J. Biochem. 119, 1100-1105 (1996)

Protection of Scallop Sarcoplasmic Reticulum ATPase from Thermal Inactivation by Removal of Calcium from High-Affinity Binding Sites on the Enzyme[1]

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Received for publication, January 8, 1996

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 E2 to E1 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, were markedly protected from the heat inactivation. The uncoupling between 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 μM 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.

Key words: Ca^{2+}-ATPase, chemiosmotic uncoupling, heat inactivation, high-affinity Ca^{2+} binding, scallop muscle sarcoplasmic reticulum.

Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) couples the energy derived from ATP hydrolysis with the transport of Ca^{2+} across the SR membrane against a concentration gradient (*1*). In the presence of Ca^{2+}, 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 Ca^{2+} externally bound to high-affinity 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, we 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 Ca^{2+} transport and ATP hydrolysis activities are rapidly lost at temperatures higher than 37°C. In the present work, we found that susceptibility of ATP hydrolysis to heat could be prevented by removing 2 mol of Ca^{2+} 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 Ca^{2+}-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

[1] This study was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

Abbreviations: AMP-PNP, adenyl-5′-imidodiphosphate; C12E9, polyoxyethylene-9-laurylether; EP, phosphoenzyme; NCD-4, *N*-cyclohexyl-*N*′-(4-dimethylamino-1-naphthyl)carbodiimide; PEP, phosphoenolpyruvate; SR, sarcoplasmic reticulum; TCA, trichloroacetic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonate.

nitrogen, and stored at −80°C. Pyruvate kinase, lactate dehydrogenase, NADH, PEP were purchased from Boehringer Mannheim. [γ-^{32}P]ATP was purchased from Amersham, $^{45}CaCl_2$ 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 $MgCl_2$, 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—Ca^{2+} transport by SR was measured in a medium containing 0.025-0.05 mg/ml SR protein, 0.1 M KCl, 5 mM $MgCl_2$, 5-10% glycerol, 40 μM $CaCl_2$, 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. Ca^{2+} 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 Ca^{2+}-transport assay except that the Ca^{2+} indicator was omitted and 2-5 μM A23187 was added to the reaction mixture. ATP hydrolysis was measured in the presence of an ATP-regenerating system (0.5 mM NADH, and 1.5 mM PEP, 0.1-1 unit of lactate dehydrogenase, and 0.2-2 units 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 Ca^{2+}-ATPase—SR, 0.05-0.1 mg/ml, was phosphorylated with 10-100 μM AT^{32}P 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 described previously (*17*).

E2-E1 transition was measured as a fluorescence change of tryptophan in Ca^{2+}-ATPase after addition of 2 mM $CaCl_2$ 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 C12E9. The suspension was centrifuged at 540,000 × *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 × *g* for 30 min to precipitate reconstituted SR vesicles.

RESULTS

Inactivation of Ca^{2+} Transport and ATP Hydrolysis by Heat Treatment of SR in the Presence of Ca^{2+}—Two milligrams/ml SR was preincubated at 38°C for various time periods in 0.1 ml of reaction medium containing 0.1 mM $CaCl_2$, 10% glycerol, 0.1 M KCl, 20 mM TES (pH 7.2), and 5 mM $MgCl_2$, then rapidly transferred into liquid nitrogen to stop the heat treatment. Ca^{2+} transport assay was carried out at 23°C under the conditions described in "EXPERIMENTAL PROCEDURES." The Ca^{2+} 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

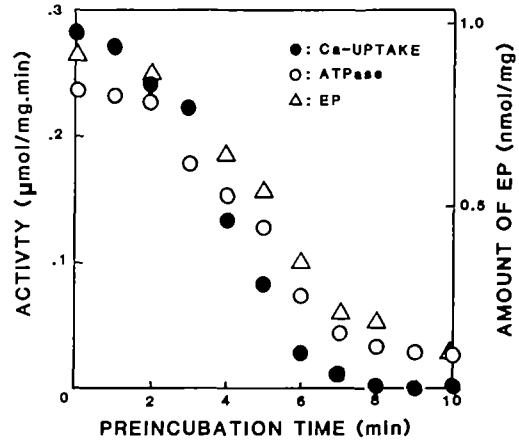

Fig. 1. **Time courses of heat inactivation of Ca^{2+}-transport, ATP hydrolysis, and EP formation.** SR was added at 38°C to the preincubation medium containing 0.1 mM $CaCl_2$ to a final concentration of 2 mg/ml. At the indicated times, 0.1 ml of the SR suspension was immediately frozen in liquid nitrogen to terminate the thermal treatment. Measurements of Ca^{2+}-transport (●), ATP hydrolysis (○), and EP formation (△) were carried out at 23°C as described in "EXPERIMENTAL PROCEDURES."

