PROTEIN-PROTEIN AND PROTEIN-LIPID INTERACTIONS IN Ca^{2+} PUMP ATPase OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM

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ABBREVIATIONS

SR	sarcoplasmic reticulum
FSR	fragmented SR
T-tubule	transverse tubule
Ca^{2+}-ATPase	Ca^{2+}, Mg^{2+}-dependent adenosine triphosphatase
EGTA	ethylene glycol bis(-aminoethyl ether)-N, N, N', N'-tetraacetic acid
EDTA	ethylenediaminetetraacetic acid
EP	phosphoenzyme
TCA	trichloroacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
E_1P	ADP-sensitive EP
E_2P	ADP-insensitive EP
ATP	adenosinetriphosphate
ADP	adenosinediphosphate
P_i	inorganic phosphate
$C_{12}E_8$	octa(ethylene glycol) monododecyl ether
$C_{12}E_9$	polyoxyethylene-9-raulylether
RSR	reconstituted SR
TES	N-Tris(hydroxymethyl)methyl-2-aminomethanesulfonate
FITC	fluorescein isothiocyanate
TNP-ATP	2'(3')-O-(2,4,6-trinitrophenyl)-ATP

GENERAL INTRODUCTION

The intracellular Ca^{2+} concentration in muscle cells is maintained at less than 1 uM in the resting state. This is 10^3-10^4 times lower than the extracellular Ca^{2+} concentration. This allows intracellular Ca^{2+} to play an essential role as a trigger for the important physiological processes. The cycle of contraction and realxation in skeletal muscle fiber is induced by the rapid increase and the subsequent decrease in the intracellular Ca^{2+} concentration.

The regulation of the intracellular Ca^{2+} concentration is carried out by Ca^{2+} pumps present in the plasma membrane and in the intracellular membrane system, the sarcoplasmic reticulum (SR), which functions as a large capacity of Ca^{2+} store.

The membrane system in muscle cells consists of the plasma membrane with its tubular infoldings (the T-system) (1) running transversely to the fiber axis and a reticular structure (SR) that forms a network surrounding the myofibrils(Fig. 1). The T-system and SR are interconnected in a junctional structure called "triad". When T-system communicates the depolarization of the plasma membrane to the interior of the muscle cell, Ca^{2+} is released from SR through a Ca^{2+} channel to bind with troponin located on thin filaments and induces muscle contraction. When Ca^{2+} is reaccumulated by SR, the muscle relaxes. These process is called as "excitation-contraction coupling".

Biochemical studies on the mechanisms for ATP hydrolysis and Ca^{2+} transport in the SR membrane system have been done mostly using fragmented SR (FSR) isolated from muscle homogenates as a microsomal fraction. The SR vesicles can uptake Ca^{2+} in the presence of Ca^{2+} and Mg-ATP in the outside medium. The SR

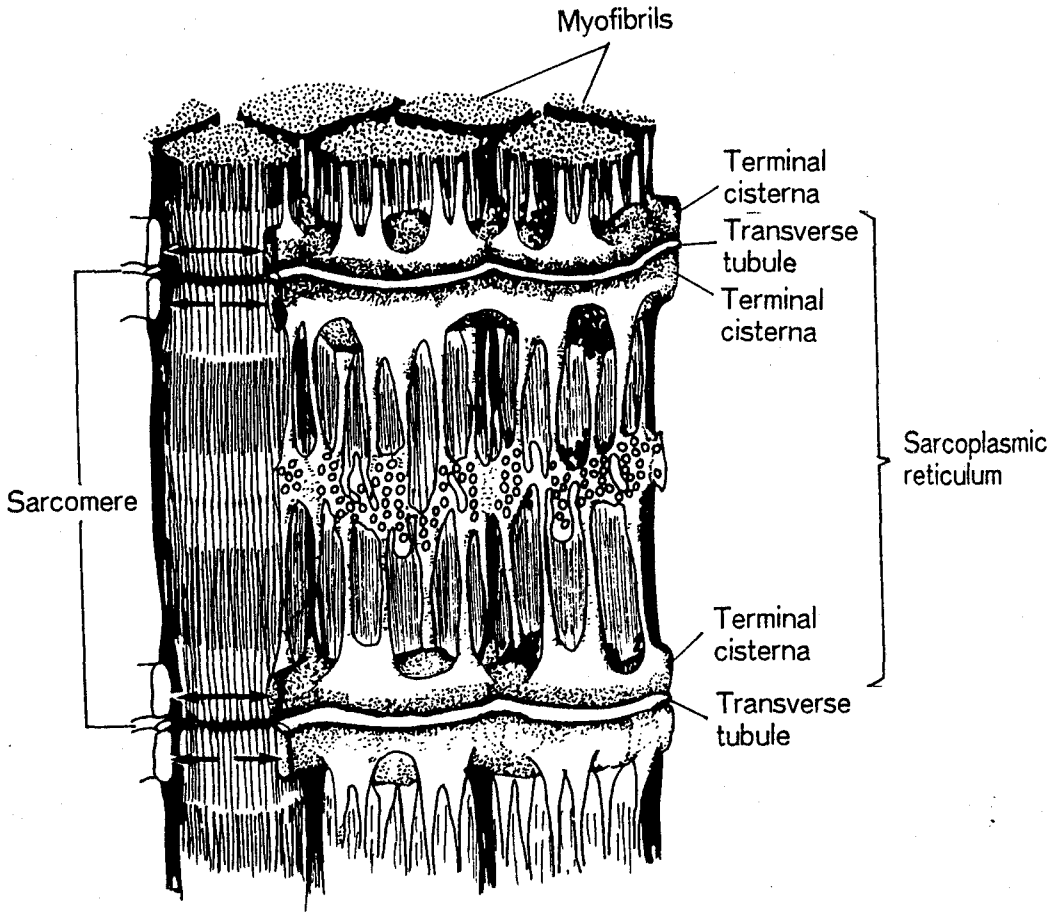

FIGURE 1. The internal membrane system of a frog sartorius muscle cell. From Peachey, L. D. (1965) J. Cell Biol., 25, 209-232.

vesicle consists of mainly phospholipids and the Ca^{2+} pump protein, Ca^{2+}-ATPase, whose molecular weight is about 110000. By using a reconstitution method from purified ATPase and excess amount of phospholipid, Racker (2) directly demonstrated that the ATPase serves as a Ca^{2+} translocator at the same time as an energy transducer.

The existance of a high-energy, phosphorylated intermediate was proposed by Hasselbach and Makinose (3) and by Ebashi and Lipman (4) who found that FSR catalized phosphate exchange between ADP and ATP in the presence of Ca^{2+} and Mg^{2+}. Later, Yamamoto and Tonomura (5) and Makinose (6) found that SR protein was phosphorylated in the presence of ATP and Ca^{2+}, and isolated the phosphorylated intermediate(EP) as an acid-stable phosphoprotein. This finding facilitated studies on the coupling mechanism between Ca^{2+} transport and the ATPase reaction in SR membrane. Transient kinetic studies on the formation and decomposition of EP have been performed by Kanazawa et al. (7), Froehlich and Taylor(8,9), and Kurzmack and Inesi(10). Sumida and Tonomura (11) directly showed that the translocation of Ca^{2+} across the SR membrane was coupled with the formation of EP. Makinose (12) and Yamada et al. (13) demonstrated that ATPase reaction of SR is reversible. Yamada and Tonomura (14) and Ikemoto (15,16) demonstrated that the apparent affinity of the enzyme for Ca^{2+} in the presence of Mg^{2+} decreased drastically when it was phosphorylated. Shigekawa and Dougherty (17) and Takisawa and Tonomura (18) reported that two kinds of EP are formed sequentially, with respect to the reactivity with ADP,

ADP-sensitive EP and ADP-insensitive EP. The reaction mechanism of coupling of ATP hydrolysis with Ca^{2+}transport across the SR membrane has been postulated so far as followings (Fig. 2). Two moles of Ca^{2+} and one mole of ATP bound to one mole of ATPase at active sites on the outer surface of the membrane (E_1 form) (19). Calcium ions are occluded by the enzyme when E_1P(ADP-sensitive EP) is formed (20). Then, affinity of the enzyme for calcium are decreased with the transition from E_1P to E_2P (ADP-insensitive EP), which results in Ca^{2+} release from the enzyme to intravesicular lumen (21). Dephosphorylation of EP results in E_2 form without bound Ca^{2+}.

To elucidate the molecular mechanism of active transport, it is essential to describe the structual features of the protein that allow coupling between the spacially distinct sites of nucleotide hydrolysis and ion transport. When SR was exposed briefly to trypsin, the ATPase protein was degraded into two fragments with molecular weights of about 55000 and 45000 (22). Further digestion degraded 55000 subfragment into two fragments with molecular weights of about 30000 and 20000 (23). And these fragments seemed to be corresponded to nucleotide binding domain (45 k fragment), phosphorylation domain (30 k fragment), and Ca^{2+} binding domain (20 k fragment). These postulations were made mostly from chemical modification studies as fluorescein isothiocyanate (FITC)labelled to 45 k fragment (24) and dicyclohexylcarbodiimide (DCCD) labelled to 20 k fragment (25) inhibited ligand binding. The Asp residue that undergoes Ca^{2+}-dependent phosphorylation by ATP is located on the 30 k fragment. Recently, the entire amino acid sequence of the

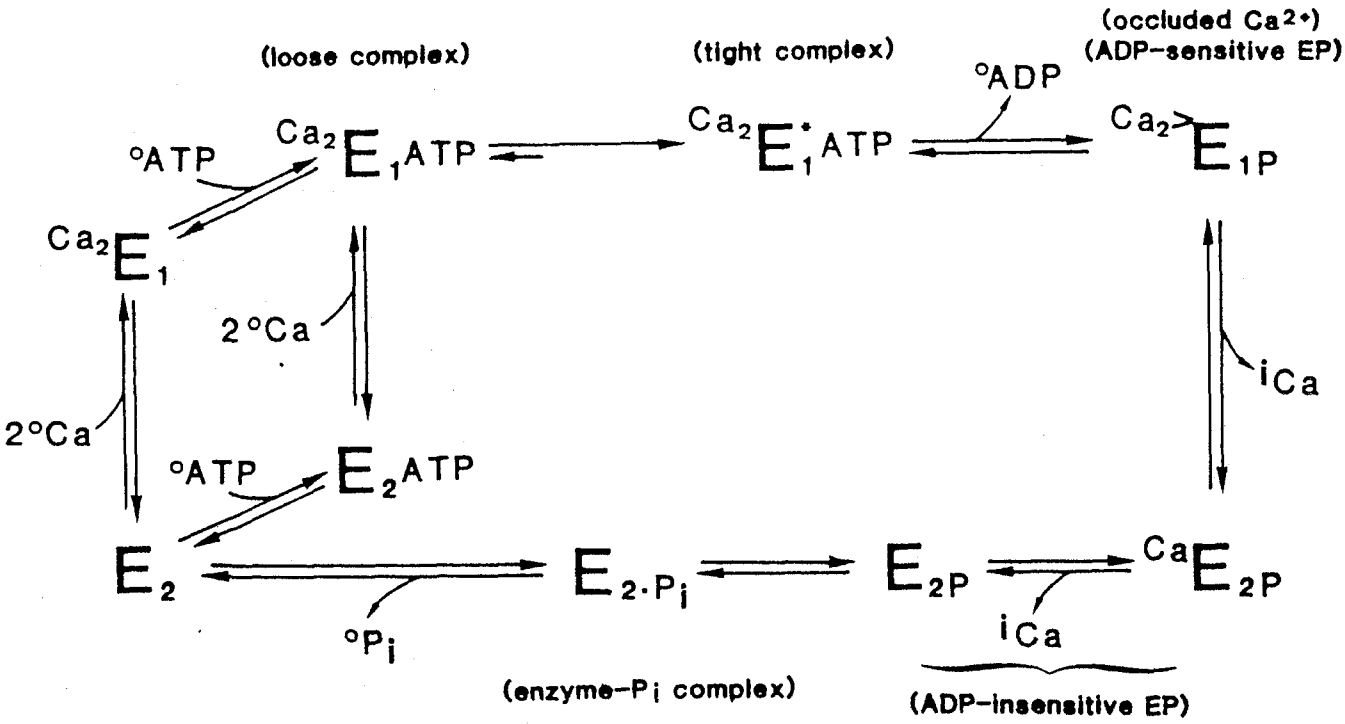

FIGURE 2. The mechanism of coupling of ATP hydrolysis with cation transport across the sarcoplasmic reticulum membrane. E_1 and E_2 are enzyme formes capable of reacting with ATP and Pi, respectively. Ca_2-E_1-ATP, and Ca_2-E_1^*-ATP represent loose and tihgt binding of ATP to the Ca-E_1, respectively. Ca_2-E_1P indicates the "occluded" Ca in the ADP-sensitive EP. E_2P and E_2Pi are ADP-sensitive EP and an enzyme-Pi complex, respectively. From Tonomura, Y. (1986). Energy-Transducing ATPases-strcuture and kinetics. Cambridge:Cambridge University Press.

Ca^{2+}-ATPase of SR has been deduced from its complementary DNA sequence (26). While this provides a prediction of secondary structure and binding domain (Fig. 3), little is yet known concerning the protein's tertiary structure and thus the exact relationship between nucleotide and transport sites. Using energy transfer between fluorescent probes attached to the specific portion of the ATPase, the nucleotide and transport sites are very close within the tertiary structure (27).

On the other hand, a great deal of experimental evidence supports the suggestion that the Ca^{2+}-ATPase exists in the membrane as an oligomer. Measuring the density of the particle on the freeze-fractured membrane, Jilka, Martonosi, and Tillack (28) and Scales and Inesi (29) concluded that the ATPase forms a tetramer in the membrane. Vanderkooi et al (30) showed that the ATPases are in near contact even when they are diluted with lipid using fluorescence energy transfer. Evidence for oligomerization of the ATPase was provided by a study of rotational movements of the ATPase molecules in the FSR by measuring the anisotropy of flash-induced photodichroism of labelled eosin thiocyanate (31). The target size molecular weight of the Ca^{2+} pump in the FSR as well as in the reconstituted SR ranged between 210000 and 250000, which was consitent with a dimeric structure (32).

Using the non-ionic detergent $C_{12}E_8$, a number of works were reported that the ATPase could be solubilized to monomeric form with retention of full enzymatic activity (33-35). Recently, Andersen, Møller and Jørgensen (36) found that 1 mol of FITC bound to the mol ATPase to inactivate the enzyme. They also found that when $C_{12}E_8$ was added to the ATPase to solubilize it to

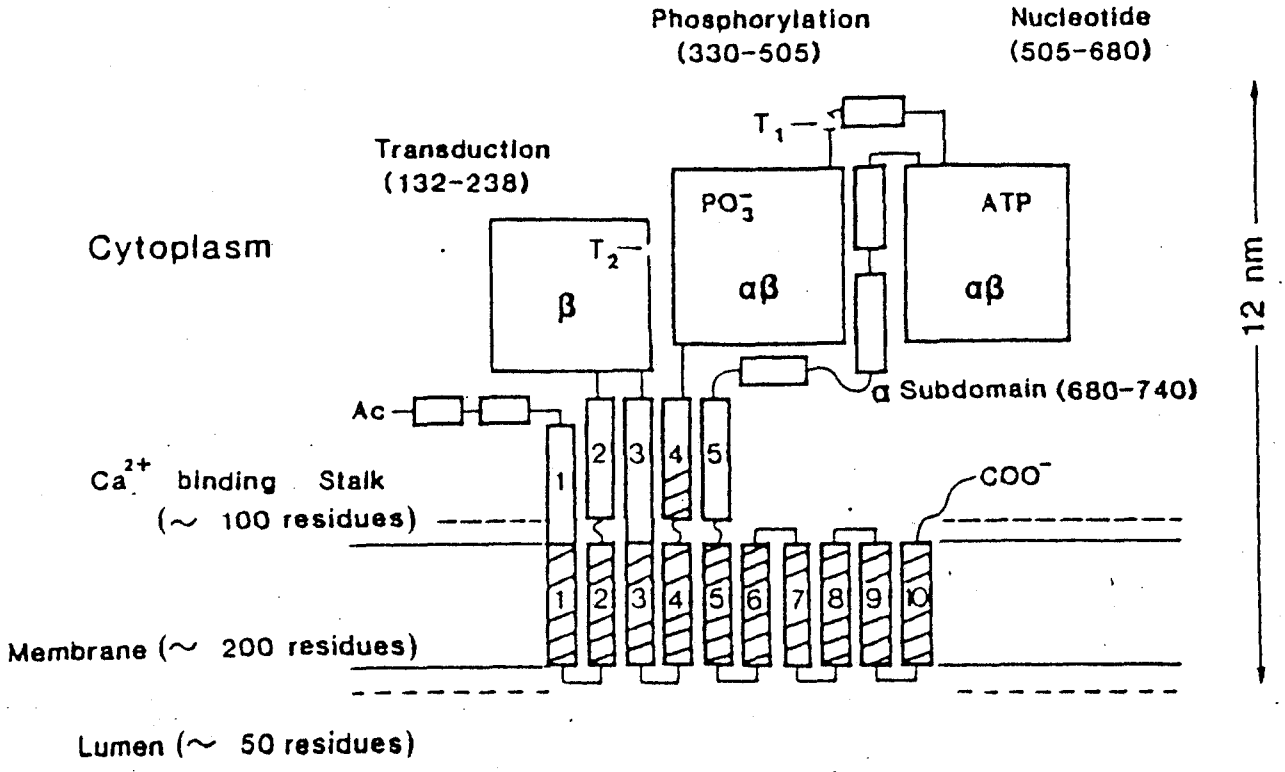

FIGURE 3. Assembly of ATPase domains. The predicted arrays of helices and the three major domains are laid out in a planar diagram. From MacLennan, D. H., Brandl,C. J., Korczak, B., and Green, N. M. (1985) Nature 316, 696-700.

the monomer, the sensitivity to FITC did not changed and the ATPase activity increased. From these results, they suggested that the functional unit of Ca^{2+} transport is the monomeric ATPase. On the contrary, Yamamoto and Tonomura (37) showed that $C_{12}E_8$-solubilized monomer ATPase hydrolized ATP with uncoupled mechainsm of the reaction and that the Mg^{2+} requirements for EP decomposition was lost. Furthermore, it was shown that the monomeric ATPases form the oligomer in accordance with a full restoration of the reaction mechanism to that of intact FSR when the detergent was removed (38). Restoration of the Mg^{2+} sensitivity was also shown in the oligomerization of monomeric ATPase in $C_{12}E_8$ solution. Thus, they concluded that the altered reaction mechanism in $C_{12}E_8$ solution is due not to the chemical effect of the detergent, but to the loss of protein-protein interaction. Watanabe and Inesi (39) suggested that the formation of E_1P results in a dissociation of the oligomeric ATPase into a monomeric form, using fluorescent energy transfer method. Thus, the possibility can not be ruled out that active Ca^{2+} transport requires an oligomeric interaction of the ATPases.

