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Temperature Sensitivity of Proteoliposomes Reconstituted from a Mixture of Scallop and Rabbit Sarcoplasmic Reticulum Ca^{2+}-ATPases[1]

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

Key words: Ca^{2+}-ATPase, molecular interaction, reconstitution, sarcoplasmic reticulum, temperature sensitivity.

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

In the preceding study, we investigated the heat inactivation of scallop Ca^{2+}-ATPase under a variety of conditions, and concluded that the thermal lability of its catalytic function may be 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, we reconstituted proteoliposomes by mixing the Ca^{2+}-ATPases 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 is no interaction between the two ATPases in the membrane, we could expect that the Ca^{2+}-transport activity of the reconstituted vesicle would additively change depending on the respective heat sensitivities. However, incubation of the vesicles at 39°C for 10 min almost completely eliminated their Ca^{2+}-transport activity, whereas heat treatment of 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*). Each SR was suspended in medium comprising 50 mM KCl, 10% glycerol, and 20 mM TES (pH 7.3), and then divided into small pieces. The

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Abbreviations: AMP-PNP, adenyl-5′-imidodiphosphate; $C_{12}E_9$, polyoxyethylene-9-laurylether; EP, phosphoenzyme; SR, sarcoplasmic reticulum; TES, *N*-tris(hydroxymethyl)methyl-2-aminomethane sulfonate.

latter were quickly frozen in liquid nitrogen and then stored at −80°C. The Ca^{2+}-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 1 ml of reconstitution medium comprising 5 mM $MgCl_2$, 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 the indicated weight ratios, the 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 the addition of 45 mg/ml $C_{12}E_9$. After several minutes, 40 mg/ml soybean asolectin solubilized in 45 mg/ml $C_{12}E_9$ was added to the medium. The SR mixture was further incubated at 20°C for 15-20 min under a continuous supply of N_2 gas, and 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 N_2 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 kg 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—The SR suspension (0.1 ml) was transferred to incubation medium comprising 5 mM $MgCl_2$, 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 times, the heat treatment was stopped by transferring the mixture to ice-cold assay medium.

Measurement of Ca^{2+} Transport—Ca^{2+} transport was measured at 23°C in an assay medium comprising SR or reconstituted vesicles at 0.02-0.1 mg/ml protein, 20-50 μM $CaCl_2$, and 0.2 mM Antipyrylazo III, as a Ca^{2+} indicator. The reaction was initiated by the addition of 0.2 mM ATP. The change in the Ca^{2+} concentration in the reaction mixture was continuously monitored by measuring the absorption of the Ca^{2+} indicator at 700 nm.

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

RESULTS

Sarcoplasmic reticulum isolated from cold water fish muscles, including scallop adductor muscle, exhibits higher sensitivity to temperature than that from mammal muscles (*8, 12, 13*). Figure 1 compares the time courses of Ca^{2+} transport by SR isolated from rabbit and scallop muscles after their pre-incubation at 39°C for 0, 5, and 10 min in medium comprising 5 mM $MgCl_2$, 0.1 M KCl, 5% glycerol, and 50 mM TES (pH 6.8). Note that the pre-incubation medium was usually contaminated by several μM Ca^{2+} under the conditions used, except when it was removed by the addition of 2-4 mM EGTA. The Ca^{2+}-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 thermolability between scallop and rabbit SR offers us a mean of directly examining the molecular interaction of Ca^{2+}-ATPase within the SR membrane.