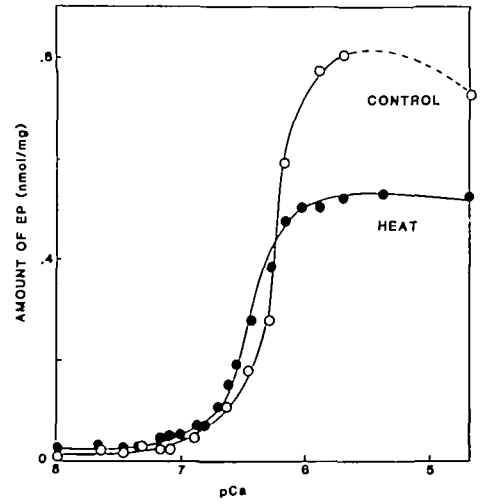

Fig. 2. **Effect of heat treatment of SR on the Ca^{2+} concentration dependence of EP formation.** SR was preincubated at 38°C (●) or 23°C (○) for 6 min in the presence of 0.1 mM $CaCl_2$, then centrifuged to wash the SR membrane. Phosphorylation of ATPase was initiated at 23°C by adding 1 mM [γ-^{32}P]ATP in the presence of 0.1 mM $CaCl_2$. Five seconds later, the reaction was terminated by addition of 5% TCA, and the amount of EP was determined as described in "EXPERIMENTAL PROCEDURES."

activity of Ca^{2+}-dependent ATPase decreased essentially in parallel to that of Ca^{2+} 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 Ca^{2+} 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 K_d and Hill number of about 0.2 μM 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 Ca^{2+}.

Protection of ATPase from Thermal Inactivation in the Presence of EGTA—The Ca^{2+}-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 E2-E1 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 E2-E1 transition on the Ca^{2+} concentration of the preincubation medium. Scallop SR was preincubated at 38°C for 10 min at different concentrations of Ca^{2+} from pCa 9 to 5. Significant enhancement of the thermal inactivation was observed by increasing the concentration of Ca^{2+} above pCa 6, while maximal activities of both ATP hydrolysis and E2-E1 transition were retained even after 12 min of thermal treatment below pCa 8. The plots of the remaining activities of ATP hydrolysis and E2-E1 transition against pCa of the preincubation medium revealed typical sigmoidal curves with a dissociation constant and Hill number of about 0.5 μM and 1.6-1.8, respectively. Essentially the same sigmoidal curve of Ca^{2+} dependence was observed for EP formation (data not shown). These observations indicate that removal of 2 mol of Ca^{2+} from the high-affinity sites on the Ca^{2+}-ATPase or fixing the enzyme in the E2 state might be required to make this enzyme resistant to heat inactivation.

Uncoupling of Ca^{2+} 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 Ca^{2+} transport activity. These results support the possibility that the thermally sensitive region for the Ca^{2+} transport is different from that for ATP hydrolysis. Uncoupling of the Ca^{2+}

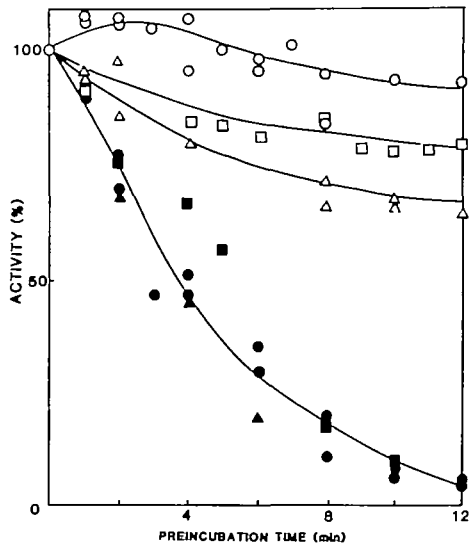

Fig. 3. **Protection of ATP hydrolysis, EP formation, and E2-E1 transition against thermal treatment of SR in EGTA.** SR was preincubated at 38°C in the presence of 5 mM EGTA (open symbols) or 0.1 mM $CaCl_2$ (closed symbols) for the indicated times. These samples were washed by centrifugation, and resuspended in the assay medium. ATP hydrolysis (○, ●), EP formation (△, ▲), and E1-E2 transition (□, ■) were measured as described in "EXPERIMENTAL PROCEDURES." All the activities were presented as a percentage of those obtained with the untreated SR.