Active transport requires the existence of two components separated by a barrier, which is normally a phospholipid bilayer. It has not been clear whether or not there is an additional specific requirement for phospholipids to the molecular mechanism of the ATPase. It was believed that all aspects of Ca^{2+} pump function are critical dependent on retention of a lipid environment consisting minimally of an annulus of 30 phospholipid molecules (40,41). However, the view that a lipid annulus is required is no longer tenable, since pump protein dissolved in

the $C_{12}E_8$ with a residual phospholipid content as low as one lipid molecule per polypeptide chain, has undiminished ATPase activity and it is capable of carrying out all the important steps of the reaction cycle (33). On the other hand, the most important difference between lipid-embedded and $C_{12}E_8$-solubilized pump protein is that the detergent solubilized protein is less stable in a Ca^{2+} free medium, where the protein is in the E_2 state. The pump protein is monomeric in $C_{12}E_8$ solution and altered properties could reflect the possible need of a dimeric structure for optimal stability and/or activity rather than a direct effect of the substitution of detergent for lipid. It is not possible at present to exclude the possibility that specific interactions with phospholipid might an important contribution to stability of the pump protein and/or its catalytic function.

The more direct evidence will be required to elucidate what is the physiological significance of the protein to protein interaction, and the protein to lipid interaction. Thus, I developed a fine procedure to reconstitute the Ca^{2+} transport from the monomeric Ca^{2+}-ATPase with excess amount of phospholipid to dilute the enzyme to be in monomeric form in the membranous state. It will provide a new information for the nature of the enzyme in the monomeric form without any perturbation of the native enzyme action caused from the detergent-protein interactions.

Previous method (42) for reconstituion of the Ca^{2+} transport from $C_{12}E_8$ solubilized monomeric ATPase was not successful enough, probably because the reconstituting medium contained 5 mM $CaCl_2$ to stabilize the solubilized ATPase.

In PART I, the effect of intra- and extra-vesicular calcium and magnesium ions on the hydrolysis of the phosphoenzyme (EP) intermediate formed in the reaction of Ca^{2+}-ATPase of the FSR were investigated. The rate constants of EP hydrolysis were measured under conditions that allowed a single turnover of ATP hydrolyzed to minimize the increase in calcium concentraion inside the vesicles. In the presence of high concentration of Mg^{2+}, as under the physiological condition, the decomposition of EP was inhibited by Ca^{2+} inside the vesicles with K_{Ca} about 5 mM and not by Ca^{2+} outside.

In PART II, I found that the solubilized enzyme can be stabilized by soybean phospholipid even in the absence of free Ca^{2+}. The findings enable it to reconstitute the Ca^{2+} transport without addition of 5 mM $CaCl_2$ when enough concentration of lipid is present. Proteoliposome thus obtained accumulated Ca^{2+} actively and efficiently with the coupling ratio of about 1.5 between Ca^{2+} uptake and ATP hydrolysis. The possibility that the ATPase transports Ca^{2+} in a dimeric form will be discussed based on the experiments using ATPase preparations extremely diluted with phospholipid in the proteoliposmome.

In the progress of elucidating the site which participate in the subunit interaction in the ATPase oligomer, ATPase protein was digested with trypin. In PART III, I found that under the non-denaturing conditions the digested fragments of the ATPase can be separated by HPLC column chromatography equilibriated with $C_{12}E_8$ only when the digestion was carried out in the presence of the detergent. The binding activity of TNP-ATP was found to be associated with low molecular weight peptides, which was

inhibited by prelabelling of FITC to the nucleotide binding site. This procedure might be effective to isolate the functional domains or sites with native configurations.

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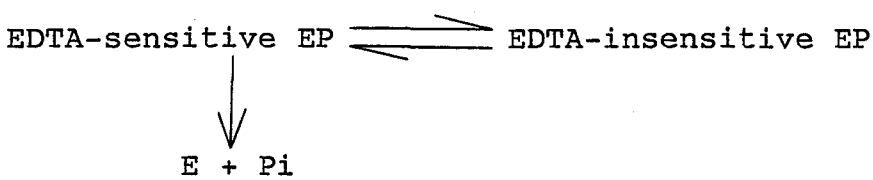
PART I

INHIBITION OF HYDROLYSIS OF PHOSPHORYLATED Ca^{2+}-ATPase OF THE SARCOPLASMIC RETICULUM BY Ca^{2+} INSIDE AND OUTSIDE THE VESICLES

ABSTRACT

The effects of intra- and extravesicular calcium and magnesium ions on the hydrolysis of phosphoenzyme (EP) intermediate formed in the reaction of Ca^{2+}, Mg^{2+}-dependent ATPase of the sarcoplasmic reticulum were investigated. The rate constants of EP hydrolysis were measured under the conditions for ATP hydrolysis that allowed a single turnover to minimize the increase in calcium concentration inside the vesicle.

The EP formed during a single turnover was hydrolyzed biphasically and could be resolved into fast- and slow-decomposing components. When free Mg^{2+} outside the vesicle was chelated by adding excess EDTA, EP could also be kinetically resolved into two components; EDTA-sensitive EP, which could be quickly decomposed by adding EDTA, and EDTA-insensitive EP, which could be stopped from decomposing by adding EDTA. The amount of EDTA-sensitive EP decreased rapidly during the initial phase after ATP addition, while that of EDTA-insensitive EP decreased slowly with the same rate constant as that of the slow-decomposing EP. These results showed that the biphasic time course of EP hydrolysis was caused by the formation of EDTA-sensitive and -insensitive EP during the reaction. The time course of EP hydrolysis could be quantitatively analyzed by the following reaction mechanism.

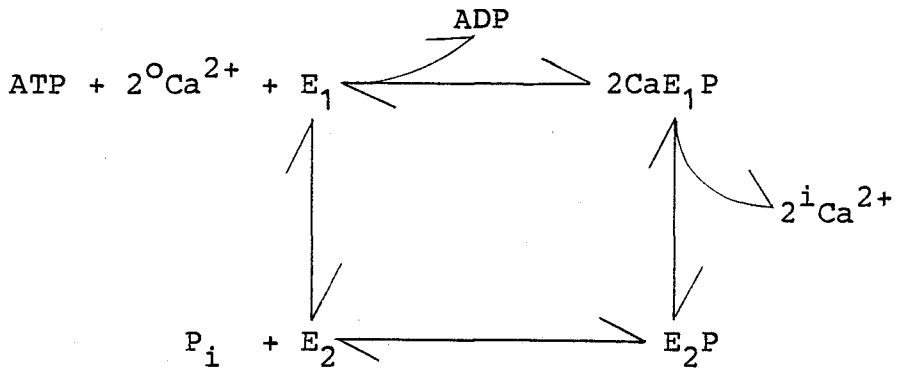


The decomposition of EDTA-insensitive EP required Mg^{2+}

outside the vesicle and was competitively inhibited by extravesicular Ca^{2+}. The decomposition of EDTA-sensitive EP was inhibited by Ca^{2+} inside the vesicle but not by outside Ca^{2+}. The linear relationships between the inverse of the rate constants of EP decomposition during the initial phase and the intravesicular $CaCl_2$ concentrations suggested that decomposition of EDTA-sensitive EP was inhibited by the binding of 1 mol of intravesicular Ca^{2+} to 1 mol of EP. Furthermore, Mg^{2+} inside the vesicle scarcely affected the inhibition of EP hydrolysis by intravesicular Ca^{2+}. These results suggested that magnesium ions are not counter-transported during active transport of calcium by SR vesicles.

INTRODUCTION

Sarcoplasmic reticulum (SR) vesicles are easily prepared from rabbit skeletal muscle without loss of the Ca^{2+} storing activity and provides one of the simplest system for studying the molecular mechanism of active transport. The major protein in the SR membrane is Ca^{2+}-ATPase, whose molecular weight is about 100,000. This enzyme serves as a Ca^{2+}-translocator at the same time as an energy transducer. The coupling mechanism between ATP hydrolysis and Ca^{2+} transport has been extensively studied by many workers (1), especially since phosphorylated intermediate (EP) was isolated as an acid-stable phosphoprotein (2,3). The simplest mechanism which has been postulated so far is as follow,



Here, suffix o and i indicate outer and inner of the SR membrane, respectively. Two moles of Ca^{2+} and one mole of ATP bound to one mole of ATPase at active sites on the outer surface of the membrane (1). Calcium ions are occluded by the enzyme when E_1P (ADP-sensitive EP) is formed (4). Then affinity of the enzyme for calcium are decreased with the transition from E_1P to E_2P

(ADP-insensitive EP), which results in Ca^{2+} release from the enzyme to intravesicular lumen (5).

There remain some important questions to be answered. The functional role of Mg^{2+} in the Ca^{2+} transport in SR is rather complicated and is not completely elucidated. The hydrolysis of EP, a rate determining step of the reaction, requires the presence of Mg^{2+} (6). However, the binding site for Mg^{2+} has not been clarified yet. Recently, Yamada and Ikemoto (7) found that EP hydrolysis is very slow when Ca-ATP is used as a substrate for phosphorylation of the enzyme. Since EP formed with Mg-ATP decomposes rapidly, Shigekawa et al. (8) proposed that the rate of EP hydrolysis is determined by metal ions derived from the metal moiety of the metal-ATP complex left bound to the substrate site. Using a leaky membrane preparation, Yamada and Tonomura (9) first showed that Ca^{2+} competitively inhibits Mg^{2+}-dependent EP hydrolysis. This finding and the fact that Ca^{2+} accumulation in the vesicular lumen inhibits the ATPase activity (10) led them to propose that Mg^{2+} required for EP hydrolysis is counter-transported during the active transport of Ca^{2+}. However, there is no direct evidence which shows that Mg^{2+} is the counter-ion of Ca^{2+} transported. Furthermore, these studies could not clarify whether the Ca^{2+}-binding site, which participates in inhibiting EP hydrolysis, is located inside or outside the membrane as leaky SR preparations were used.

In the present study, I examined the effects of intra- and extravesicular Ca^{2+} and Mg^{2+} on EP hydrolysis using isolated SR vesicles which have membranes essentially impermeable to Ca^{2+} (11). Kinetic studies on EP hydrolysis showed that decomposition

of EP formed from Mg-ATP as a substrate is inhibited by intravesicular Ca^{2+}. Since Mg^{2+} inside the vesicle had little effect on the inhibition of EP hydrolysis by Ca^{2+}, our results suggested that Mg^{2+} is not counter-transported during active transport of Ca^{2+} (12).

EXPERIMENTAL PROCEDURE

SR vesicles, prepared from rabbit skeletal muscle as described previously (13), were suspended in 0.3 M sucrose and 1 mM HEPES at pH 7.1, frozen in liquid nitrogen and stored at -80 °C.

The phosphorylation reaction was started by adding [γ-^{32}P]ATP to the enzyme solution. The final reaction mixture consisted of 1 mg/ml SR vesicles, 0.1 μM [γ-^{32}P]ATP, 0.1 M KCl, 0.162 M sucrose, 0.49 mM $CaCl_2$, 0.5 mM EGTA, and 50 mM Tris maleate at pH 7.0 and 0 °C, unless otherwise indicated. The reaction was quenched by adding TCA solution to give final concentrations of 5% (w/v) TCA, 0.4 mM ATP, and 0.4 mM Pi. The amounts of [^{32}P]EP formed and [^{32}P]Pi produced during the reaction were determined as described previously (14).

For Ca^{2+} loading, SR vesicles (20 mg/ml) were incubated overnight at 0 °C in media containing 0.1 M KCl, 0.162 M sucrose, various concentrations of $MgCl_2$ and $CaCl_2$, and 50 mM Tris maleate at pH 7.0. The concentration of $CaCl_2$ outside the vesicle was diluted 20 hold with the same buffer except that various concentrations of EGTA and $CaCl_2$ were included to give a final free Ca^{2+} concentration of 10 μM.

Analog simulations were run on an ALS-200X Hitachi Analog/Hybrid Computer to test the validity of kinetic models as well as to evaluate kinetic rate constants of intermediate steps from the data.

The concentrations of free Ca^{2+}, Mg^{2+}, and Mg-ATP, Ca-ATP were calculated using the following apparent binding constants:

Ca^{2+} to EGTA, 5.87×10^7; EDTA, 7.28×10^7; ATP, 3.66×10^3; and Mg^{2+} to EGTA, 2.07×10^2; EDTA, 9.25×10^5; ATP, 9.19×10^3, at pH 7 and an ionic strength of 0.1 (15).

[γ-^{32}P]ATP was synthesized as described by Johnson and Walseth (16). ^{32}Pi was obtained from the Japan Radioisotope Association.

RESULTS

Biphasic time course of EP hydrolysis during a single turnover —— To determine the rate constants of EP hydrolysis with minimal increase in the Ca^{2+} concentration inside the SR vesicle, the Ca^{2+}-ATPase was phosphorylated with ATP at a concentration lower than that of the enzyme phosphorylation site. When the reaction was started by adding 0.1 μM ATP to the solution containing 1 mg/ml SR, 0.1 M KCl, 1 mM $MgCl_2$, and 10 μM free Ca^{2+}, about 90% of the ATP added was consumed in 2 s for the phosphorylation (Fig. 1A). Thereafter, EP hydrolyzed spontaneously without showing a lag phase. The amount of ATP remaining, which was estimated by subtracting the amounts of EP formed and Pi produced from the amount of ATP added, decreased with time to reach a constant value (about 5% of the ATP added) at 20 s after the start of the reaction. When the data shown in Fig. 1A were replotted in the form of ln[EP] versus time (Fig. 1B), two straight lines appeared. During 10 s after the start of the reaction, the rate constant obtained from the slope of the straight line was 0.099 s^{-1}. It decreased with time to a constant value of 0.019 s^{-1} at 30 s after the start of the reaction. The amount of slow-decomposing component, obtained by extrapolating the slow phase of EP decomposition to the time just after the start of the reaction, was about 0.023 nmol/mg which was 23% of the amount of ATP added.

Effect of Ca^{2+} outside the vesicle on EP hydrolysis —— Using the leaky SR membrane, Ikemoto (17) showed that EP hydrolysis could be instantaneously stopped by adding a few millimoles per

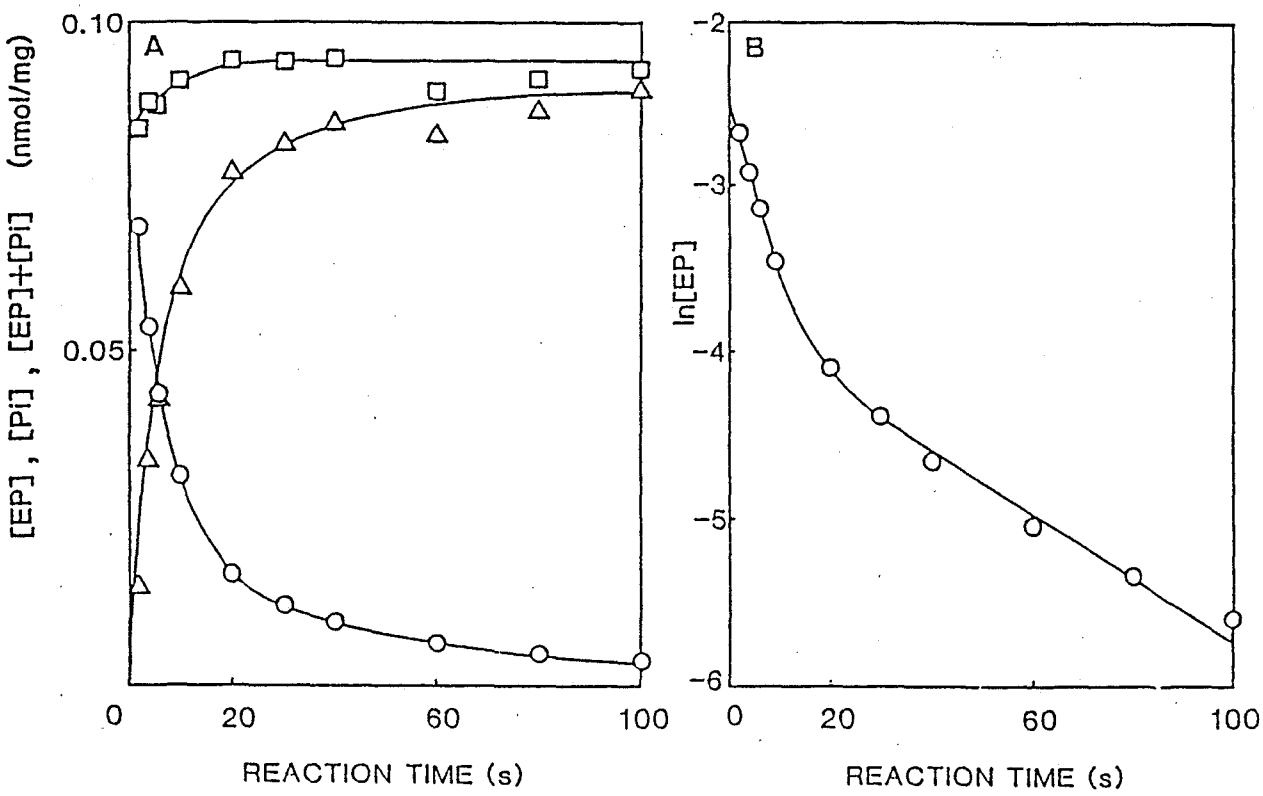


Fig. 1. Time course of the decomposition of EP formed at low ATP concentration. A. Phosphorylation was carried out at 0 °C with 1 mg/ml SR vesicles in 1 mM $MgCl_2$, 0.49 mM $CaCl_2$, 0.5 mM EGTA (the concentration of free Ca^{2+} was about 10 μM), 0.1 M KCl, 50 mM Tris maleate (pH 7.0), 0.162 M sucrose, and 0.1 μM [γ-^{32}P] ATP. At different times after addition of ATP, the reaction was quenched with TCA and the amounts of [^{32}P]EP (○) and ^{32}Pi liberated (△) were determined. The sums of EP and Pi are shown by □ . B. Logarithms of the amounts of EP shown in 1A are plotted against time.

