has higher sensitivity to temperature than those from mammal muscles (8,12,13). Figure 1 compares the time courses of Ca²⁺-transport by SR isolated from rabbit and scallop muscles after their pre-incubation at 39°C for 0, 5, and 10 min in medium containing 5 mM MgCl₂, 0.1 M KCl, 5% glycerol, and 50 mM TES (pH 6.8). Noted that the pre-incubation medium is usually contaminated with μ M Ca²⁺ under the conditions used, except when Ca²⁺ is removed by addition of 2-4 mM EGTA. The Ca²⁺transport activity of rabbit SR was unaffected by the heat treatment for up to 10 min, while the activity of scallop SR completely disappeared within 5 min.

Such a big difference in the thermolability between scallop and rabbit SR offers me the means to directly examine molecular interaction of Ca²⁺-ATPase within the SR membrane. In the following experiments, I reconstituted proteoliposomes by mixing heat-labile scallop SR and heat-resistant rabbit SR to investigate the effects of thermal treatment upon Ca^{2+} -transport by the proteoliposomes. Figure 2 presents the time courses of Ca²⁺-transport by the reconstituted vesicles before and after their incubation at 39°C for 10 min. The velocity of the Ca²⁺-transport to decrease with increasing reaction time, probably due to the formation of some membrane potential accompanied by Ca²⁺ movements across the reconstituted membrane. The transport activity of the proteoliposomes reconstituted with rabbit SR alone (panel a) was only slightly decreased by pre-incubation at 39°C for 10 min, while the activity of the proteoliposomes reconstituted with scallop SR completely disappeared with the treatment (panel c). For the panel b data, proteoliposomes were reconstituted with a 1:1 mixture of scallop and rabbit SR by protein weight. Surprisingly, when they were incubated for 10 min at 39°C, Ca²⁺-transport activity decreased to almost the same level as that observed for the control vesicles reconstituted with scallop SR alone (panel c). If I assume that the Ca²⁺-transport can be independently catalyzed by the scallop and rabbit Ca²⁺-ATPases in the reconstituted membrane, almost the same activity as observed for the heated vesicles which contain rabbit SR alone should remain after the heat treatment (compare between upper traces in panel a and b). Furthermore, it does not seem that Ca²⁺-transport activity of the untreated vesicles was additively changed by mixing rabbit and scallop SR. These results suggest that scallop and rabbit Ca²⁺-ATPase



Fig. 1. Effects of heat treatment on Ca²⁺ transport by rabbit and scallop SR. SR was added at 39°C to preincubation medium containing 5 mM MgCl₂, 0.1 M KCl, 5% glycerol, and 50 mM TES (pH 6.8) to a final concentration of 1 mg/ml. At 0 (a), 5 (b), and 10 min (c), the SR suspension was rapidly cooled in ice water to stop the heat treatment. Ca²⁺-transport was initiated at 23°C by addition of 0.2 mM ATP (\downarrow) to the assay solution containing 50 μ M CaCl₂, 5 mM oxalate, 5 mM MgCl₂, 0.1 M KCl, 5% glycerol, and 50 mM TES (pH 6.8) in the presence of 0.2 mM Antipyrylazo III and 20 μ g/ml of SR.



Fig. 2. Effect of heat treatment on Ca²⁺ transport by proteoliposomes reconstituted from scallop and rabbit SR. Proteoliposomes were reconstituted as described in "EXPERIMENTAL PROCEDURES" by mixing scallop and rabbit SR at the protein weight ratio of 0:1 (a), 1:1 (b), and 1:0 (c). The proteoliposomes were suspended in the preincubation medium and thermally treated as given in Fig. 1, for 0 (lower traces) and 10 min (upper traces). Ca²⁺ transport by the proteoliposomes was measured at 23°C under the conditions described in Fig. 1. (\downarrow), addition of ATP.

molecules exist in the reconstituted membrane mainly as an oligomer, and that interaction between them is essential for the enzyme to catalyze Ca^{2+} -transport. Heat denaturation of the scallop Ca^{2+} -ATPase may result in destruction of the oligomeric interaction.