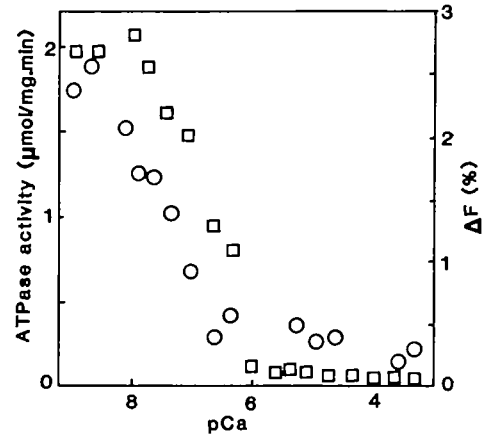

Fig. 4. **Ca^{2+} concentration dependence of heat sensitivity of Ca^{2+}-ATPase and E2-E1 transition.** SR was preincubated at 38°C for 10 min in the presence of various concentrations of Ca^{2+}. Ca^{2+}-ATPase (□) and E2-E1 transition (○) activities were measured as described in "EXPERIMENTAL PROCEDURES."

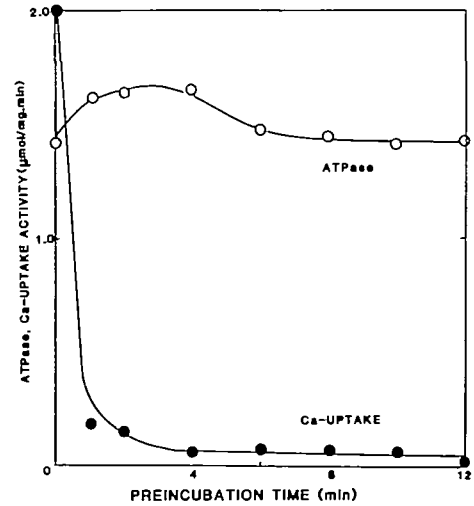

Fig. 5. **Uncoupling of ATP hydrolysis from Ca^{2+} transport after thermal treatment of SR in the presence of EGTA.** SR was preincubated at 38°C in the presence of 2 mM EGTA for the indicated times. ATP hydrolysis (○) and Ca^{2+} transport (●) were measured as described in "EXPERIMENTAL PROCEDURES."

transport from the ATPase reaction may lead to an increase in the Ca^{2+} permeability of the SR membrane. In order to test this possibility, we examined the leakiness of the SR membrane to Ca^{2+} 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 $^{45}Ca^{2+}$ by incubation with 10 mM $^{45}Ca^{2+}$ for 5 h at 0°C, or for 2 h at 23°C. After the Ca^{2+} 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.

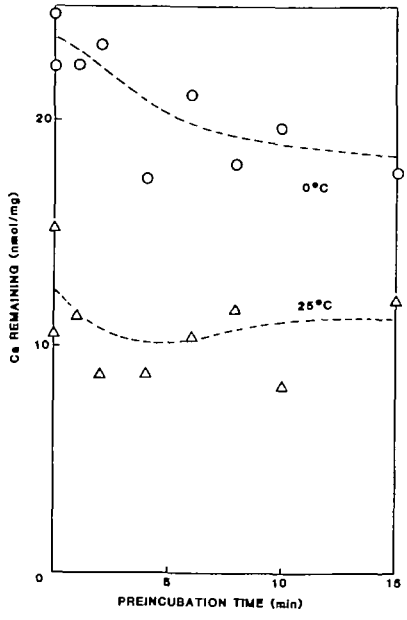

Fig. 6. **Effects of heat treatment on the Ca^{2+} permeability of SR membrane.** SR was treated at 38°C for the indicated times as presented in Fig. 5, then the SR vesicles were loaded with 10 mM $^{45}Ca^{2+}$ at 0°C for 5 h (○) or at 25°C for 2 h (△). The SR suspension was transferred into 20 volumes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

The amount of $^{45}Ca^{2+}$ 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 Ca^{2+} 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 Ca^{2+} transport with no effect on ATPase activity. With rabbit SR, however, neither ATP hydrolysis nor Ca^{2+} transport was destroyed by incubation with Ca^{2+} 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.

Role of Membrane Structure in Thermal Stabilization of Ca^{2+}-ATPase in EGTA—To test whether or not EGTA protection of Ca^{2+}-ATPase from heat inactivation requires some membrane structure of SR, we compared the thermal lability of the ATPase solubilized in C12E9 and the recon-

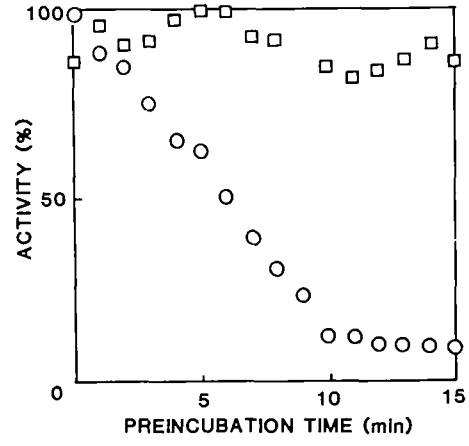

Fig. 8. **Effects of thermal treatment of SR in the presence of AMP-PNP on the ATP hydrolysis and Ca^{2+} transport.** SR (5 mg/ml) was preincubated at 38°C in the presence of 0.1 mM $CaCl_2$, and 1 mM AMP-PNP for the indicated time, then washed by centrifugation to remove the nucleotide. ATP hydrolysis (□) and Ca^{2+} transport (○) were measured as presented in Fig. 5.