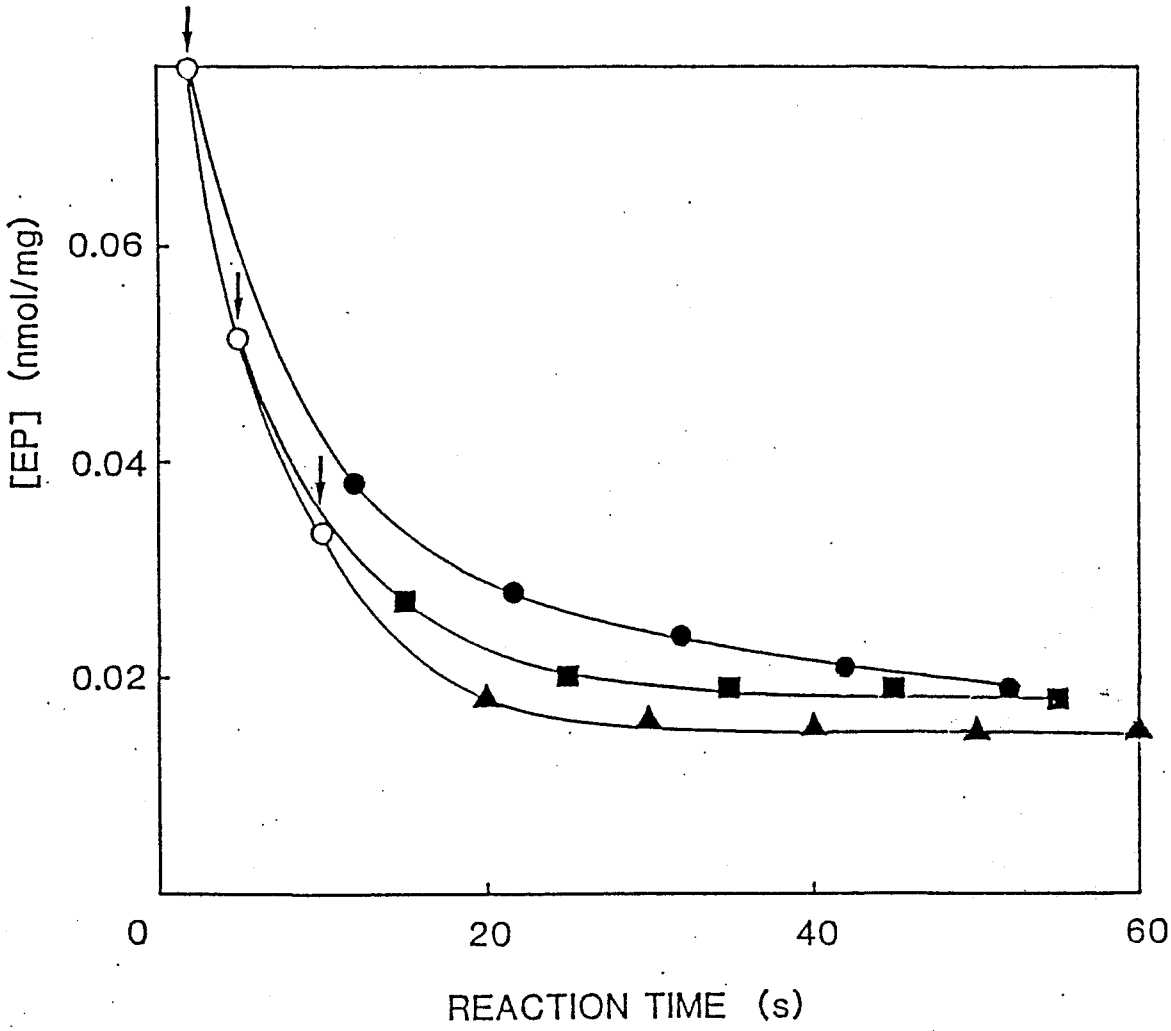


Fig. 2. Effect of addition of high $CaCl_2$ concentration on EP hydrolysis during the initial phase of the reaction. The enzyme was phosphorylated as given in the legend of Fig. 1A except that $CaCl_2$ and EGTA were 0.1 and 0.112 mM, respectively. At 2 (●), 5 (■), or 10 s (▲) after the start of the reaction, 5 mM $CaCl_2$ was added to the reaction medium (↓) and further decomposition of EP was measured. For the control (○), $CaCl_2$ was not added.

liter of Ca^{2+} to the reaction medium. In case of tight SR vesicles, EP hydrolysis could not be halted instantaneously when 5 mM $CaCl_2$ was added outside the vesicle (see Fig. 2). The rate decreased and reached zero at about 30 s after addition of $CaCl_2$. The amount of EP which could be prevented from hydrolyzing by adding 5 mM $CaCl_2$, remained almost constant within 10 s after the start of the reaction. Since the total amount of EP decreased rapidly in a control experiment, the amount of EP which could not be stopped from hydrolyzing by the addition of $CaCl_2$, decreased rapidly with reaction time. When 5 mM $CaCl_2$ was added at 60 s after the start of the reaction, EP hydrolysis was immediately stopped (data not shown).

Figure 3 shows the decomposition rate of EP after various concentrations of $CaCl_2$ had been added at 20 s after the start of the reaction. EP decomposition clearly obeyed first-order kinetics, and the apparent rate constant (k_d) decreased from 0.016 to 0.007 s^{-1} as the concentration of $CaCl_2$ added increased from 0.1 to 0.6 mM.

By varying the Mg^{2+} concentration in the phosphorylation medium, the k_d values after addition of various concentrations of $CaCl_2$ were determined as described in Fig. 3. Figure 4 shows the plot of k_d^{-1} versus the reciprocal of the $MgCl_2$ concentration (from 0.5 to 10 mM) at 0.1, 0.2, 0.4, and 0.6 mM of $CaCl_2$ added to the external medium. As shown in the figure, k_d^{-1} allowed a linear relationship to the reciprocal of the $MgCl_2$ concentration. The inset of Fig. 4 shows a plot of the slope in Fig. 4 against external $CaCl_2$ concentrations. The slope increased linearly with the concentration of $CaCl_2$ added outside the vesicle. Therefore,

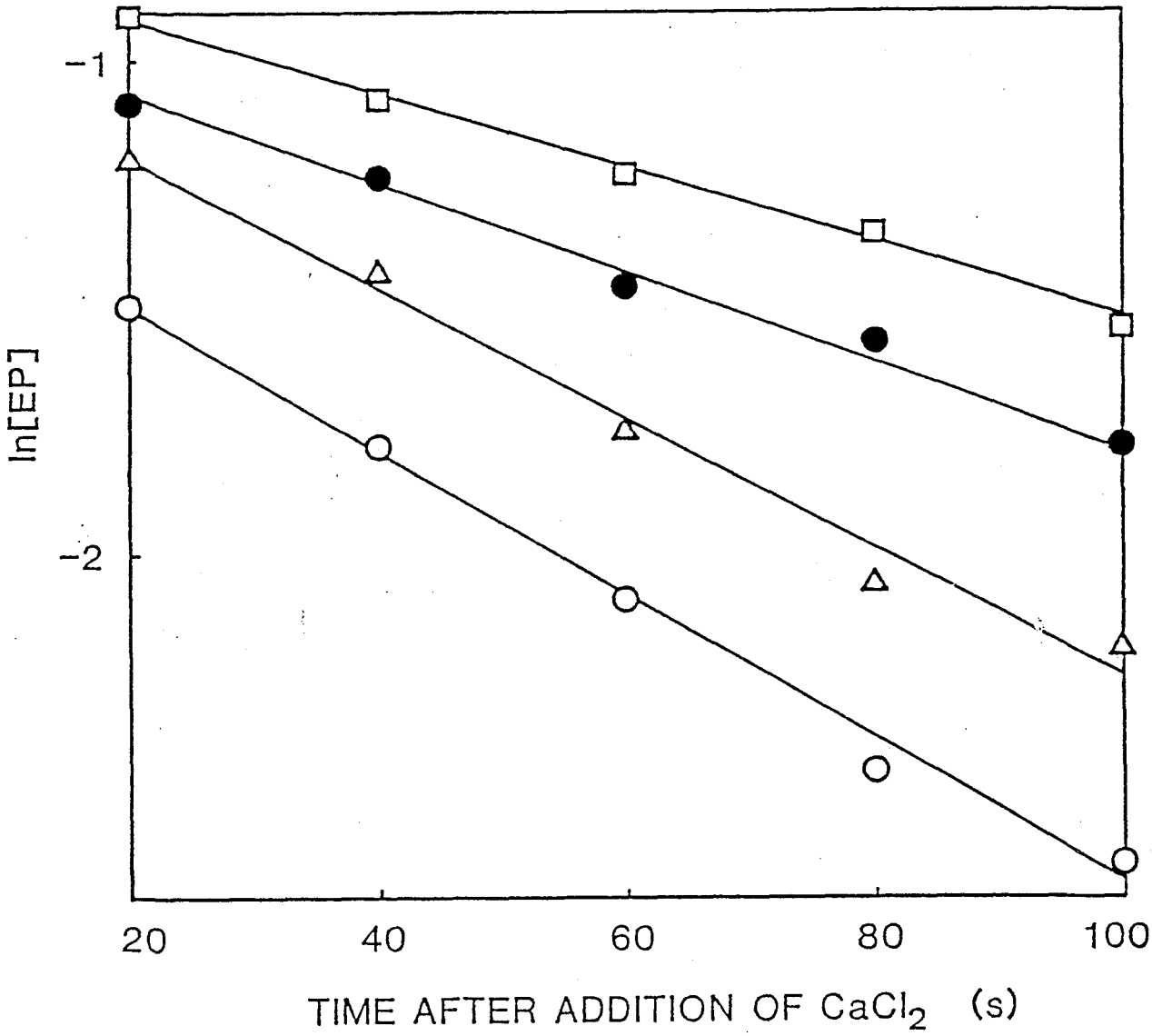


Fig. 3. Inhibition of EP decomposition by $CaCl_2$ added outside the SR vesicle. Phosphorylation was carried out with 0.2 mg/ml SR vesicles in the presence of 0.75 mM $MgCl_2$, 5 μM free Ca^{2+}, 0.5 μM ATP, 0.3 M sucrose, and the other conditions described in Fig. 1A. At 20 s after the start of the reaction, 0.1 (○), 0.2 (△), 0.4 (●), or 0.6 (□) mM $CaCl_2$ was added and the decomposition of EP was measured.

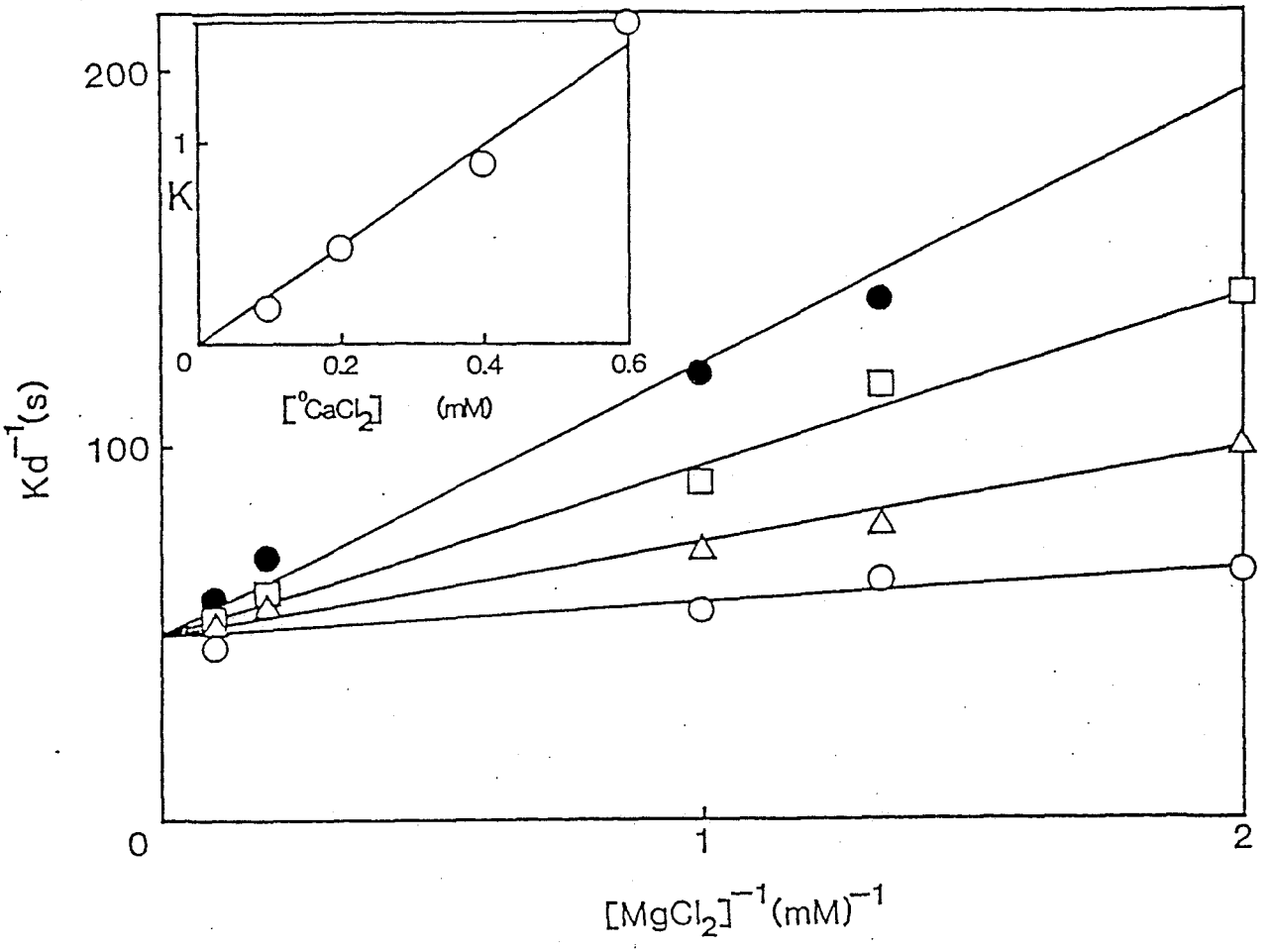


Fig. 4. Dependence of the rate constants of EP decomposition on the $CaCl_2$ concentration outside the vesicle at various $MgCl_2$ concentrations. Enzyme phosphorylation was carried out as described in the legend of Fig. 3, except that the concentration of $MgCl_2$ was varied and the EP decomposition after addition of various concentrations of $CaCl_2$ was measured. Rate constants (k_d) were determined from the slope of the semilogarithmic plot of EP decomposition as shown in Fig. 3. The k_d^{-1} was plotted against the inverse of the $MgCl_2$ concentration at various concentrations of $CaCl_2$ added. $CaCl_2$ was added to the external medium at 0.1 (○), 0.2 (△), 0.4 (□), 0.6 mM (●). The inset shows the dependence of the apparent affinities (K) of EP for Mg^{2+} on the extravesicular $CaCl_2$ concentration. The apparent affinities for Mg^{2+} were obtained as the slope of k_d versus $[MgCl_2]^{-1}$ plot in Fig. 4.

the relationship of the k_d obtained with the $CaCl_2$ and $MgCl_2$ concentrations was expressed as

$$k_d = k_{d\ max} / (1 + K_{Mg}[Ca^{2+}] / K_{Ca}[Mg^{2+}]).$$

The values of $k_{d\ max}$ and K_{Mg}/K_{Ca} were determined to be 0.02 s^{-1} and 2.48, respectively.

Effect of intravesicular Ca^{2+} on EP hydrolysis —— Ca^{2+} added to the external medium did not inhibit EP decomposition during the initial 10 s after the start of the phosphorylation (see Fig. 2). I, therefore, investigated the effect of intravesicular Ca^{2+} on the EP hydrolysis during the initial phase of the reaction. Intravesicular $CaCl_2$ concentration was varied by incubating the vesicles with various concentrations (from 0 to 15 mM) of $CaCl_2$ added to the medium. The external $CaCl_2$ concentration was maintained at 10 μM using Ca-EGTA buffer. As shown in Fig. 5, decomposition of EP obeyed first-order kinetics during 10 s after the start of the reaction and the apparent rate constant, k_d decreased from 0.110 to 0.028 s^{-1} when intravesicular $CaCl_2$ concentration was increased from 1 to 20 mM. As shown in Fig. 6, the plot of k_d^{-1} versus intravesicular $CaCl_2$ concentration was found to be linear. Therefore, the relationship between k_d and the inside Ca^{2+} concentration could be given by

$$k_d = k_{d\ max} / (1 + [Ca^{2+}] / K_{Ca}).$$

The k_d values were obtained at various concentrations of $MgCl_2$. The $MgCl_2$ concentraton in the Ca^{2+}-loading medium was adjusted to

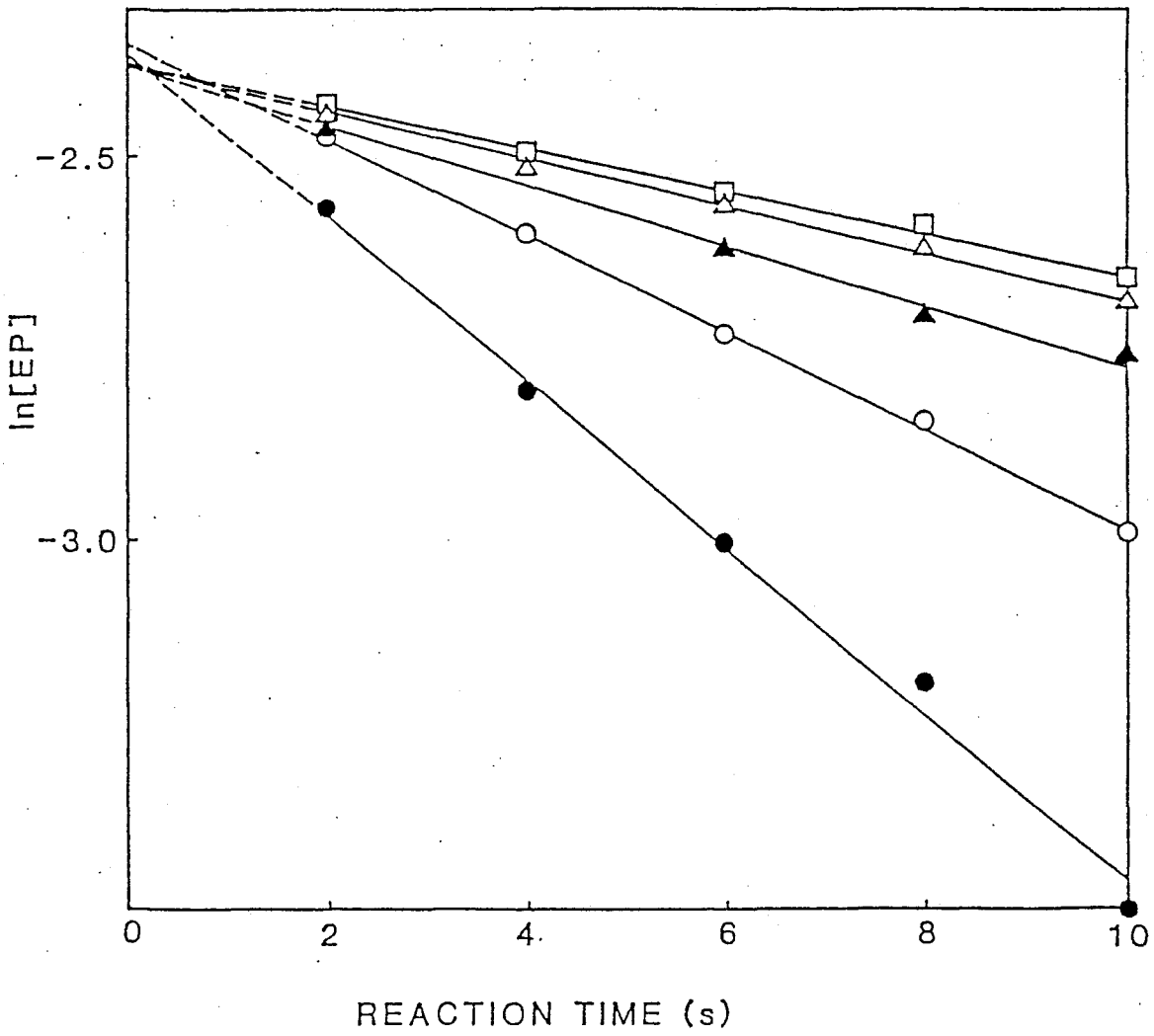


Fig. 5. Inhibition of EP decomposition by $CaCl_2$ inside the SR vesicle. SR vesicles, 20 mg/ml were preloaded with various amounts of $CaCl_2$ in the presence of 2 mM $MgCl_2$ as described in "EXPERIMENTAL PROCEDURE." Enzyme phosphorylation was carried out soon after dilution. The reaction medium contained 1 mg/ml SR vesicles loaded with $CaCl_2$, 0.1 μM ATP,and 2 mM $MgCl_2$, and the concentration of free Ca^{2+} outside the vesicle was adjusted to about 10 μM using EGTA-Ca buffer. Other conditions were the same as those described in Fig. 1A. $CaCl_2$ concentrations inside the SR vesicle were 1 (●), 5 (○), 10 (▲), 15 (△), and 20 mM (□).