Another possible explanation for the susceptibility of the reconstituted vesicles to heat inactivation is that the thermal treatment may specifically increase Ca^{2+} permeability of the membrane *via* the scallop Ca^{2+} -ATPase. In the experiments presented in Fig. 3, I measured the Ca^{2+} efflux from proteoliposomes reconstituted with scallop and rabbit SR at different protein weight ratios. These vesicles were loaded with Ca^{2+} during the reconstitution in the presence of 10 mM CaCl₂. The Ca²⁺-loaded proteoliposomes were further incubated at 39°C for 10 min. Panel a, b, and c of Fig. 3 show the time courses of Ca^{2+} release from the respective proteoliposomes reconstituted with rabbit SR alone, a 1:1 mixture of scallop and rabbit SR, and scallop SR alone, after addition of 5 μ M A23187. Upon addition of the ionophore, Ca^{2+} was rapidly released from the proteoliposomes and reached the steady level within 30 sec. From the calibration curve of the Ca^{2+} concentration in panel d, about 15 μ M Ca²⁺ was released from each sample of reconstituted vesicles.

The amount of Ca^{2+} released from the thermally treated vesicles was about 20% lower than that from untreated vesicles possibly due to loss of internal Ca^{2+} during the treatment. The reduction in the amount of Ca^{2+} release after the heat treatment was seen in the vesicles reconstituted with rabbit SR alone (panel a) as well as in those containing scallop SR (panel b and c). These results do not support the possibility of heat inactivation of Ca^{2+} -transport of proteoliposomes being caused by a rise in Ca^{2+} leakage of the membrane *via* the scallop Ca^{2+} -ATPase.

As shown in Fig. 4, the susceptibility of the reconstituted proteoliposomes to heat inactivation increased as a function of the protein weight ratio of scallop SR to rabbit one, and appeared to reach the maximal level at a ratio higher than 1 (Fig. 4, inset). These observations support the possibilities that the scallop and rabbit Ca^{2+} -ATPases form a dimer in the reconstituted membrane at the molar ratio of 1:1 and that the molecular interaction between them plays an important role in the active transport of Ca^{2+} across the membrane. I decided to test whether or not the scallop Ca^{2+} -ATPase could still form an oligomer in the reconstituted membrane even after the SR was thermally treated. Figure 5 shows the results for proteoliposomes reconstituted by



Reaction time (min)

Fig. 3. Ca²⁺ release from the Ca²⁺-loaded proteoliposomes after addition of Ca²⁺ ionophore. Proteoliposomes were reconstituted by mixing scallop and rabbit SR at a ratio of 0:1 (a), 1:1 (b), and 1:0 (c) in the solution described in "EXPERIMENTAL PROCEDURES" except for substitution of 10 mM CaCl₂ for oxalate. The Ca²⁺-loaded proteoliposomes were incubated for 10 min at 23°C (upper traces) and 39°C (lower traces). They were diluted with 50-fold of the assay medium containing 0.2 mM Antipyryl azo III. Ca²⁺ release was measured at 23°C after addition of 5 μ M A23187 (+) using the calibration curve as shown in panel d.



Fig. 4. Dependence of the heat sensitivity of proteoliposomes on the protein weight ratio of scallop to rabbit SR in the reconstituted membrane. A. Proteoliposomes were reconstituted is described in "EXPERIMENTAL PROCEDURES" by mixing scallop and rabbit SR at a protein weight ratio of 0:1 (1), 0.4:1 (2), 0.6:1 (3), 1:1 (4), and 2:1 (5), respectively. Each sample was incubated at 39°C for 0 (lower traces) and 10 min (upper traces), and the time courses of Ca²⁺ transport were measured as given in Fig. 1. B. Heat inactivation was represented as a percentage and plotted against the protein weight ratio of scallop to rabbit SR. They were determined from the Ca²⁺ concentration difference at 1 min after addition of ATP (\downarrow) between the time courses of Ca²⁺transport in the control (lower traces) and heat-treated samples (upper traces). mixing pre-heated scallop SR with native rabbit SR at the protein weight ratios of 0:1 (panel a), 1:1 (panel b), and 2:1 (panel c). In each case, the control vesicles were reconstituted with native scallop and rabbit SR at the respective ratios, then these vesicles were incubated for 10 min at 39°C. It is clear from panel b or c of Fig. 5 that heat treatment of scallop SR before reconstitution did not inhibit the Ca²⁺-transport activity, whereas heat treatment after reconstitution strongly inhibited it. The former vesicles possess transport activity as high as those vesicles reconstituted with rabbit SR alone (panel a). On the other hand, heat treatment of rabbit SR either before or after reconstitution had virtually no effect on the Ca²⁺-transport as shown in panel a. These observations indicate that heat treatment of scallop SR before reconstitution resulted in failure of molecular interaction among the Ca²⁺-ATPases. In such a case, native Ca²⁺-ATPase of rabbit SR forms a functional dimer by itself in the reconstituted membrane.