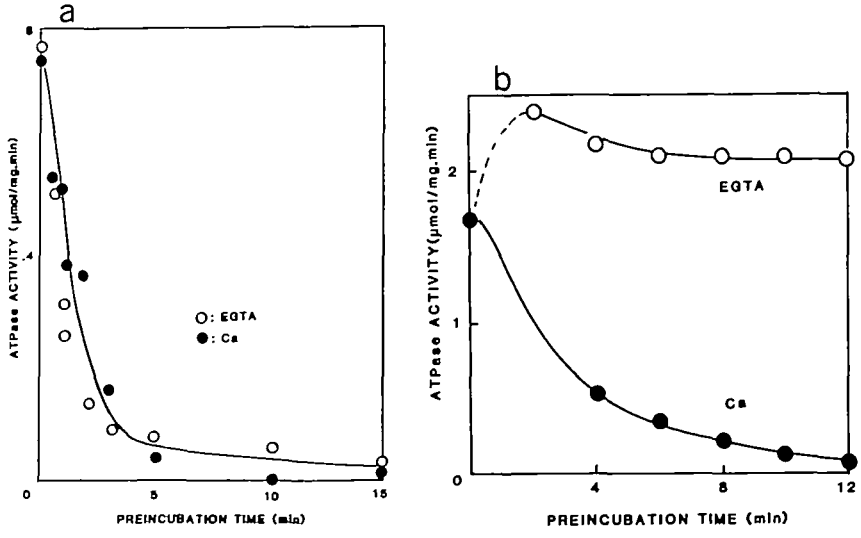

Fig. 7. **a: Inactivation of the solubilized ATPase by thermal treatment in the presence and absence of Ca^{2+}.** SR at 2 mg/ml was solubilized with 30 mg/ml C12E9, then preincubated in the presence of 0.1 mM $CaCl_2$ (●) or 2 mM EGTA (○) at 38°C for the indicated times. The SR suspension was added to 20 volumes of assay medium. ATPase activity was measured at the final Ca^{2+} concentration of 0.1 mM as described in "EXPERIMENTAL PROCEDURES." **b: Restoration of thermal resistance of the Ca^{2+}-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 $CaCl_2$ (●) or 5 mM EGTA (○) for the indicated times. ATPase activity of the reconstituted SR was measured as described in (a).

stituted 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 Ca^{2+}-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 Ca^{2+} transport with complete retention of ATP hydrolytic activity. The Ca^{2+}-ATPase activity shows strong resistance to the heat treatment in spite of the presence of 0.1 mM Ca^{2+}. Other nucleotides (ADP and AMP) up to 10 mM showed no effect on the SR functions (data not shown). We examined the effects of other reagents such as β-mercaptoethanol, dithiothreitol, vanadate, and polyclonal antibody against the Ca^{2+}-ATPase purified from scallop SR, but they failed to offer protection against heat inactivation of SR catalytic functions.

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 Ca^{2+} transport and ATPase activities of scallop SR were almost completely lost within 10 min of incubation at 38°C in the presence of Ca^{2+}. Based on preliminary tests, it was considered that the thermal treatment of SR directly affects the Ca^{2+}-ATPase in the SR membrane, because the thermal inactivation was still observed when Ca^{2+}-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 high-affinity 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 E2 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 E2 state (*22-25*). In addition, the heat treatment of SR with 1 mM AMP-PNP, which stimulates the conversion of the enzyme from the E2 to the E1 state (*26*), completely protected Ca^{2+}-ATPase from thermal lability (Fig. 8).

As shown in Fig. 7a, when SR membrane was destroyed by C12E9, the Ca^{2+}-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 Ca^{2+}-ATPase requires not only removal of Ca^{2+} from the enzyme but also correct location in the SR membrane.

Based on these considerations, we 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 spectrometric 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 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 Ca^{2+} at physiological concentrations. They observed that nonhydrolyzable nucleotides affected the crystal formation. These observations are consistent with our present results. Moreover, our previous study (*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 Ca^{2+}-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, we 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.

We thank Dr. Seiki Kuramitsu of Osaka University for valuable discussions regarding this work.

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