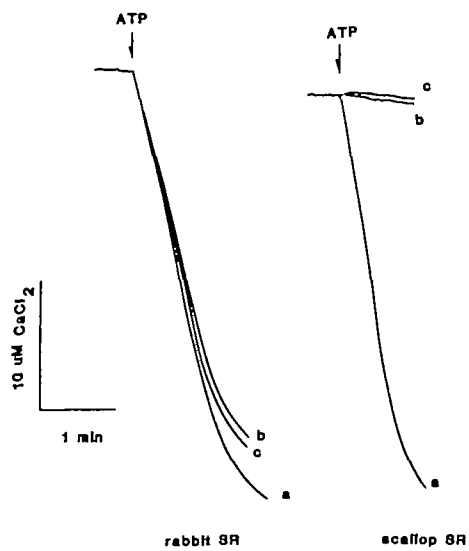

Fig. 1. **Effects of heat treatment on Ca^{2+} transport by rabbit and scallop SR.** SR was added at 39°C to preincubation medium comprising 5 mM $MgCl_2$, 0.1 M KCl, 5% glycerol, and 50 mM TES (pH 6.8) to a final concentration of 1 mg/ml. At 0 (a), 5 (b), and 10 min (c), the SR suspension was rapidly cooled in ice water to stop the heat treatment. Ca^{2+}-transport was initiated at 23°C by the addition of 0.2 mM ATP (↓) to the assay solution containing 50 μM $CaCl_2$, 5 mM oxalate, 5 mM $MgCl_2$, 0.1 M KCl, 5% glycerol, and 50 mM TES (pH 6.8) in the presence of 0.2 mM Antipyrylazo III and 20 μg/ml of SR.

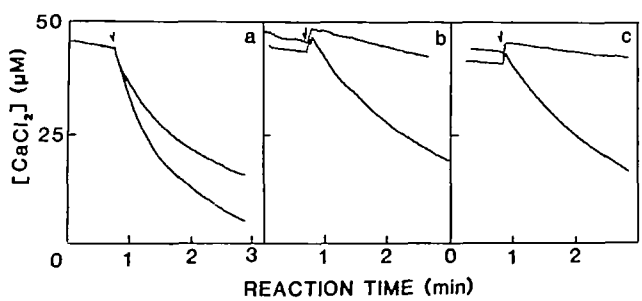

Fig. 2. **Effect of heat treatment on Ca^{2+} transport by proteoliposomes reconstituted from scallop and rabbit SR.** Proteoliposomes were reconstituted as described under "EXPERIMENTAL PROCEDURES" by mixing scallop and rabbit SR at protein weight ratios of 0:1 (a), 1:1 (b), and 1:0 (c). The proteoliposomes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

In the following experiments, we 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 Ca^{2+} transport by the reconstituted vesicles before and after their incubation at 39°C for 10 min. The velocity of the Ca^{2+} transport decreased with increasing reaction time, probably due to the formation of some membrane potential accompanied by Ca^{2+} movement across the reconstituted membrane. The transport activity of the proteoliposomes reconstituted with rabbit SR alone (panel a) only slightly decreased on pre-incubation at 39°C for 10 min, while the activity of the proteoliposomes reconstituted with scallop SR completely disappeared with the same 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, the Ca^{2+}-transport activity decreased to almost the same level as that observed for the control vesicles reconstituted with scallop SR alone (panel c). If we assume that the Ca^{2+} transport can be independently catalyzed by the scallop and rabbit Ca^{2+}-ATPases in the reconstituted membrane, almost the same activity as observed for the heated vesicles containing rabbit SR alone should remain after the heat treatment (compare the upper traces in panels a and b). Furthermore, it does not seem that Ca^{2+} transport activity of the untreated vesicles was additively changed by mixing rabbit and scallop SR.

These results suggest that scallop and rabbit Ca^{2+}-ATPase 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 the Ca^{2+} permeability of the membrane *via* the scallop Ca^{2+}-ATPase. In the experiments presented in Fig. 3, we 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 $CaCl_2$. The Ca^{2+}-loaded proteoliposomes were further incubated at 39°C for 10 min. Panels a, b, and c in Fig. 3 show the time courses of Ca^{2+} release from the proteoliposomes reconstituted, respectively, with rabbit SR alone, a 1:1 mixture of scallop and rabbit SR, and scallop SR alone, after the addition of 5 μM A23187. Upon addition of the ionophore, Ca^{2+} was rapidly released from the proteoliposomes and reached a steady level within 30 s. As judged from the calibration curve of the Ca^{2+} concentration in panel d, about 15 μM Ca^{2+} was released from each sample of reconstituted vesicles.