In the preceding study (7), I found that when scallop SR was heated in the presence of 2 to 5 mM EGTA, the Ca²⁺-dependent ATP hydrolysis, as well as EP formation was almost completely protected against heat inactivation, although Ca²⁺- transport activity was completely lost. Since the protection was not seen when SR was heated in the presence of a free Ca²⁺ concentration higher than 0.5 μ M, I concluded that removal of Ca²⁺ from the high-affinity binding sites of the scallop ATPase resulted in complete protection against heat inactivation. The difference in thermolability of scallop ATPase between in the presence and absence of Ca²⁺ may be attributed to differences in protein structure. It would be interesting to know if the scallop Ca²⁺-ATPase can still interact with rabbit Ca²⁺-ATPase to form a dimer in the membrane when scallop SR is thermally treated in the presence of EGTA, then reconstituted it into proteoliposomes.

As shown in panel b of Fig. 6, the resulting proteoliposomes exhibited Ca^{2+} transport activity as high as that of the control vesicles which were reconstituted from native scallop and rabbit SR (panel a). The transport activity of the proteoliposomes was inhibited, in the similar manner to the control vesicles, by subsequent incubation at 39°C in the presence of Ca^{2+} (upper curve in panel a, b). For the experiments in panel c, proteoliposomes were reconstituted by mixing the native rabbit SR with scallop SR which had been heated in Ca^{2+} instead of EGTA. These vesicles similarly showed high Ca^{2+} -transport activity, but did not show any inhibition of the activity by further treatment at high temperature in the presence of Ca^{2+} (panel c). These findings suggest that the heat treatment of scallop SR in the presence of Ca^{2+} may prevent not only the activity of Ca^{2+} -transport but also the interaction between the ATPase molecules. On the other hand, heat treatment of SR in the absence of Ca^{2+} may fully protect the molecular interaction among the Ca^{2+} -ATPases in the reconstituted membrane.



Fig. 5. Reconstitution of heat-resistant proteoliposomes from pre-heated scallop SR and native rabbit SR. Lower traces: SR was heated in the presence of Ca²⁺ for 10 min before reconstitution. Proteoliposomes were reconstituted by mixing heat-inactivated scallop SR with intact rabbit SR at a ratio of 0:1 (a), 1:1 (b), and 2:1 (c). Upper traces: Proteoliposomes were reconstituted from native scallop and rabbit SR at the respective ratios given above, then incubated at 39°C for 10 min. The time courses of Ca²⁺ transport were measured under the conditions described in Fig. 1. (\downarrow), addition of ATP.



Fig. 6. Reconstitution of heat-labile proteoliposomes from scallop SR pre-heated in EGTA and native rabbit SR. Proteoliposomes were reconstituted by mixing native rabbit SR with native scallop SR (a), with SR pre-heated in the presence (b) or absence of 2 mM EGTA (c), at a protein weight ratio of 1:1. Ca^{2+} - transport activity was measured after heat treatment of these samples in the presence of 0.1 mM CaCl₂ for 0 (lower traces) and 10 min (upper traces). (\downarrow), addition of ATP.

DISCUSSION

Much work has been done on the molecular interaction among the Ca^{2+} -ATPases in the SR membrane by adopting a variety of methods and techniques such as electron microscopic observations (14-16), saturation transfer EPR (17-20), fluorescence spectroscopy (21, 22), chemical cross linking methods (23-25), and kinetic analysis (5, 27-31). However, the precise interactions remain poorly understood due to critical restrictions on the investigation conditions.