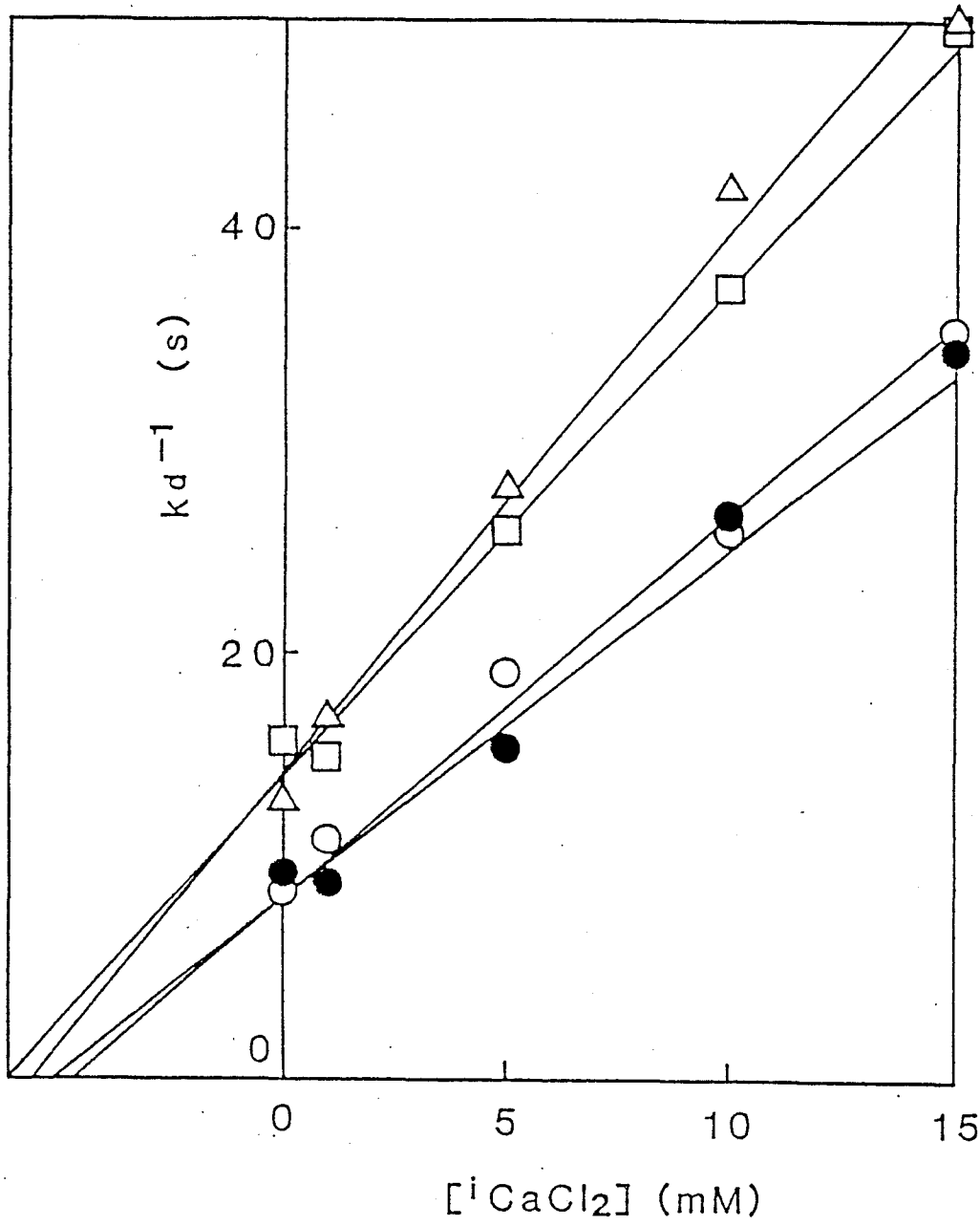


Fig. 6. Dependence of the rate constants of EP decomposition on $CaCl_2$ inside the vesicle at various $MgCl_2$ concentrations. SR vesicles were preloaded with various amounts of Ca^{2+} and Mg^{2+} as described in "EXPERIMENTAL PROCEDURE." The reaction was carried out as described in Fig. 5, except that the $MgCl_2$ concentration was varied. Rate constants (k_d) were determined from the slope of ln[EP] versus time as shown in Fig. 5. The k_d^{-1} was plotted against the concentration of $CaCl_2$ loaded inside the vesicle. $MgCl_2$ concentration both inside and outside the vesicle were 1 (○), 2 (●), 5 (△), 10 mM (□).

that of the phosphorylation medium. K_{Ca} values were 4.7, 5.2, 5.7, 6.2 mM at 1, 2, 5, 10 mM $MgCl_2$, respectively. Thus, the inhibition of EP decomposition by inside Ca^{2+} appeared to be little affected by Mg^{2+} in the range of 1 to 10 mM. It should also be noted that $k_{d\ max}$ decreased as the $MgCl_2$ concentration increased.

EDTA-sensitive and -insensitive EP —— When the enzyme was phosphorylated with ATP in the presence of $MgCl_2$, the EP hydrolysis could not be halted instantaneously by chelating free Mg^{2+} with excess EDTA (6, 18, 19). Figure 7 shows the time course of EP hydrolysis during the initial 10 s after EDTA addition. The EP hydrolysis was clearly accelerated up to 0.33 s^{-1} from the control value of 0.11 s^{-1} without EDTA addition.

Figure 8 shows the time courses of EP decomposition after the addition of 5 mM EDTA at 10, 30, 60, 120 s after phosphorylation of the enzyme. EP hydrolysis occurred immediately after EDTA addition then almost stopped within 20 s. These results indicated that EP could be resolved kinetically into two fractions; EP which can be more quickly hydrolyzed by EDTA addition, i.e., EDTA-sensitive EP, and EP which has its hydrolysis stopped by EDTA addition, i.e., EDTA-insensitive EP. In Fig. 8, the amount of EDTA-insensitive EP was determined by extrapolating the very slow phase of EP decomposition which appeared after addition of EDTA to the time when EDTA was added. At 10 s after the start of phosphorylation, EDTA-insensitive EP accounted for 36% of the total EP. The ratio of EDTA-insensitive EP to total EP increased with time to reach more than 70% at 1 min after the start of the reaction.

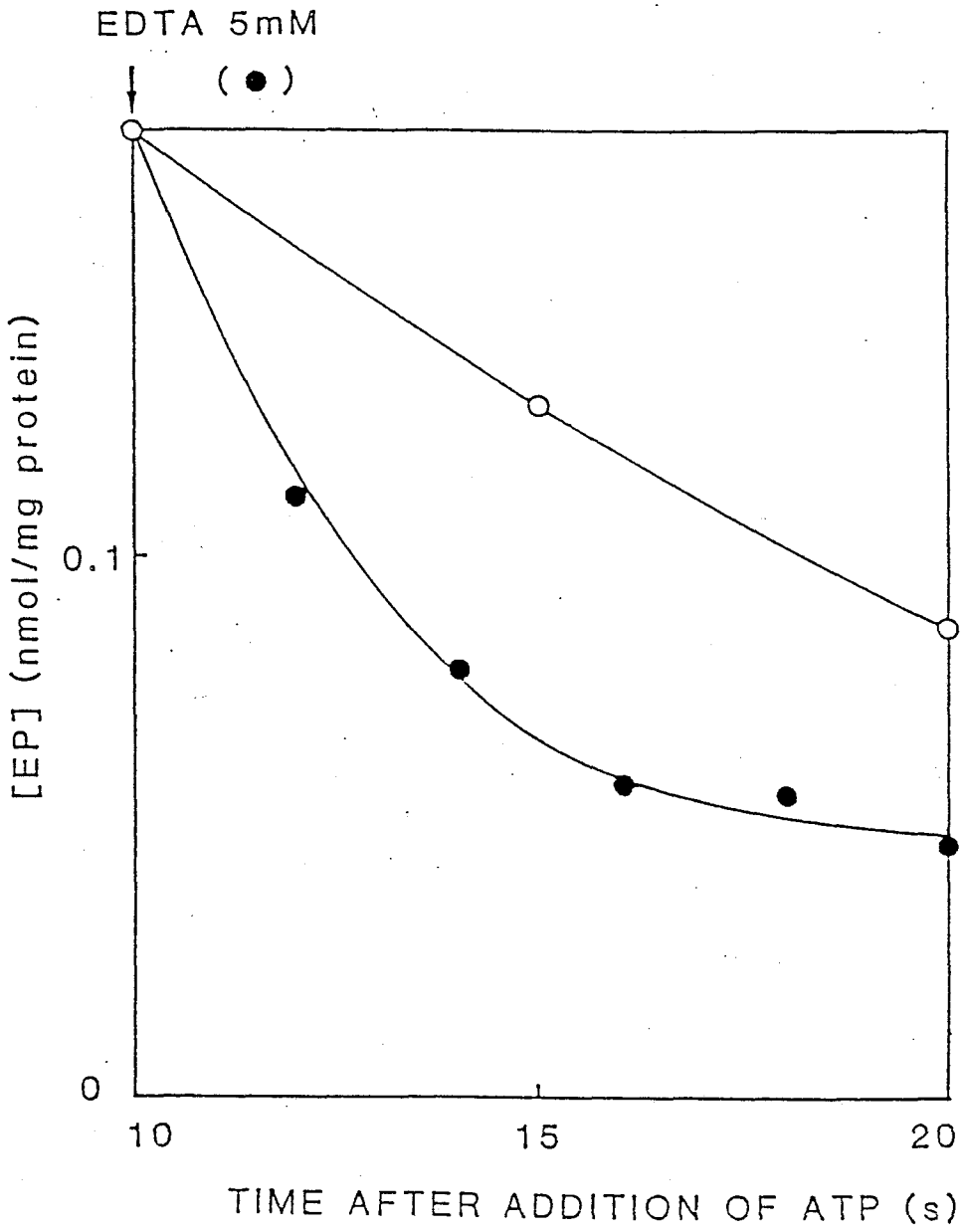


Fig. 7. Acceleration of EP decomposition by addition of EDTA. Enzyme phosphorylation was carried out as described in the legend of Fig. 1A, except that the concentrations of ATP, $CaCl_2$, and EGTA were 0.5 μM, 0.1 mM, and 0.112 mM, respectively. At 10 s after addition of ATP, EDTA was added to the reaction mixture to give final concentration of 5 mM. At various times after the EDTA addition, the reaction was quenched with TCA to determine the amount of EP present (●). In the control experiment the amount of EP (○) was measured without adding EDTA.

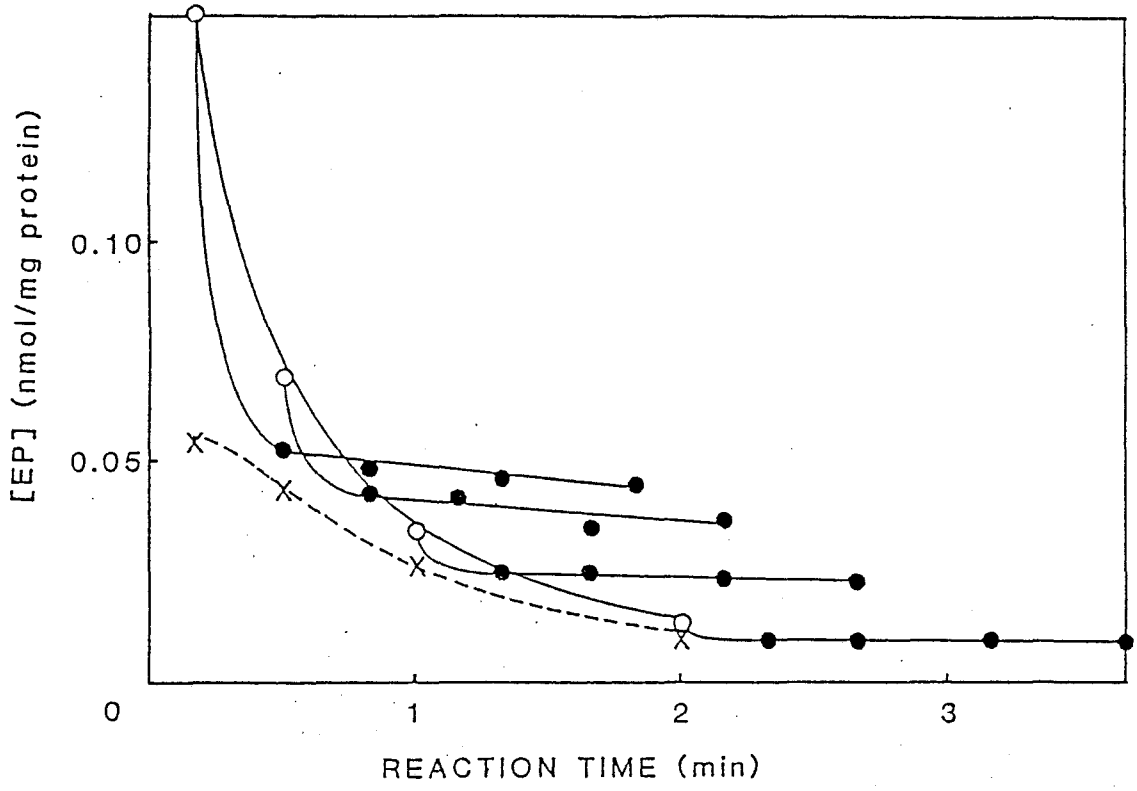


Fig. 8. Time courses of the decomposition of EP and EDTA-insensitive EP in the presence of 1 mM $MgCl_2$. Phosphorylation was carried out with SR vesicles as described in the legend of Fig. 1A, except that the concentrations of ATP and sucrose were 0.5 μM and 0.3 M, respectively. The reaction was quenched with TCA at 10, 30, 60, or 120 s and the amount of EP (○) was measured. At each time, 5 mM EDTA was added to the reaction mixture as described in Fig. 7 and further decomposition of EP was followed at different times after the EDTA addition (●). The amounts of EDTA-insensitive EP (X) at the time that EDTA was added were estimated by extrapolating of the slow phase of EP decomposition to the time when EDTA was added. The solid (○) and broken lines were obtained from the simulation of total EP and EDTA-insensitive EP, respectively, according to Scheme (1) in the text. The rate constants used are listed in Table I.

The correlation between the slow-decomposing EP and EDTA-insensitive EP was clearly shown when the phosphorylation was done at 0.1 mM $MgCl_2$. As shown in Fig. 9, EDTA-insensitive EP accounted for 60% of the total EP at 10 s, and more than 80% at 1 min after the start of the reaction. EDTA-insensitive EP decomposed with a definite lag phase of about 30 s, after which it decomposed exponentially at a rate constant of 0.009 s^{-1}, the same rate as that of slow-decomposing EP.

Formation of EDTA-insensitive EP from EDTA-sensitive EP —— Figure 10 shows the time courses of the formation of EDTA-sensitive and -insensitive EP during the initial phase of the reaction. The SR (0.15 mg/ml) was phosphorylated with an excess amount of ATP (10 μM) in order to maintain the amount of EP during the reaction and 0.75 μM Ca^{2+} ionophore (A23187) was included in the reaction medium to prevent a rise in Ca^{2+} concentration inside the vesicle. As shown in the figure, total EP reached a steady level (2.5 nmol/mg) within 2 s after ATP addition in the presence of 1 mM $MgCl_2$. In the presence of 0.1 mM $MgCl_2$, the amount of total EP increased slightly after 2 s.

On the other hand, the amount of EDTA-insensitive EP increased with time to reach a constant value (0.85 and 0.40 nmol/mg at 0.1 and 1 mM $MgCl_2$, respectively) at about 20 s after the start of the reaction. The time courses of the formation of EDTA-insensitive EP obeyed first-order kinetics yielding rate constants of 0.13 s^{-1} and 0.15 s^{-1} in the presence of 0.1 and 1 mM $MgCl_2$, respectively. Extrapolation of each time course of the formation of EDTA-insensitive EP to zero time gave the initial

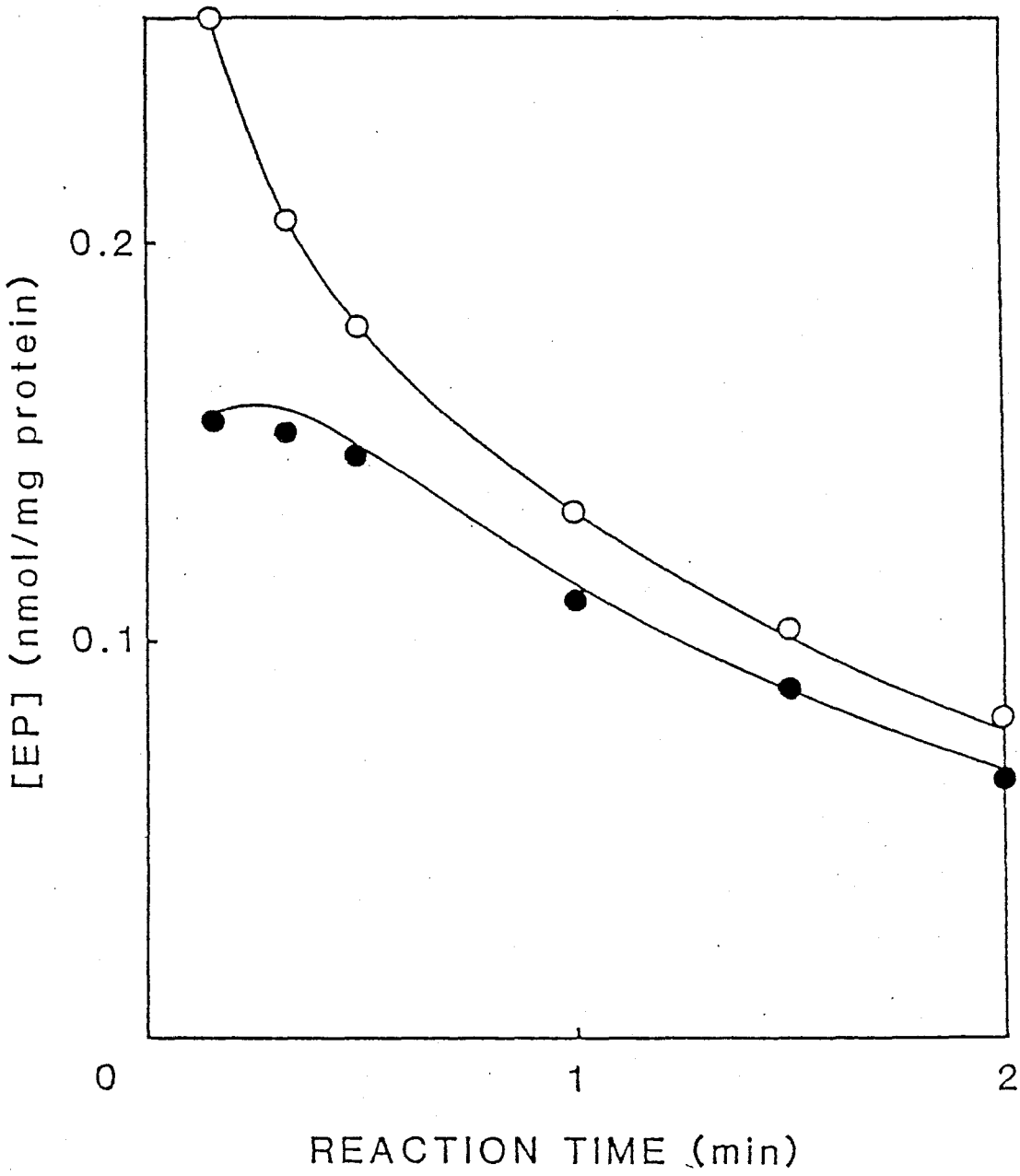


Fig. 9. Time courses of the decomposition of EP and EDTA-insensitive EP in the presence of 0.1 mM $MgCl_2$. The enzyme was phosphorylated as given in the legend for Fig. 8, except that the $MgCl_2$ concentration was 0.1 mM, and the amount of EP (○) was measured. The amounts of EDTA-insensitive EP (●) were estimated as described in Fig. 8. The solid lines were obtained from simulation of total EP and EDTA-insensitive EP according to Scheme (1). The rate constants used are listed in Table I.