The amount of Ca^{2+} released from the thermally treated

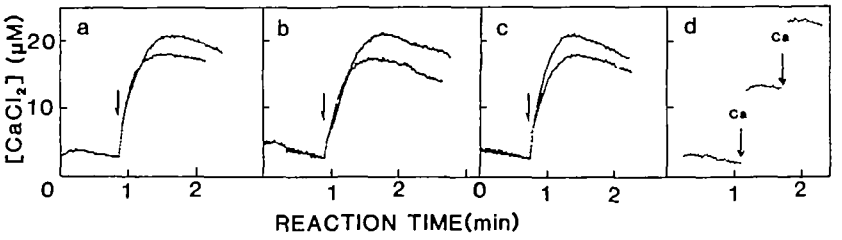

Fig. 3. **Ca^{2+} release from Ca^{2+}-loaded proteoliposomes after the addition of a Ca^{2+} ionophore.** Proteoliposomes were reconstituted by mixing scallop and rabbit SR at ratios of 0:1 (a), 1:1 (b), and 1:0 (c) in the solution described under "EXPERIMENTAL PROCEDURES" except for substitution of 10 mM $CaCl_2$ for oxalate. The Ca^{2+}-loaded proteoliposomes were incubated for 10 min at 23°C (upper traces) or 39°C (lower traces). They were diluted with 50-fold of the assay medium containing 0.2 mM Antipyrylazo III. Ca^{2+} release was measured at 23°C after the addition of 5 μM A23187 (↓) using the calibration curve in panel d.

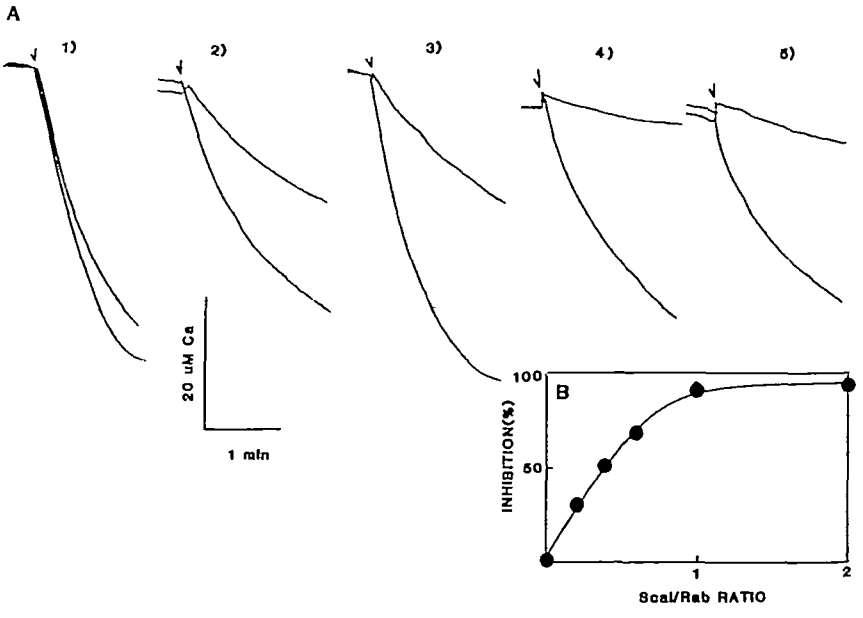

Fig. 4. **Dependence of the heat sensitivity of proteoliposomes on the protein weight ratio of scallop to rabbit SR in the reconstituted membrane.** A: Proteoliposomes were reconstituted as described under "EXPERIMENTAL PROCEDURES" by mixing scallop and rabbit SR at protein weight ratios of 0:1 (1), 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) or 10 min (upper traces), and the time courses of Ca^{2+} transport were measured as described in Fig. 1. B: Heat inactivation is represented as a percentage and plotted against the protein weight ratio of scallop to rabbit SR. Intensity of the inactivation is determined from the difference in the Ca^{2+} level at 1 min after the addition of ATP (↓) between the time courses of Ca^{2+} transport in the control (lower traces) and heat-treated samples (upper traces).

vesicles was about 20% lower than that from untreated vesicles, possibly due to loss of internal Ca^{2+} during the treatment. A 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 (panels b and c). These results do not support the possibility that the heat inactivation of Ca^{2+} transport by proteoliposomes is caused by an increase in Ca^{2+} leakage through 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 to rabbit SR, and appeared to reach the maximal level at a ratio higher than 1 (Fig. 4B). 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.