In the present study, I examined the temperature effects on the Ca²⁺-transport by reconstituted proteoliposomes consisting of heat-labile scallop SR and heat-stable rabbit one. If there is no interaction between their Ca²⁺-ATPase molecules in the reconstituted membrane, incubation of the vesicles at 39°C for 10 min in the presence of Ca²⁺ will inhibit only the Ca²⁺-transport catalyzed by the scallop Ca²⁺-ATPase, but not that by the rabbit one. In fact, however, heat treatment of the proteoliposomes resulted in almost complete attenuation of Ca²⁺-transport, indicating that it inactivated not only the scallop but also the rabbit SR Ca²⁺-ATPase (Fig. 2). Similar phenomenon was observed with the proteoliposomes reconstituted from the mixture of purified Ca²⁺-ATPases of scallop and rabbit SR (Data not shown). This observation supports our previous work (7) which suggests that the thermal treatment directly affects scallop Ca²⁺-ATPase.

It is unlikely that the heat denaturation of scallop Ca^{2+} -ATPase would increase the Ca^{2+} permeability of the reconstituted membrane to reduce the Ca^{2+} -transport activity, since the amount of Ca^{2+} released from the vesicles upon addition of a Ca^{2+} ionophore was slightly smaller in the thermally treated vesicles than the untreated ones (Fig. 3). In addition, the difference of the amount of Ca^{2+} release from the vesicles containing scallop and rabbit Ca^{2+} -ATPase was almost the same as that from the control vesicle containing rabbit Ca^{2+} -ATPase alone. These results well agreed with our previous observation that heat treatment of scallop SR in the presence of EGTA strongly inhibits Ca^{2+} -transport activity without a rise in Ca^{2+} permeability of the SR membrane (7).

The susceptibility of the proteoliposomes to heat inactivation increased as a function of the protein weight ratio of scallop to rabbit SR in the reconstituted membrane, and appeared to reach maximum at a ratio higher than 1 (Fig. 4). These

results suggest that, in the reconstituted membrane, each mol of Ca^{2+} -ATPases of scallop and rabbit SR may dominantly form a dimer as a minimum functional unit, and that the dimeric interaction between them may be involved in the catalytic cycle of Ca^{2+} transport. Heat treatment of reconstituted vesicles at 39°C for 10 min may easily destroy the dimeric interaction in the functional unit *via* denaturation of scallop Ca^{2+} -ATPase.

As shown in Fig. 5, when the scallop SR was heated in the presence of Ca^{2+} before reconstituting vesicles, the resulting vesicles still possessed Ca²⁺-transport activity as high as those reconstituted from rabbit SR alone. This finding can be interpreted that, since heat-denatured scallop ATPase does not form a dimer in the membrane, the remaining rabbit ATPase molecules will form a dimer by themselves to serve the Ca^{2+} pump. In the preceding study (7), I found that removal of Ca^{2+} by EGTA from the high-affinity sites on the scallop ATPase fully protected ATPase reaction, but not Ca²⁺-transport activity, against heat inactivation. In addition, several authors including as have suggested that removal of Ca²⁺ from the enzyme may stabilize it in an oligomeric form (7, 16, 23, 26). As shown in Fig. 6b, heat sensitive proteoliposomes were obtained when scallop SR was previously heated in EGTA, then reconstituted it into vesicle together with the same amount of rabbit intact SR. Since catalytic activity of scallop Ca²⁺-ATPase had been lost by the treatment (7), the Ca²⁺-transport into a vesicle would be catalyzed by rabbit ATPase in the reconstituted membrane. This phenomenon is interpreted that, scallop Ca²⁺-ATPase may be still capable to interact with rabbit one, and that the interaction was easily destroyed by the subsequent treatment of the vesicles at high temperature in the presence of Ca^{2+} . Work is underway to identify and characterize the heat-sensitive domain as well as the molecular interaction domain on scallop Ca²⁺-ATPase by adopting genetic method.

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