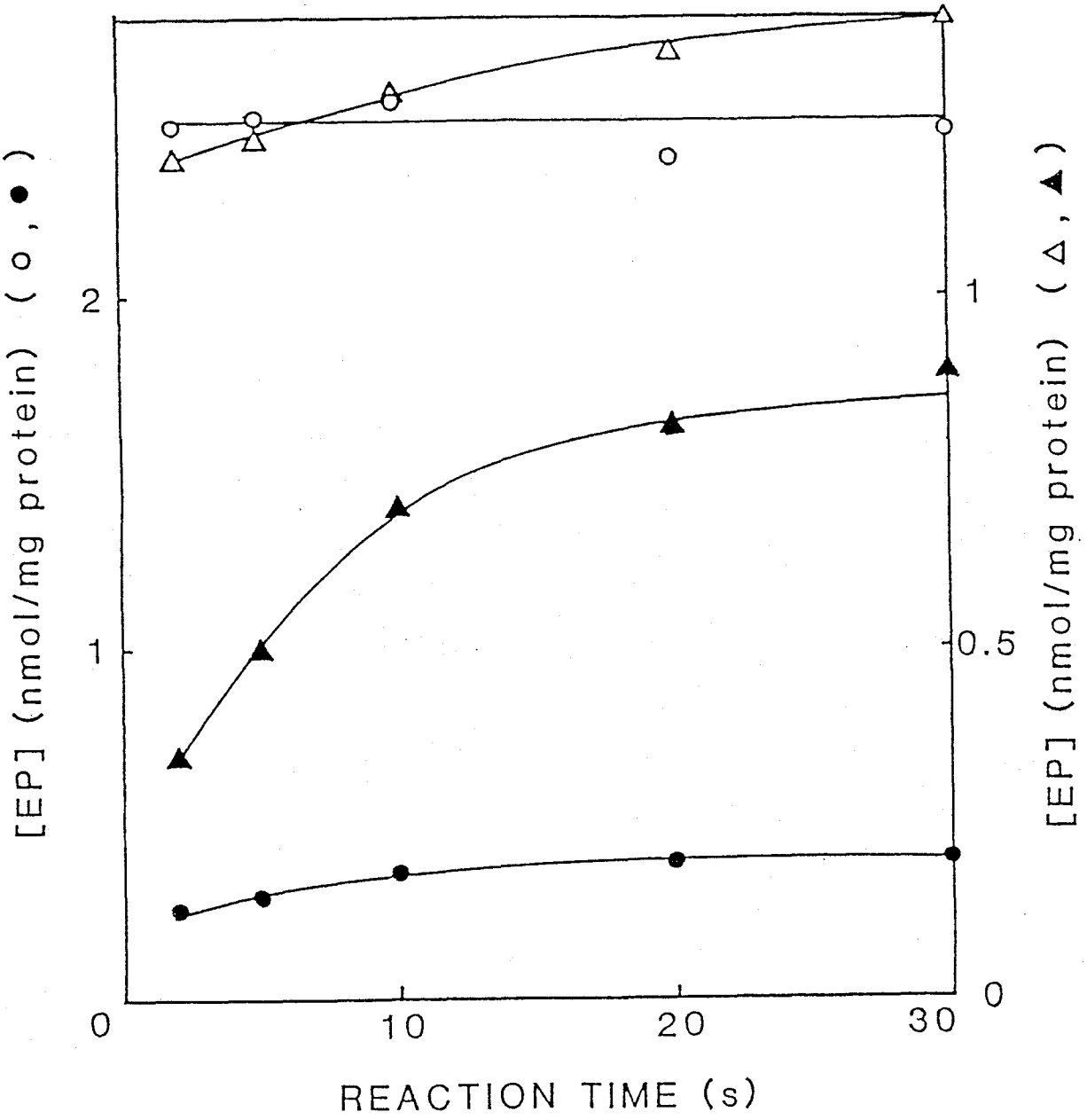


Fig. 10. Formation of EDTA-insensitive EP from EDTA-sensitive EP during the initial phase of the reaction. Phosphorylation was started by adding 10 μM ATP to the solution containing 0.15 mg/ml SR vesicles, 0.75 μM A23187, 0.1 M KCl, 0.162 M sucrose, 50 mM Tris maleate (pH 7.0), and various concentrations of $MgCl_2$ and free Ca^{2+} as indicated below. The amounts of EP (●, ▲) and EDTA-insensitive EP (○, △) were determined as described in Fig. 8. ○, ●, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.093 mM EGTA to give a free Ca^{2+} concentration of 10 μM; △, ▲, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.102 mM EGTA to give a free Ca^{2+} concentration of 5 μM.

values, which were about 6% of total EP formed in both cases.

The ratio of EDTA-insensitive EP to the total EP at 10 s after the start of the reaction during a single turnover decreased as $MgCl_2$ concentration increased. They were 60, 55, 37, and 36% at 0.1, 0.2, 0.5 and 1 mM $MgCl_2$, respectively (cf. Figs. 8, 9). In Fig. 10, the ratios measured at the steady state were 61 and 16% in the presence of 0.1 and 1 mM $MgCl_2$, respectively.

DISCUSSION

In the present study, I examined the effects of intra- and extravesicular Ca^{2+} and Mg^{2+} on EP hydrolysis to clarify whether the site which participates in regulating the rate of EP hydrolysis is located inside or outside the SR membrane. I used isolated SR vesicles with membranes essentially impermeable to Ca^{2+} (11). Thus, I could easily control the Ca^{2+} concentration on either side of the membrane. The enzyme was phosphorylated under the condition where the Mg-ATP concentration, calculated as described in "EXPERIMENTAL PROCEDURE", was more than 94% of that of the total metal-ATP complex in order to prevent EP formation from Ca-ATP. The rate constants of EP hydrolysis were measured under the conditions where ATP is hydrolyzed by SR in a single cycle of the reaction in order to minimize the rise in the concentration of intravesicular Ca^{2+} due to active Ca^{2+} transport. As shown in Fig. 1, about 90% of the ATP added was consumed within 2 s after the start of the reaction. Since the EP hydrolysis occurred without a lag phase, the remaining ATP had little effect on the rate of EP hydrolysis during the initial phase of the reaction. In the presence of more than 1 mM $MgCl_2$, the amount of ATP remaining during the initial phase was less than 5% of the ATP added (cf. Fig. 5). In the presence of less than 1 mM $MgCl_2$, I also observed that about 5% of the ATP added remained unhydrolyzed at 2 min after the start of the reaction. A similar amount of ATP remained in the experiments of Fig. 3. Since the amount of ATP remaining was almost constant throughout the experiment of Fig. 3, the slow decomposition of EP is

probably not caused by the formation of EP from the remaining ATP.

EP was hydrolyzed biphasically during a single turnover and was composed of fast-decomposing EP and slow-decomposing EP. EP formed during a single turnover could also be separated into two kinds of EP by adding excess EDTA to chelate free Mg^{2+} in the medium (Figs. 8, 9). The amount of EDTA-sensitive EP decreased rapidly during the initial phase of the reaction and the amount of EDTA-insensitive EP decreased slowly with the same rate constant as that of the slow-decomposing EP. These results indicated that fast- and slow-decomposing EP closely correspond to the EDTA-sensitive and -insensitive EP, respectively. It has been reported that the EP formed is partially hydrolyzed after addition of excess EDTA to chelate free Mg^{2+} (6, 18, 19). Two possible explanations for the effect of EDTA were, Mg^{2+} might remain bound to activate EP hydrolysis (18) or the Mg^{2+}-activated state of EP suitable for hydrolysis is retained after the removal of the bound Mg^{2+} (19). Since my data showed that the rate of EP hydrolysis in the presence of EDTA is much larger than that in its absence, the effect of EDTA could not be explained only by assuming that Mg^{2+} is tightly bound to EP. It should be also added that the rapid decomposition of EP after EDTA addition is not due to the ATP formation from EP and ADP, since the corresponding amount of Pi was liberated with the decrease in EP after the EDTA addition (data not shown). Similar results has been reported by Takakuwa and Kanazawa (19) who performed their experiments in the presence of low concentration of ATP.

Biphasic decomposition of EP during a single turnover

reaction can be quantitatively analyzed by the following scheme

$$\text{EDTA-sensitive EP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{EDTA-insensitive EP} \quad (1)$$

$$\text{EDTA-sensitive EP} \xrightarrow{k_d} E + Pi$$

where k_1, k_{-1}, k_d are rate constants. As shown by the broken and solid lines in Fig. 8 and Fig. 9, a reasonable fit to the time course of total EP and EDTA-insensitive EP could be achieved based on this scheme. The time courses could not be simulated by assuming that EDTA-insensitive EP decomposes directly into enzyme and Pi. EDTA-insensitive EP was assumed to be formed from EDTA-sensitive EP because EDTA-insensitive EP during a single turnover did not decompose single-exponentially (see Figs. 8, 9) and decomposed slowly after almost all of the EDTA-sensitive EP had decomposed (see Figs. 8, 9). The sequential formation of EDTA-insensitive EP from EDTA-sensitive EP is also supported by the time courses of their formation shown in Fig. 10. Since the estimated amount of EDTA-insensitive EP was not negligible at the time when ATP was added, the possibility remains that EDTA-insensitive EP was formed directly via the E-ATP complex.

Ca^{2+} added to the external medium inhibited hydrolysis of slow-decomposing EP. Since the EDTA-insensitive EP is decomposed at a rate identical to that of slow-decomposing EP, it is reasonable to assume that the decomposition of EDTA-insensitive EP was inhibited by Ca^{2+} added to the external medium. The k_d^{-1} versus $[MgCl_2]^{-1}$ plot gave a family of straight lines intersecting at a common intercept on the k_d^{-1} axis for each Ca^{2+} concentration (Fig. 4). K_{Mg} increased in parallel with the

concentration of Ca^{2+} added. The simplest explanation for these results is that Ca^{2+} competitively inhibited the binding of Mg^{2+} which is required for EP hydrolysis. Clearly, the binding site is located on the outer surface of the vesicles, since EP hydrolysis was inhibited by extravesicular Ca^{2+} and EDTA added to the external medium. In order to clarify the role of Mg^{2+} in the decomposition of EDTA-insensitive EP, analog simulations were performed to evaluate the rate constants from the time courses of the decomposition of total EP and those of EDTA-insensitive EP at various concentrations of $MgCl_2$. The sets of k_1 and k_{-1} values obtained are summarized in TABLE I. When Mg^{2+} concentration increased, the ratio of k_1/k_{-1} decreased and the equilibrium between EDTA-sensitive and -insensitive EP shifted to EDTA-sensitive EP. These results could then be reasonably explained by the following scheme:

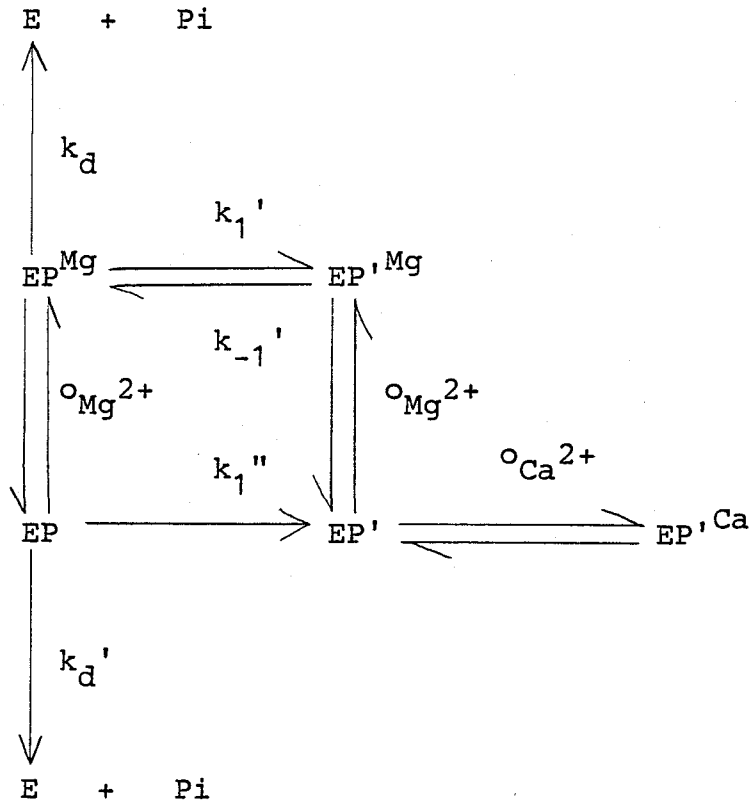


TABLE I. Rate constants obtained from simulation of the time courses of the decomposition of total EP and EDTA-insensitive EP. Rate constants were obtained from simulations of the decomposition of total EP and EDTA-insensitive EP at various Mg^{2+} concentrations with an analog computer according to Scheme (1).

$MgCl_2$ (mM)	k_1	k_{-1} (s^{-1})	k_1 / k_{-1}
0.1	0.030	0.014	2.14
0.2	0.022	0.017	1.29
0.5	0.014	0.019	0.74
1.0	0.013	0.030	0.43

where EP and EP' designate EDTA-sensitive and -insensitive EP, respectively; $^{o}Ca^{2+}$ and $^{o}Mg^{2+}$ are free Ca^{2+} and Mg^{2+} in the external medium of the vesicle, and EP^{Mg}, EP'^{Mg}, and EP'^{Ca} designate intermediates with bound Mg^{2+} or Ca^{2+} which are exposed to the external medium. It can be assumed that k_{-1}'' is zero since the decomposition of EDTA-insensitive EP was halted by chelating free Mg^{2+} with EDTA. k_d' is assumed to be higher than k_d since the addition of EDTA caused a rapid decomposition of EDTA-sensitive EP. In the presence of a high Mg^{2+} concentration, the equilibrium shifts to Mg^{2+}-bound forms and the rate of EP hydrolysis is determined by k_1' and k_{-1}'. When the Mg^{2+} concentration is decreased, the equilibrium shifts to Mg^{2+}-unbound forms and the rate of EP decomposition decreases since k_{-1}'' is zero. Extravesicular Ca^{2+} binds to the site on EDTA-insensitive EP competitively with Mg^{2+} to inhibit its hydrolysis.

Intravesicular Ca^{2+} inhibited the hydrolysis of EP during the initial phase of the reaction. This agrees with the report that the increase in the concentration of intravesicular Ca^{2+} due to active transport coupled with ATP hydrolysis led to a fall in the Ca^{2+} uptake and ATPase activity (10). Furthermore, the linear relationship between k_d^{-1} and the concentration of $CaCl_2$ inside the vesicle (Fig. 6) suggested that EP hydrolysis was inhibited by the binding of 1 mol of Ca^{2+} inside the vesicle to 1 mol of EP at 0 °C. The coupling ratio between Ca^{2+} transport and ATP hydrolysis has been reported to be 1 at 0 °C (20). Therefore, the Ca^{2+} involved in the inhibition of the hydrolysis of fast-

decomposing EP was assumed to be the Ca^{2+} which is transported across the membrane into the SR lumen. If this assumption is true, my experimental results also suggested that EP can be hydrolyzed only after the Ca^{2+} is released into the SR lumen. A similar conclusion has been reached by Takakuwa and Kanazawa (19). It should be noted that the K_{Ca} values are calculated assuming that the Ca^{2+} concentration inside the vesicle is equal to that added to the preincubation medium. In the presence of 0.1 M KCl, a linear relationship is assumed to exist between the Ca^{2+} concentration inside the vesicle and that added to the incubation medium. However, the concentration of free Ca^{2+} inside the vesicle has been reported to differ from that of Ca^{2+} added to the incubation medium (21). In addition, the Ca^{2+} concentration inside the vesicle in the absence of added Ca^{2+} was calculated to be 0.2 mM, since the SR preparations contained about 10 nmol free Ca^{2+} per mg protein. Therefore, the obtained K_{Ca} values are nominal.

In the present study, I also observed that Mg^{2+} scarcely affected the inhibition of EP hydrolysis by intravesicular Ca^{2+}. This contradicts the report by Yamada and Tonomura (9) who reported that Ca^{2+} competitively inhibited Mg^{2+}-dependent EP hydrolysis. Since the Mg^{2+}-dependent decomposition of EDTA-insensitive EP is competitively inhibited by extravesicular Ca^{2+}, they might have measured the decomposition rate of EDTA-insensitive EP. Actually, their K_{Mg} / K_{Ca} value is very similar to that for EDTA-insensitive EP. Based on their kinetic study on EP hydrolysis, they proposed that Mg^{2+} is counter-transported during Ca^{2+} transport. This possibility was later

refuted by Chiesi and Inesi (22) who showed that Mn^{2+}, an analog of Mg^{2+}, is not counter-transported during Ca^{2+} uptake. Since Mn^{2+} is also an analog of Ca^{2+}, the experiments of Chiesi and Inesi (22) could not exclude the possibility that Mg^{2+} is counter-transported during Ca^{2+} uptake. My present kinetic study on EP hydrolysis clearly shows that Mg^{2+} cannot serve as a counter-ion of Ca^{2+}.

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PART II

PROTEIN-PROTEIN AND PROTEIN-LIPID INTERACTIONS IN Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM

ABSTRACT

The method has been developed for reconstituting the Ca^{2+} transport from monomeric Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) solubilized with nonionic detergent polyoxyethylene-9-raulylether ($C_{12}E_9$). In the previous method (R. Andoh, and T. Yamamoto (1985) J. Biochem. 97, 877-882), 5 mM $CaCl_2$ was added to the reconstituting medium to stabilize the ATPase. However, 5 mM Ca^{2+} inside the vesicles found to inhibit Ca^{2+} transport activity in reconstituted proteoliposomes as well as in SR vesicles. It was found that 5-30 mg/ml phospholipid stabilized EP formation capability of monomeric ATPase for more than 30 minutes after solubilization in octa(ethylene glycol)-monododecylether ($C_{12}E_8$) or $C_{12}E_9$ in the absence of free Ca^{2+}. Therefore, active Ca^{2+} transport could be reconstituted without added $CaCl_2$ from the monomeric ATPase in the presence of soybean asolectin and oxalate in the reconstituting medium. The proteoliposomes accumulated large amount of Ca^{2+} in the presence of ATP at initial rates up to about two-fold of that of native SR vesicles with the coupling ratio between Ca^{2+} uptake and ATP hydrolysis of about 1.5 at 25 C. Calcium specific ionophore, A-23187 strongly inhibited Ca^{2+} uptake by the reconstituted vesicles, however, A-23187 itself did not cause any Ca^{2+} accumulation in the absence of ATP.