We decided to determine 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 mixing pre-heated scallop SR with native rabbit SR at 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, and then the vesicles were incubated for 10 min at 39°C. It is clear from panels b and c in Fig. 5 that heat treatment of scallop SR before reconstitution did not inhibit the Ca^{2+}-transport activity, whereas heat treatment after reconstitution strongly inhibited it. The former vesicles possessed as high transport activity as the 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 Ca^{2+} 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 Ca^{2+}-ATPases. In such a case, native Ca^{2+}-ATPase of rabbit SR forms a functional dimer by itself in the reconstituted membrane.

In the preceding study (7), we found that when scallop SR was heated in the presence of 2 to 5 mM EGTA, Ca^{2+}-dependent ATP hydrolysis, as well as EP formation was almost completely protected against heat inactivation, although Ca^{2+}-transport activity was completely lost. Since this protection was not seen when SR was heated in the presence of a free Ca^{2+} concentration higher than 0.5 μM, we concluded that removal of Ca^{2+} 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 Ca^{2+} may be attributed to differences in protein structure. It would be interesting to know if the scallop Ca^{2+}-ATPase can still interact with rabbit Ca^{2+}-ATPase to form a dimer in the membrane when scallop SR is thermally treated in the presence of EGTA and then reconstituted into proteoliposomes.

As shown in panel b in Fig. 6, the resulting proteoliposomes exhibited as high Ca^{2+}-transport activity as that of the control vesicles reconstituted from native scallop and rabbit SR (panel a). The transport activity of the proteoliposomes was inhibited, in a similar manner to in the case of the control vesicles, on subsequent incubation at 39°C in the presence of Ca^{2+} (upper curves in panels a and 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 on further treatment at high temperature in the presence of Ca^{2+} (panel c).

These findings suggest that the heat treatment of scallop

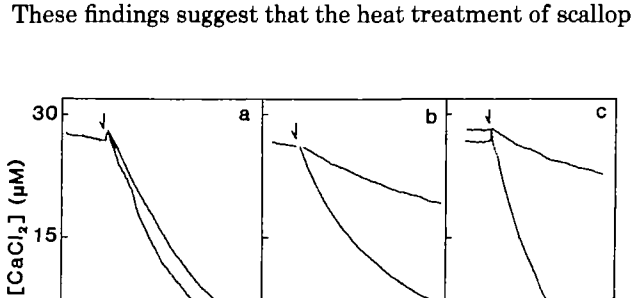

Fig. 5. **Reconstitution of heat-resistant proteoliposomes from pre-heated scallop SR and native rabbit SR.** Lower traces: SR was heated for 10 min in the presence of Ca^{2+} before reconstitution. Proteoliposomes were reconstituted by mixing heat-inactivated scallop SR with intact rabbit SR at ratios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