When proteoliposomes were reconstituted by widely varying weight ratio of lipid to SR protein from 10 to 1333 with constant

lipid concentration(20 mg/ml), the Ca^{2+} storing capacity decreased from 53.3 to 3.5 nmol/ mg phospholipid. The effect of lipid dilution on the Ca^{2+} storing capacity of the proteo-liposomes was discussed in terms of the functional unit of Ca^{2+} transport.

INTRODUCTION

The methods of reconstitution of membrane proteins into artificial membranes have been widely used to investigate the processes in which they assemble into a membrane to form the unit for the physiological functions, or to provide information about relationships between subunit structures and catalytic functions of the emzymes. Many studies have been reported on the reconstitution of Ca^{2+} transport in SR membrane. Racker (1) demonstrated that Ca^{2+} transport activity was restored when the Ca^{2+}-ATPase purified by the method of MacLennan (2) was mixed with soybean lecithin in the presence of cholate, which was removed by dialysis. There have been reported a variety of method for the reconstitution by using different kinds of detergent such as cholate (1,3), deoxycholate (4-6) Triton X-100 (7), octaethylene glycol dodecylether ($C_{12}E_8$) (8,9).

A great deal of experimental evidence supports the suggestion that the Ca^{2+}-ATPase exists in the membrane as an oligomer. $C_{12}E_8$ or polyoxyethylene-9-raulylacid ($C_{12}E_9$) is one of the most useful detergents for the studies on the oligomeric interaction or on the reconstitution of the SR Ca^{2+}-ATPase. Because, these nonionic detergents solubilize the ATPase into a monomeric form with retention of the enzymatic activity(10). The ATPase solubilized with excess $C_{12}E_8$ shows the similar activity of EP formation(12) and accelerated activity of ATP hydrolysis(13)

compared to those of the membrane bound ATPase. It was also shown that phosphorylated monomeric ATPase could change in affinity for Ca^{2+}(14). On the other hand, Yamamoto and Tonomura(11,12) suggested that the reaction mechanism of the monomeric Ca^{2+}-ATPase in the presence of $C_{12}E_8$ was uncoupled type and quite different from that of the ATPase in SR membrane. They have shown that the monomeric ATPases form an oligomer in accordance with a full restoration of the reaction mechanism to that of intact one when $C_{12}E_8$ was removed from the solubilized ATPase by the incubation with the hydrophobic resin, Bio-beads SM-2 (11,12,15). In addition, Ca^{2+} transport activity could be partially restored when soybean lecithin was added before the removal of the detergent. Therefore, it is still remained unkown how many polypeptide chains are in a functional unit for Ca^{2+} transport.

In the previous paper (8), however, the reconstitution of Ca^{2+} transport from $C_{12}E_8$ solubilized ATPase has not been enough successful. Probably it was due to the high concentration of Ca^{2+} which was added to stabilize the soluble Ca^{2+}-ATPase might remain inside the reconstituted vesicle and might inhibit the ATPase reaction. In fact, it was directly demonstrated that high concentration of calcium inside the intact SR vesicles strongly inhibits the ATPase catalytic reaction cycle by preventing the hydrolysis of the phosphoenzyme(PART I).

In the present study, it was found that the enzymatic

activity of the $C_{12}E_8$- or $C_{12}E_9$-solubilized Ca^{2+}-ATPase could be stabilized in the presence of soybean phospholipid even in the absence of free Ca^{2+}. Based on these findings, I have for the first time provided a fine procedure for the reconstitution of active Ca^{2+} transport from the monomeric ATPase solubilized in $C_{12}E_9$. I measured the Ca^{2+}-storing capacity as a function of lipid/protein ratio in the proteoliposome. The effect of lipid dilution could be fitted to the theoretical curve which was obtained by assuming that the minimal functional unit of the Ca^{2+} pump is a dimeric ATPase.

EXPERIMENTAL PROCEDURES

Preparation of SR vesicles

SR vesicles, prepared from rabbit skeletal muscle as described previously (16) were suspended in 0.1 M KCl, 0.3 M sucrose and 5 mM Hepes at pH 7, quickly frozen in liquid nitrogen and stored at -80 °C.

Reagents

Soybean L-α-lecithin was purchased from Sigma Chemical Co., Ltd. Asolectin was obtained from Associated Concentrates. $C_{12}E_8$ and $C_{12}E_9$ was obtained from Nikko Chemical Co., Ltd. Bio-beads SM-2 was obtained from Bio-Rad and repeatedly washed with methanol, water, and ethanol. A-23187 was obtained from Calbiochem-Behring Co. Antipyrylazo III was purchased from Nakarai Chemicals, LTD. [γ-^{32}P]ATP was synthesized as described by Johnson and Walseth (17). ^{32}Pi was obtained from the Japan Radioisotope Association. Other chemicals were of analitical grade.

Loading Ca^{2+} into SR vesicles

2 mg/ml of SR vesicles were loaded with $CaCl_2$ by incubation for various times in standard medium at 20 °C which contained 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 M KCl, 20 % glycerol and 40 mM TES(pH 7.5).

Reconstitution of proteoliposomes

Reconstitution of Ca^{2+} pump protein into phospholipid vesicles was carried out essentially with the procedure of Andoh and Yamamoto (8) with some modifications. Without added $CaCl_2$, 0.015-2 mg of SR protein/ml was solubilized at 25 °C with 50 mg

$C_{12}E_9$/ml in the solubilizing solution, so that the Ca^{2+}-ATPases exsits mainely in the monomeric form. It contained 0.15 M potassium-oxalate, 5 % glycerol, and 30 mM Hepes (pH 7.0). In a minute, 5-120 mg/ml of asolectin which was sonicated to clarity at 0 C in 30 mM Hepes (pH 7.0) was added to the solubilized SR. To remove the detergent, the mixture was vigorously stirred at 25 °C in the presence of 0.1-0.2 g/ml of Bio-beads SM-2. After various length of the incubation, Bio-beads were removed by filtrating the mixture.

In some experiments, oxalate outside the proteoliposomes was removed. The suspension was passed through a column containing 5 ml of Sephacryl G-50 equilibrated with the assay medium, according to the column centrifugation method of Penefsky(18).

Measurements of Ca^{2+} uptake

In the experiments of Ca^{2+} loaded SR vesicles or Ca^{2+}-trapped proteoliposomes, Ca^{2+} uptake was measured under the condition of 0.1 mg/ml SR protein, 0.5 mM $^{45}CaCl_2$, 0.4 mM EGTA, 5 mM $MgCl_2$, 20 % glycerol, 50 mM Tris maleate (pH 7.0), and 0.3 mM ATP at 25 °C. At appropriate times, 1 ml aliquots of the reaction mixture were diluted with 5 ml of a stop solution which contained 5 mM EGTA, 5 mM $MgCl_2$, 0.1 M KCl, 20 mM Tris maleate (pH 7.0), and 20 % glycerol. They were immediatly passed through a Millipore filter (0.45 μm in pore size). The filters were dried and the radioactivity of ^{45}Ca remained in the filters was measured in the scintillation fluid with a liquid scintillation counter (Beckman Moel LS-150).

Ca^{2+} uptake by the reconstituted proteoliposomes (RSR) was usually measured at 25 °C after dilution of RSR by 1/10-1/50 with

assay medium without potassium-oxalate. Extravesicular free Ca^{2+} concentration was photometrically measured by the Ca^{2+} indicator dye , antipyrylazo III, which gives good signal to noise characteristics, appropriate affinity for Ca^{2+}, high selectivity, and fast response(19). 2.5 ml of the assay mixture which contained 0.4-10 ug/ml RSR, 0.25 M KCl, 5-8 mM $MgCl_2$, 5 % glycerol, 30 mM Hepes (pH 7), 100 μM antipyrylazo III, was added to a cuvett. $CaCl_2$ was added in 6.25 μl increments, also for calibrating for Ca^{2+} concentration, from 10 mM standard stock solution to the final concentration of 25-100 μM. The reaction was initiated by adding 0.3-1 mM ATP from 237 mM stock solution. Changes in calcium concentration were monitored continuously by Shimazu UV-300 dual wavelength spectrophotometer by measuring the absorbance at 720 nm and subtracting the absorbance at 790 nm.

Measurements of ATP hydrolysis and phosphoenzyme level

ATP hydrolysis and phosphoenzyme formation by RSR was measured in the same assay mixture for Ca^{2+} uptake but with [γ-^{32}P]ATP and without antipyrylazo III. For the measurements of ATP hydrolysis, the reaction was quenched at 30 sec by adding TCA solution to give final concentration of 5 % (w/v) TCA, 0.4 mM ATP, and 0.4 mM Pi. The amount of $^{32}P_i$ produced during the reaction were determined as described elsewhere(20).

Proteoliposomes were phosphorylated with 0.2 mM [γ-^{32}P]ATP for 5 sec at 0°C in order to avoid the AT^{32}P diffusion into the liposome lumen across the membrane. Double amounts of $C_{12}E_9$ to asolectin present was added after quenching by 5 % TCA with 5 mM nonradioactive ATP and P_i in the final concentrations, and vigorously stirred at 0°C before PCA washing.

The amounts of [^{32}P]EP formed were determined as described previously (20).

Simulation of Ca^{2+} transport by monomeric or dimeric ATPase

ATP-dependent Ca^{2+} storing capacity of the proteoliposomes might be depended on the concentration ratios of asolectin to SR ATPase. It was simulated according to following equations. Assumptions are: 1) ATPases are distributed to liposomes in monomeric form with the same probability and the Ca^{2+} storing capacity of each liposome is uniformly constant. 2) All the ATPases in the medium are intactly incorporated into the liposome membrane with equal probability in orientation, alternatively, right-side out or inside out. 3) Dimeric ATPases can be formed only from ATPases which are incorporated to the same liposome membrane in the same orientations. The probability P(x) that x copies of ATPases are incorpolated to one liposome is expressed by Poisson distribution as

$$P(x) = \frac{(n / L)^x}{x !} * \exp (- n / L)$$

where n and L are the total number of ATPases and that of liposomes in the medium, respectively. When monomeric ATPase is the minimal functional unit for Ca^{2+} uptake, the liposome uptakes Ca^{2+} with the probability $Q_m(x) = 1 - 1 / 2^x$. For about dimeric ATPases, the probability $Q_d(x) = 1 - (1 + x) / 2^x$. Then, total number of liposomes which can uptake Ca^{2+}, L_m and L_d, for each cases are

$$L_m = \sum_{x=1}^{n} \{ Q_m(x) * P(x) * L \}$$

and

$$L_d = \sum_{x=1}^{n} \{ Q_d(x) * P(x) * L \},$$

respectively.

Electron Microscopy

Proteoliposomes were examined by an electron microscopy, after negative staining. An aliquot of RSR solution was placed on carbon coated, polyvinyl formal films on 150-mesh copper grids and stained with 1 % uranylacetate. A JEOL 100-S electron microscope was used with an accelerating voltage of 80 kV.

Others

The amount of nonionic detergent $C_{12}E_9$ was assayed by the method of Garewal(21). The amount of phospholipid was determined by the method of Hess and Derr (22). Protein concentration was determined by the biuret reaction calibrated by nitrogen determination.

RESULTS

Inhibition of Ca^{2+} uptake by calcium loaded inside the SR vesicles or reconstituted proteoliposomes-----In Fig. 1, SR vesicles were preincubated for various lengthes of time in a solution contained 5 mM $CaCl_2$, and the time courses of Ca^{2+}-uptake by these Ca^{2+}-loaded SR vesicles were compared with that of by the proteoliposome reconstituted in the presence of 5 mM $CaCl_2$ under the condition as described in the previous paper(8). As shown in the figure, both the rate and extent of Ca^{2+}-uptake by SR vesicles decreased with increasing the preloading time and approximately 40 % of the initial activity was lost at 8 hour incubation with 5 mM $CaCl_2$. This activity seemed to be unaffected by further incubation for up to 25 hour. In addition, the rate and extent of Ca^{2+} transport by the proteoliposome were similar to those of the SR vesicles preloaded with 5 mM $CaCl_2$. These results suggested that the relatively low Ca^{2+} uptake activity of the reconstituted membrane is due to the high concentration of Ca^{2+} which remained inside the proteoliposomes. Therefore, in order to obtain the reconstituted membrane with a high Ca^{2+}-transport activity, it is important to examine the other reconstitution conditions in which 5 mM $CaCl_2$ could be excluded from the medium with retension of full activity of the Ca^{2+}-ATPase.

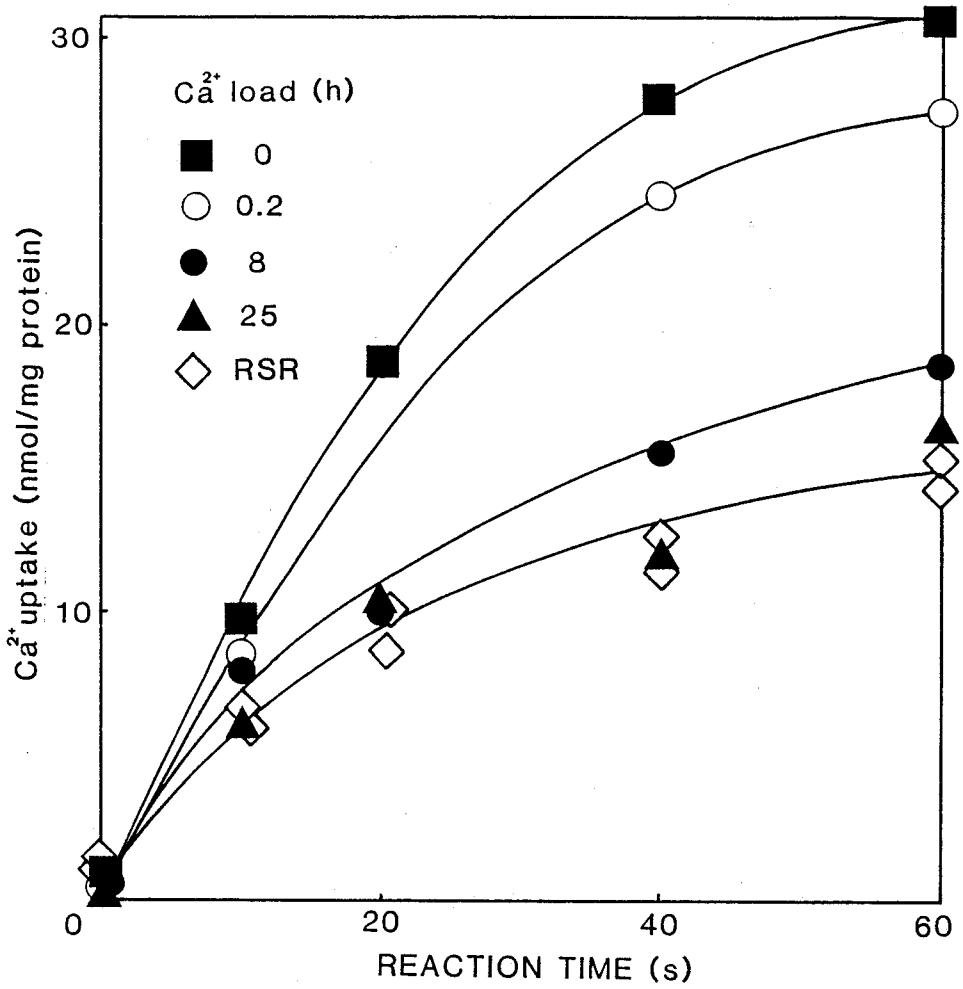

FIGURE 1. Effect of preloading of SR with Ca^{2+} on the Ca^{2+} uptake activity. 2 mg/ml intact SR vesicles were incubated in the medium containing 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 M KCl, 20 % glycerol, and 40 mM TES (pH7.5) at 20 °C for 0 (■), 0.2 (○), 8 (●), and 25 (▲) hours. (◇), SR vesicles reconstituted in the presence of 5 mM $CaCl_2$ as described by Andoh and Yamamoto (8), in which potassium-oxalate was absent. For Ca^{2+} uptake measurements, each membrane suspension were diluted by 10 fold with assay medium containing a small amount of $^{45}Ca^{2+}$, 0.4 mM EGTA, 5 mM $MgCl_2$, 20 % glycerol, and 50 mM Tris maleate (pH 7.0). The reaction was initiated by adding 0.3 mM ATP at 25 °C. The reaction was stopped by dilution with "stop-solution" at the times indicated, and the amount of $^{45}Ca^{2+}$ accumlated was determined.