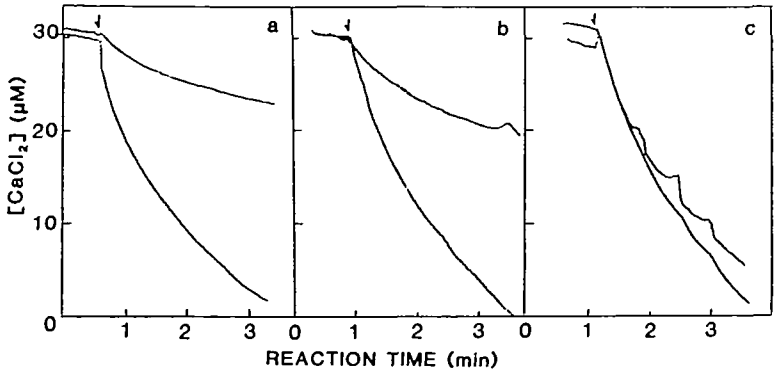

Fig. 6. **Reconstitution of heat-labile proteoliposomes from scallop SR pre-heated in EGTA and native rabbit SR.** Proteoliposomes were reconstituted by mixing native rabbit SR with native scallop SR (a), or with the SR pre-heated in the presence (b) or absence of 2 mM EGTA (c), at a protein weight ratio of 1:1. Ca^{2+}-transport activity was measured after heat treatment of these samples in the presence of 0.1 mM $CaCl_2$ for 0 (lower traces) or 10 min (upper traces). (J) addition of ATP.

SR in the presence of Ca^{2+} may prevent not only the ability 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 between the Ca^{2+}-ATPases in the reconstituted membrane.

DISCUSSION

Much work has been done on the molecular interaction between the Ca^{2+}-ATPases in the SR membrane, with a variety of methods and techniques such as electron microscopic observation (*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 interaction remains poorly understood due to critical restrictions of the investigation conditions.

In the present study, we examined the effect of temperature on the Ca^{2+} transport by reconstituted proteoliposomes consisting of heat-labile scallop SR and heat-stable rabbit one. If there is no interaction between these Ca^{2+}-ATPase molecules in the reconstituted membrane, incubation of the vesicles at 39°C for 10 min in the presence of Ca^{2+} will only inhibit the Ca^{2+} transport catalyzed by the scallop Ca^{2+}-ATPase, *i.e.* not that by the rabbit enzyme. In fact, however, heat treatment of the proteoliposomes resulted in almost complete abolition of Ca^{2+}-transport, indicating that it inactivated not only the scallop but also the rabbit SR Ca^{2+}-ATPase (Fig. 2). A similar phenomenon was observed with the proteoliposomes reconstituted from the mixture of purified Ca^{2+}-ATPases of scallop and rabbit SR (data not shown). This observation supports our previous work (*7*), which suggested that the thermal treatment directly affects scallop Ca^{2+}-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 the addition of a Ca^{2+} ionophore was slightly smaller for the thermally treated vesicles than the untreated ones (Fig. 3). In addition, the difference in the level of Ca^{2+} release from the vesicles containing scallop and rabbit Ca^{2+}-ATPases was almost the same as that from the control vesicles 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 an increase in the 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 the maximum at a ratio higher than 1 (Fig. 4). These results suggest that, in the reconstituted membrane, each molecule of the Ca^{2+}-ATPases of scallop and rabbit SR may predominantly 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 the reconstitution of vesicles, the resulting vesicles still possessed as high Ca^{2+}-transport activity as those reconstituted with rabbit SR alone. This finding can be interpreted as 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*), we found that removal of Ca^{2+} with EGTA from the high-affinity sites on the scallop ATPase fully protected the ATPase reaction, but not Ca^{2+}-transport activity, against heat inactivation. In addition, several authors including us have suggested that removal of Ca^{2+} 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, and then reconstituted into vesicles together with the same amount of intact rabbit SR. Since the catalytic activity of scallop Ca^{2+}-ATPase was lost on the treatment (*7*), the Ca^{2+} transport into a vesicle would be catalyzed by rabbit ATPase in the reconstituted membrane. The phenomenon illustrated in Fig. 6b is interpreted as that scallop Ca^{2+}-ATPase may still be able to interact with the rabbit enzyme, and that the interaction is easily destroyed on the subsequent treatment of the vesicles at high temperature in the presence of Ca^{2+}. In Fig. 6c, scallop SR was heated in Ca^{2+} before the reconstitution of vesicles. The resulting vesicles lost the heat sensitivity, probably due to a lack of interaction with rabbit Ca^{2+}-ATPase in the reconstituted membrane.

Work is underway to identify and characterize the heat-sensitive domain as well as the molecular interaction domain of scallop Ca^{2+}-ATPase by means of a genetic method.

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