Stabilization of solubilized Ca^{2+}-ATPase by phospholipid-----It was reported in earlier papers (11-13) that SR ATPase solubilized in a high concentration of $C_{12}E_8$ or Triton X-100 irreversibly lost the EP formation activity as a function of incubation time when the Ca^{2+} concentration in the solubilizing medium was below 1 mM. However, it was found that the irreversible inactivation of the $C_{12}E_8$-solubilized Ca^{2+}-ATPase in the absence of free Ca^{2+} was prevented by the addition of phospholipid to the incubation medium. In the experiments shown in Fig. 2a, 0.1 mg/ml SR membrane was solubilized with 50 mg/ml $C_{12}E_8$ in the presence of 2 mM EGTA to remove free Ca^{2+} and incubated at 25 °C with various concentrations of soybean lecithin. Without addition of phospholipid to the SR membrane, EP formation activity was decreased to about 20 % of the initial activity during 1 hour of incubation after the solubilization of SR membrane. The rate of inactivation of EP formation was decreased with increasing the concentration of phospholipid in the incubation medium. Most of the EP formation activity was retained for more than 30 minutes of incubation, when higher than 5 mg/ml phospholipid was present in the medium. More than 60 % of the initial activity was still remained for 1 hour after initiating the incubation when phospholipid was present at concentrations higher than 10 mg/ml. In the presence of 5 mM Ca^{2+}, the enzymatic activity was essentially unaffected by the addition of phospholipid at concentrations from 10 to 30 mg/ml(Fig. 2b). Thus, the $C_{12}E_8$

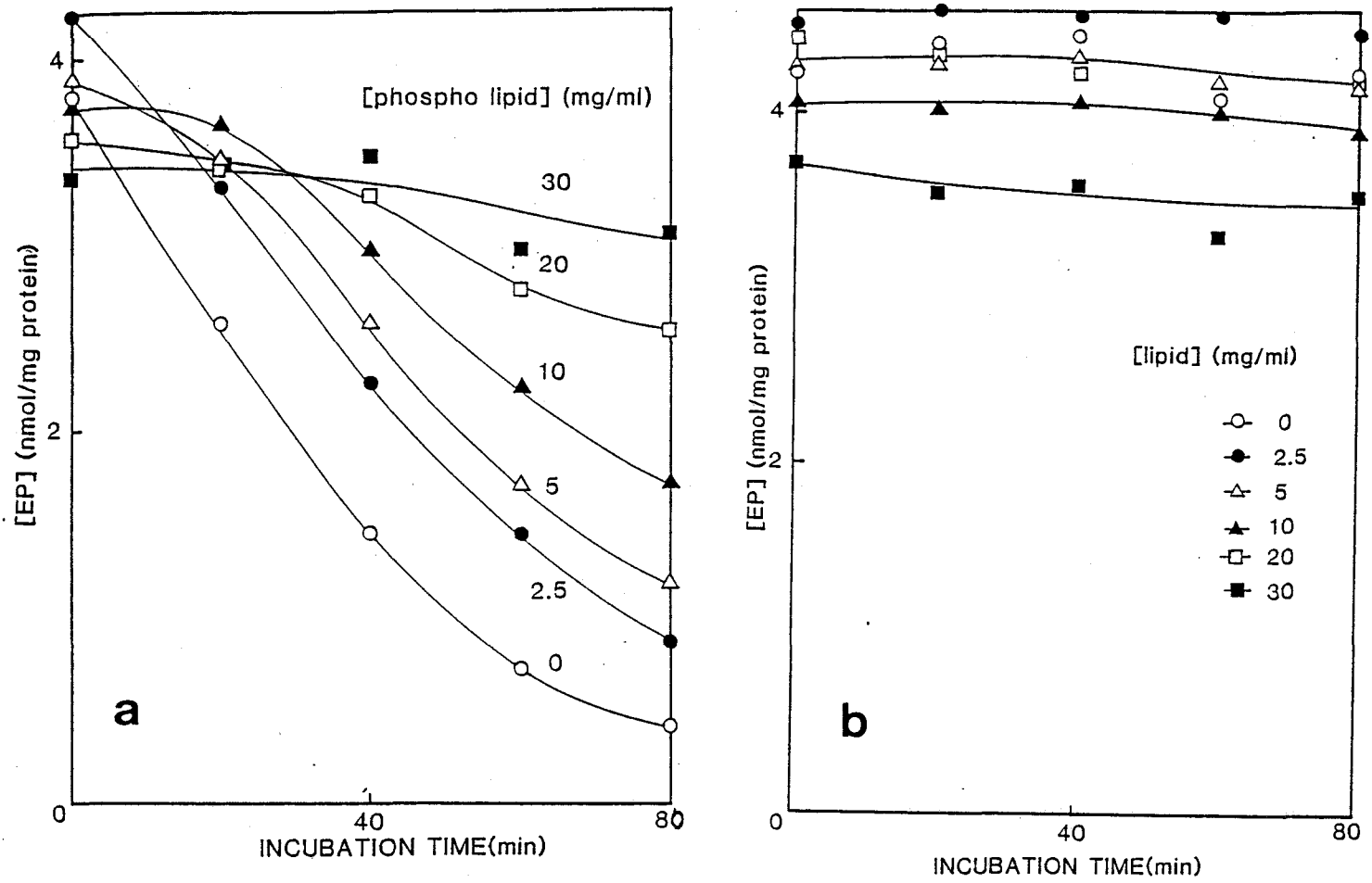

FIGURE 2. Stabilization of phosphorylation activity of solubilized Ca^{2+}-ATPase by phospholipid. a: 0.1 mg/ml SR vesicles were solubilized with 50 mg/ml $C_{12}E_8$ in the Ca^{2+} free medium which contained 2 mM EGTA, 0.1 M KCl, 5 mM $MgCl_2$, 20 % glycerol, 50 mM TES (pH 7.5), and various concentrations of soybean lecithin. After the time of incubation at 25 °C, 7 mM $CaCl_2$ was added to stabilize the enzyme and the suspension were diluted by 10 fold as in FIGURE 1, but without $^{45}Ca^{2+}$. EP formation was carried out by adding 0.2 mM [γ-^{32}P]ATP to the medium and the amount of $E^{32}P$ was determined as described in "EXPERIMENTAL PROCEDURES." b: Same experiments as a, except that 5 mM $CaCl_2$, instead of 2 mM EGTA, was initially presented in the incubation medium and subsequent addition of 7 mM $CaCl_2$ was omitted. For a and b, the concentration of added lecithin were 0 (○), 2.5 (●), 5 (△), 10 (▲), 20 (□), 30 mg/ml (■).

solubilized Ca^{2+}-ATPase can also be stablilized for a long time in the presence of soybean phospholipid instead of $CaCl_2$. Since most of the detergent in the medium is removed by Bio-beads SM-2 within 30 minutes of incubation(see below), $CaCl_2$ can be omitted from the reconstitution medium without loss in the enzymatic activity if sufficient concentration of phospholipid was added immediately after the solubilization. Similar effect of phospholipid was also observed with $C_{12}E_9$-solubilized Ca^{2+}-ATPase (data not shown). In the present study, the Ca^{2+} transport was reconstituted from monomeric Ca^{2+}-ATPases solubilized with excess $C_{12}E_9$(50 mg/ml) and asolectin added after solubilization.

Kinetic Properties of the Reconstituted Proteoliposomes----Figure 3 shows the time course of reconstitution of Ca^{2+} uptake after the addition of Bio-beads to the mixture of the $C_{12}E_9$-solubilized ATPase and soybean asolectin. The concentration of $C_{12}E_9$ decreased rapidly from 50 to 9.5 or to 1.5 mg/ml during 30 minutes or 1 hour of the incubation, respectively. In accordance with the decrease in the detergent concentration, Ca^{2+}-transport activity was increased with time. At 30 minutes, Ca^{2+} uptake was already restored partially. The rate of Ca^{2+} transport reached to the maximum about 1 hour after initiation of the incubation, then it was decreased slowly by further incubation with Bio-beads.

It is well known that accumulation of Ca^{2+} by intact SR vesicles is greately enhanced by the addition of oxalate to the reaction mixture outside SR vesicles(23). On the other hand,

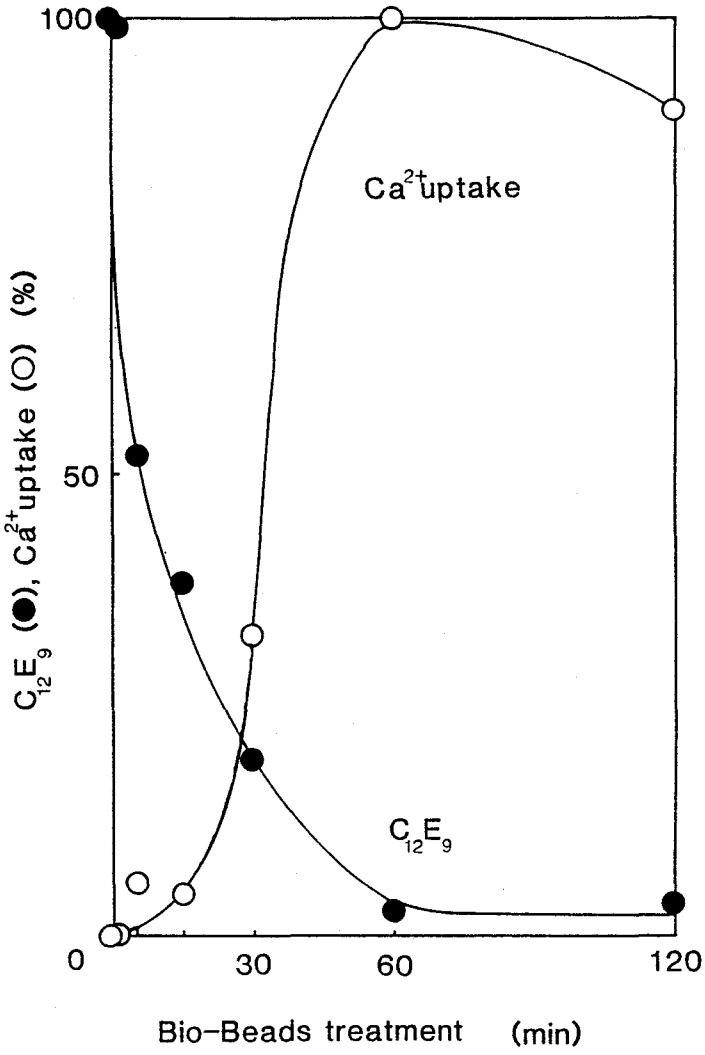

FIGURE 3. Time course of reconstitution of Ca^{2+} uptake from solubilized Ca^{2+}-ATPase and soybean asolectin. 0.1 mg/ml SR vesicles solubilized with 50 mg/ml $C_{12}E_9$ in the solubilizing solution was treated with Bio-Beads to remove the detergent after addition of 20 mg/ml asolectin. At various times of incubation, Bio-Beads was excluded by filtration and the rates of Ca^{2+} uptake were measured (○) using antipyrylazo III as the Ca^{2+} indicator dye after dilution by 10 fold with assay medium as described under "EXPERIMENTAL PROCEDURES". The concentration of $C_{12}E_9$ (●) left in the filtrate was also determined. Maximum Ca^{2+} uptake rate was 2.36 μmol/mg/min.

Ca^{2+} uptake of the reconstituted SR was not accelerated by the external addition of oxalate. To obtain proteoliposomes with high activity of Ca^{2+}-transport, oxalate had to be added before tight vesicles were formed. This can be explained that the population of proteoliposomes having the anion channel woulad be much lower than that of intact SR vesicles when large amount ofphospholicid was added to dilute bilayer lipid of SR membrane. In Fig. 4, proteoliposomes were prepared by varing the oxalate addition time after the start of Bio-Beads treatment. Time courses of Ca^{2+} uptake by the proteoliposomes were measured by monitoring free Ca^{2+} with Ca^{2+} indicator dye antipyrylazo III. The proteoliposomes showed only a low rates and extents of Ca^{2+} uptake when 50 mM oxalate was added at 30 minutes of Bio-Beads incubation. On the other hand, 2-3 fold of enhancement in both rates and extents of Ca^{2+} uptakd was observed when oxalate was added within 15 minutes after the start of Bio-Beads treatment. Furthermore, the initial rates of Ca^{2+} uptake of the reconstituted membrane was dependent on the concentration of oxalate inside the vesicle (Fig. 5). The proteoliposomes were prepared in the presence of various concentration of oxalate in the reconstitution medium. The initial rates of Ca^{2+} uptake was measured as in Fig. 3. As shown in Fig. 5, the rate increased as a function of oxalate concentration and reached the maximum rate at about 100 mM oxalate. Therefore, in the following experiments, I added 150 mM oxalate to the reconstituting medium in the present study.

To show that the Ca^{2+} uptake by the proteoliposomes was not

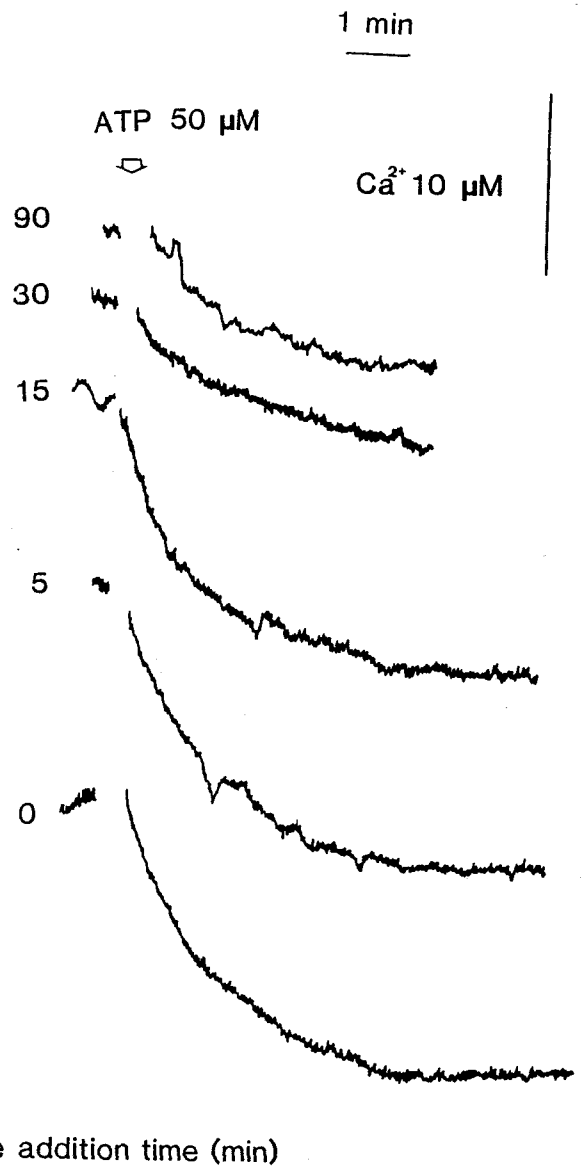

FIGURE 4. Dependence of oxalate addition time during the Bio-Beads treatment on Ca^{2+} uptake by proteoliposomes. Proteoliposomes were reconstituted as described in the legend to FIGURE 3 except that potassium-oxalate was initially omitted. At various times of Bio-Beads incubation indicated, 50 mM potassium-oxalate was added and Bio-Beads treatment was continued up to 90 min. After dilution of the proteoliposomes by 50 fold with the assay medium, they were assayed for Ca^{2+} uptake by continuously monitoring the concentration of Ca^{2+} with antipyrylazo III, in addition of 20 μM $CaCl_2$ and then 50 μM ATP.

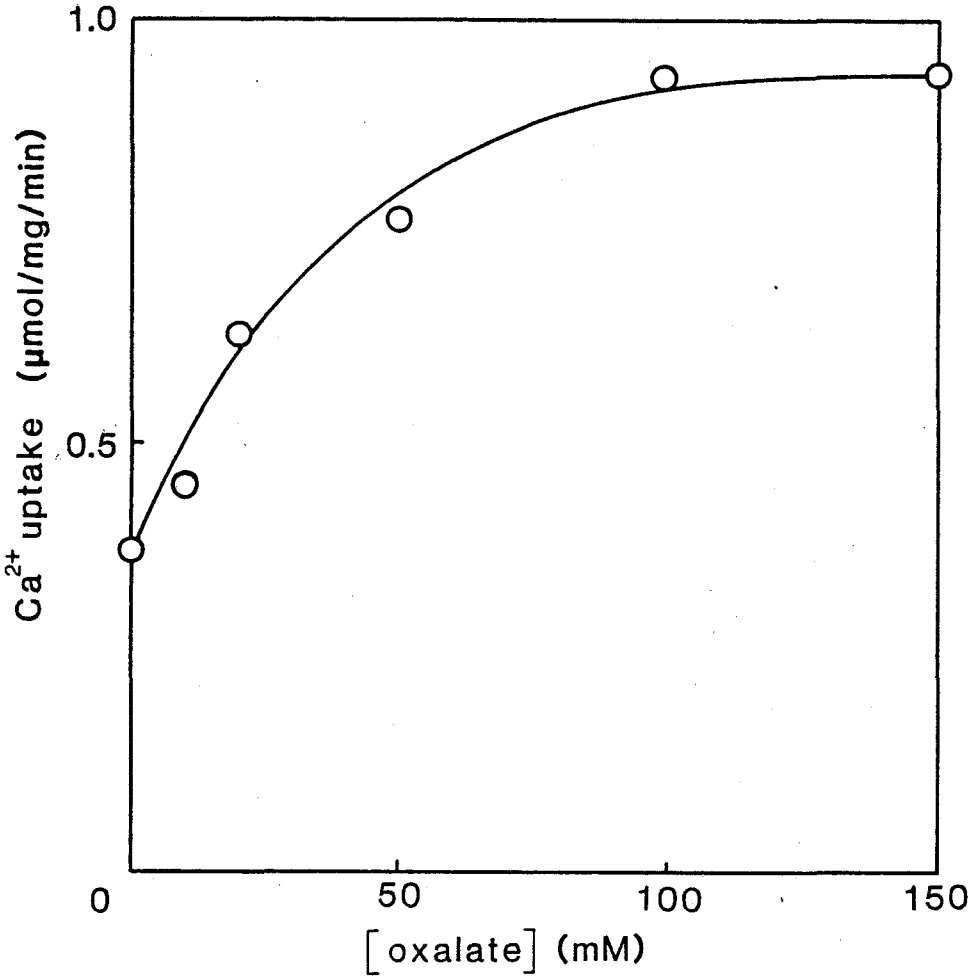

FIGURE 5. The dependence of Ca^{2+} uptake initial rate on the concentration of oxalate in the reconstituting medium. The proteoliposomes were reconstituted from 1 mg/ml SR vesicles solubilized with 50 mg/ml $C_{12}E_9$ and 20 mg/ml soybean asolectin in the presence of various concentrations of potassium-oxalate indicated. After dilution of the proteoliposomes by 100 fold, the rate of Ca^{2+} uptake was measured from the time dependent change in antipyrylazo III absobance. The reaction medium contained 0.01 mg/ml proteoliposomes, 0.25 M KCl, 5 mM $MgCl_2$, 5 % glycerol, 30 mM Hepes (pH 7.0), 100 uM antipyrylazo III, 25 μM $CaCl_2$, and 50 μM ATP.

artefact caused by the oxalate addition, the effect of calcium ionophore on the Ca^{2+} uptake activity was tested. Fig. 6 shows the time course of Ca^{2+} uptake monitored by antipyrylazo III. Ca^{2+} uptake by the proteoliposomes was observed only when ATP was added(Fig. 6 right), which indicated that the Ca^{2+} uptake by the proteoliposomes was ATP dependent. On the other hand, when 10 μM A-23187, calcium ionophore, was added in stead of ATP to proteoliposome to make the membrane leaky, no uptake of Ca^{2+} was observed (Fig. 6 left). These results indicate that the Ca^{2+} accumulation by the proteoliposomes was not caused by the passive influx of Ca^{2+} to form precipitation with oxalate inside the vesicle, but by the pumping activity of the enzyme. On the other hand, A-23187 strongly inhibited the ATP dependent Ca^{2+}-accumulation by the proteoliposomes(Fig. 6 left).

Figure 7 shows the time courses of the ATP-dependent Ca^{2+} uptake by proteoliposomes which were reconstituted from 0.1 mg/ml of the SR protein and various concentrations of asolectin in the presence of 150 mM oxalate. The rate of Ca^{2+} accumulation into the proteoliposome increased as a function of the weight ratio of phospholipid to SR protein. ATP-independent decrease in the free Ca^{2+} was observed, but the rate was negligiblly small(less than 4 %) compared to the initial rates of ATP- dependent Ca^{2+} uptake by the proteoliposomes.

As shown in Fig. 8, both the rates of Ca^{2+} uptake and ATP hydrolysis by proteoliposomes were dependent on the concentration of asolectin in the reconstitution medium. When proteoliposomes were reconstituted with various concentrations of the

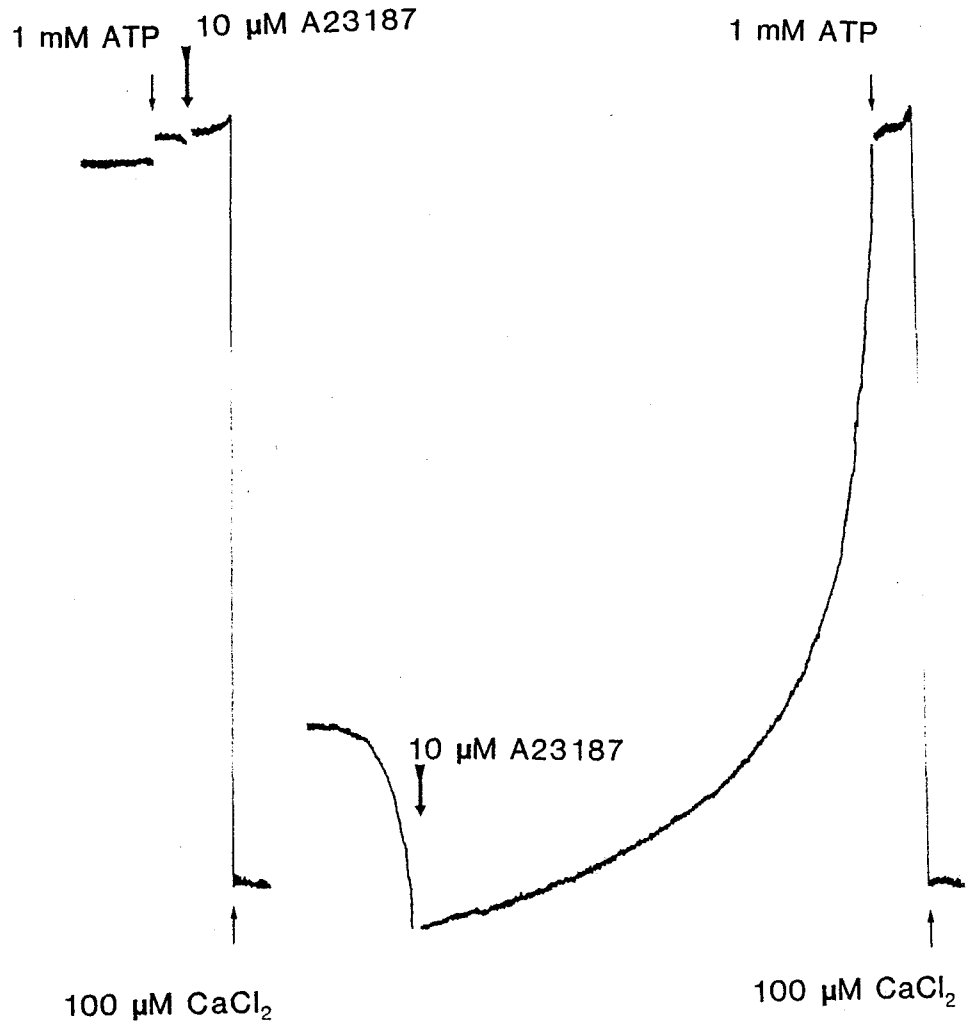

FIGURE 6. Inhibition of Ca^{2+} uptake by proteoliposomes by calcium ionophore. 1 mg/ml SR vesicles were reconstituted with 20 mg/ml $C_{12}E_9$ and 30 mg/ml asolectin. The time course of the Ca^{2+} concentration changes were monitored in the assay medium as described in FIGURE 3. In the presence of 100 μM $CaCl_2$, 1 mM ATP was added to the assay medium to initiate the reaction. After (right) or before (left) the addition of ATP, 10 μM A-23187 was added from 5 mM stock in ethanol.

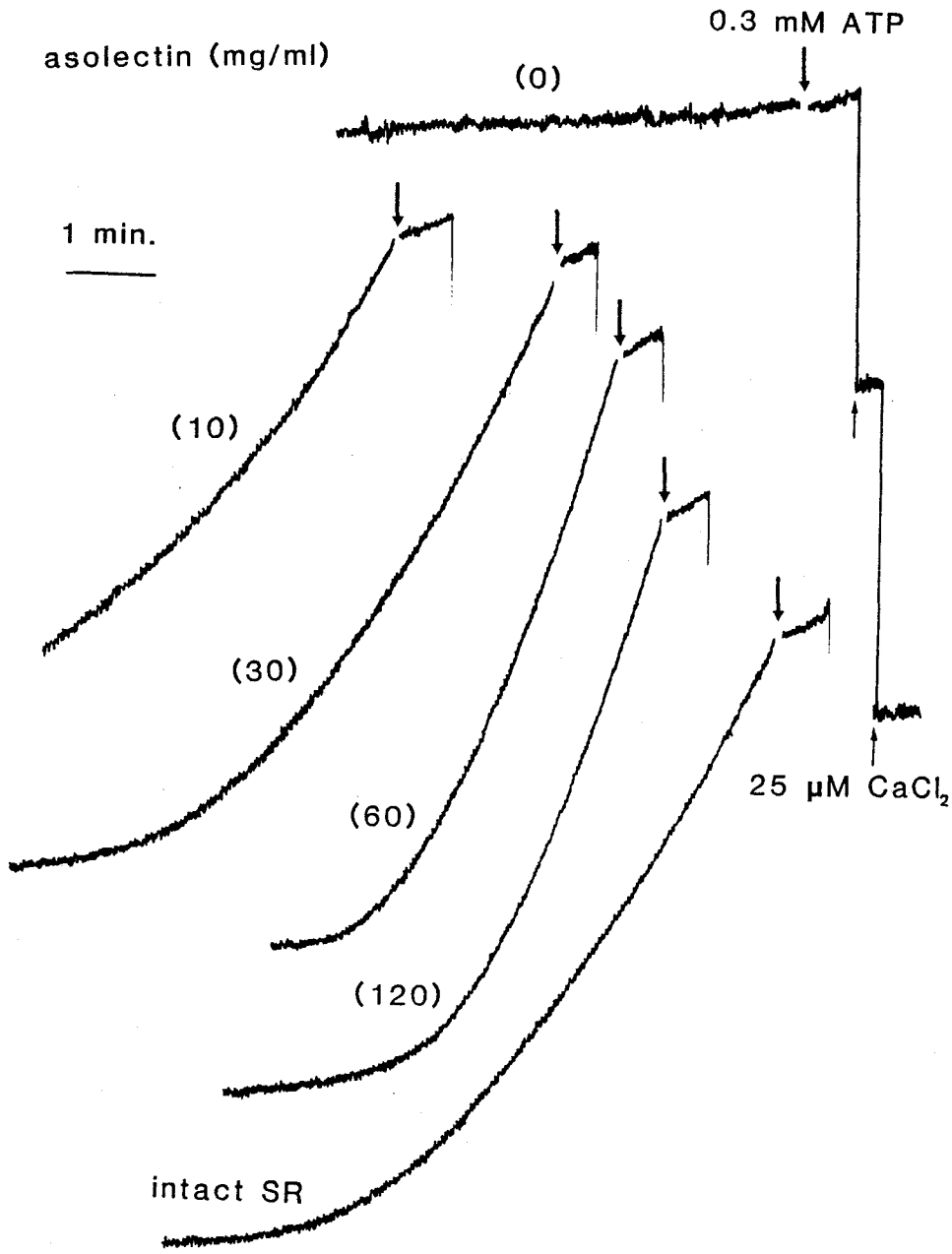

FIGURE 7. Time course of Ca^{2+} uptake by reconstituted proteoliposomes. 0.1 mg/ml SR vesicles were reconstituted with 50 mg/ml $C_{12}E_9$ and various concentration of asolectin indicated. They were diluted by 10 fold with the assay medium and assayed for Ca^{2+} uptake as described in FIGURE 3 by addition of 50 μM $CaCl_2$ and 0.3 mM ATP at 25 °C. For comparison, the Ca^{2+} uptake by intact SR vesicles in the same protein concentration as those of the proteoliposomes was also monitored in the assay medium.

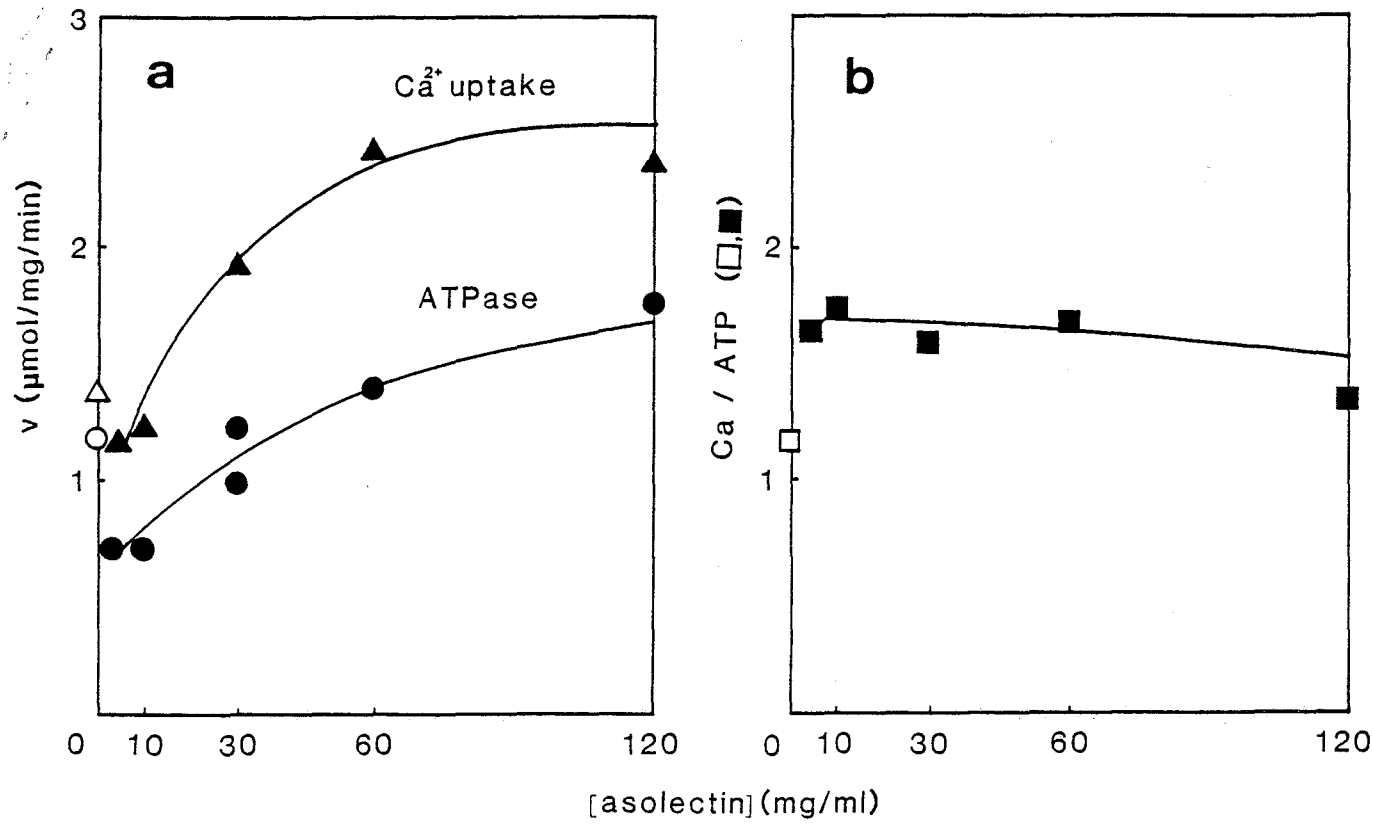

FIGURE 8. Dependence of coupling ratio of Ca^{2+} transport by proteoliposomes on the asolectin concentration. a: Proteoliposomes were prepared as in FIGURE 7 at various concentrations of asolectin (5-120 mg/ml) and assayed at 25 °C after dilution by 10 fold with the assay medium. The initial rates of Ca^{2+} uptake by proteoliposomes (▲) and intact SR vesicles (△) were measured as described in FIGURE 3. The initial rates of ATP hydrolysis by RSR (●) and intact SR vesicles (○) were measured in the same condition except that [γ-^{32}P]ATP was added to the assay medium in the absence of antipyrylazo III. The amount of $^{32}P_i$ produced was determined as described under "EXPERIMENTAL PROCEDURES." b: The coupling ratio, (Ca^{2+} uptake)/(ATP hydrolysis) in initial rates were plotted against the concentraion of asolectin. (■) proteoliposomes; (□) intact SR vesicles.

phospholipid from 5 to 120 mg/m in the presence of 0.1 mg/ml SR protein, the rates of Ca^{2+} uptake and ATP hydrolysis increased from 1.2 to 2.4 μmol and 0.7 to 1.8 μmol/mg protein/min, respectively. While, the coupling ratio between Ca^{2+} uptake and ATP hydrolysis was about 1.5, which was independent of the lipid concentrations widely ranged from 5 to 120 mg/ml. These values were compatible to or greater than those of intact SR vesicles (1.4 μmol Ca^{2+} uptake and 1.2 μmol ATP hydrolysis/mg protein/min, and the coupling ratio of 1.2 under our condition). Fig. 9 shows the steady state levels of phosphoenzyme(EP) for both intact SR and reconstituted proteoliposomes. In the intact SR, the level of EP at steady state of the ATPase reaction was about 4.2 nmol/mg protein, while only half of the amount of EP(2.1 nmol/mg protein) was formed in the reconstituted proteoliposomes. The level of EP was independent of the concentration of phospholipid in the reconstitution medium in the range from 5 to 120 mg/ml. To examine whether or not ATPases were denatured in the reconstitution process, I measured the EP level of the proteoliposomes which had been partially solubilized again with $C_{12}E_9$. The levels of EP of them became the same as that of intact SR vesicles (Fig. 9). These results indicate that Ca^{2+}-ATPase were reasonablly reconstituted without loss of enzyme activity. The results also indicate that Ca^{2+}-ATPase were incorporated into the liposome membrane at random orientations, inside-out or rightside-out, so that only half of the enzyme catalytic sites would react with external ATP and Ca^{2+}.

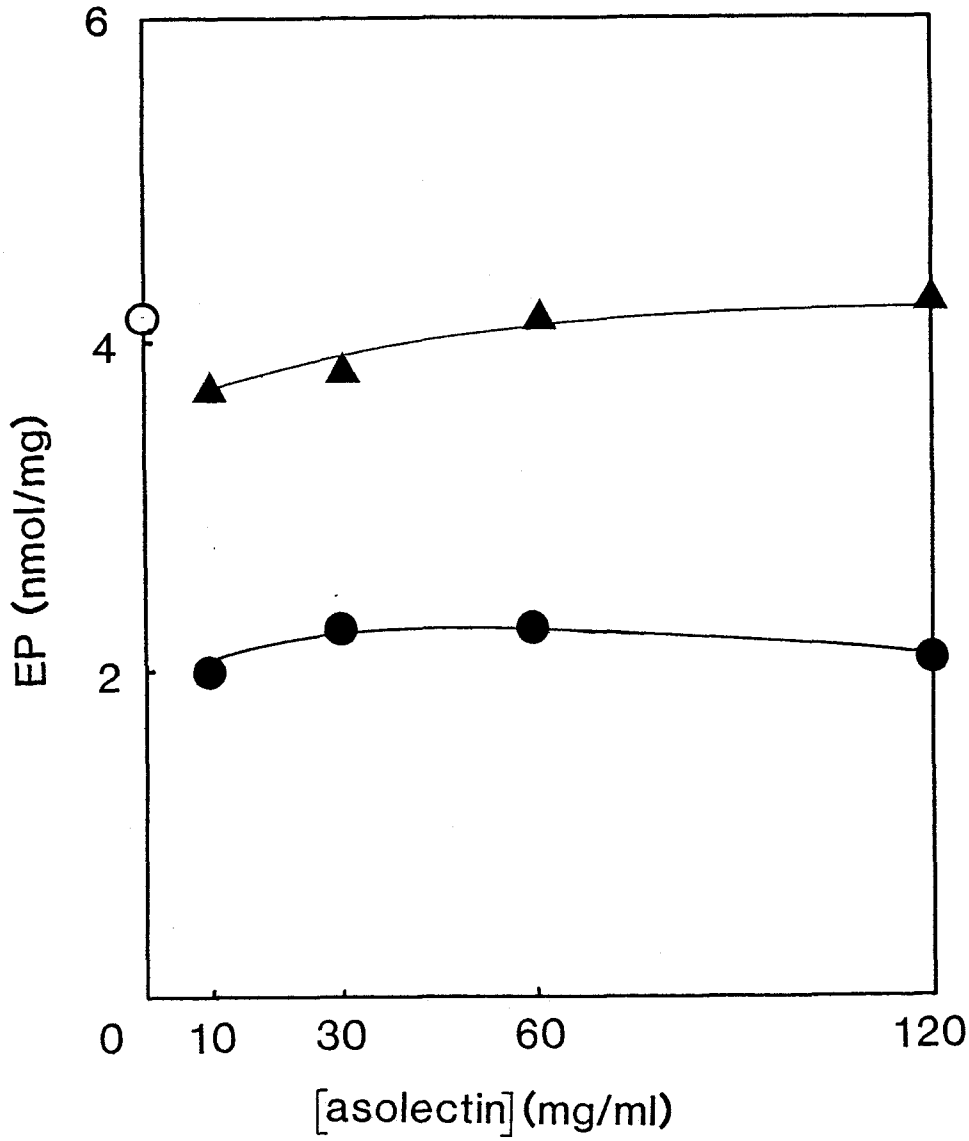

FIGURE 9. Dependence of phosphoenzyme formation by proteoliposomes on the asolectin concentration. Proteoliposomes at various concentrations of asolectin were prepared as in FIGURE 7 and diluted by 5 fold with the assay medium. Phosphorylation was carried out at 0 C for 5 sec. in the assay medium with 100 μM $CaCl_2$ and 0.2 mM [γ-^{32}P]ATP for RSR (●) and for intact SR vesicles (○). The reaction was quenched with 5 % TCA and the amounts of [^{32}P]EP was determined as described under "EXPERIMENTAL PROCEDURES". EP levels (▲) were also measured after addition of 2.5, 5, 15, 30, 50 mg/ml $C_{12}E_9$ to the proteoliposomes of 5, 10, 30, 60, 120 mg/ml asolectin at reconstitution, respectively.

Structural characterization of the reconstituted proteoliposomes-----Figure 10 shows the electron micrograph of the proteoliposomes which were negatively stained with 1 % uranium acetate. The proteoliposomes were prepared from 0.015 mg/ml SR protein solubilized with 50 mg/ml $C_{12}E_9$ and 20 mg/ml asolectin as described in "EXPERIMENTAL PROCEDURES". It was observed that most of the reconstituted vesicles were formed with a single wall of lipid bilayer and the diameter of the vesicle varied from about 80 to 160 nm. These values were cclose to the average diameter of liposome which was estimated of the basis of column chromatography on Sephacryl S-1000 by using various size of Immutex as markers (data not shown). The size were also close to the reported value by Ueno et al. (24) who used $C_{12}E_8$ and Bio-Beads coumn in reconstitution. Although the ratios in concentrations (mg/ml) between the lipid to protein were varied from 20 to 1333, the average diameters remained the same.

In Fig. 11, I measured the ATP dependent Ca^{2+}-storing capacity of the proteoliposomes which was defined as maximum amount of Ca^{2+} (nmol) accumulated by the proteoliposomes per phospholipid (mg). The proteoliposomes were reconstituted with 20 mg/ml asolectin with varying concentration of SR protein from 2 to 0.015 mg/ml. The extra-liposomal oxalate was removed by column centrifugation method. In the case of these samples, no ATP-independent decrease in the concentration of Ca^{2+} was obserbed. The recovery of the lipid and protein was more than 96 % and 90 % in the proteoliposomes,respectively.

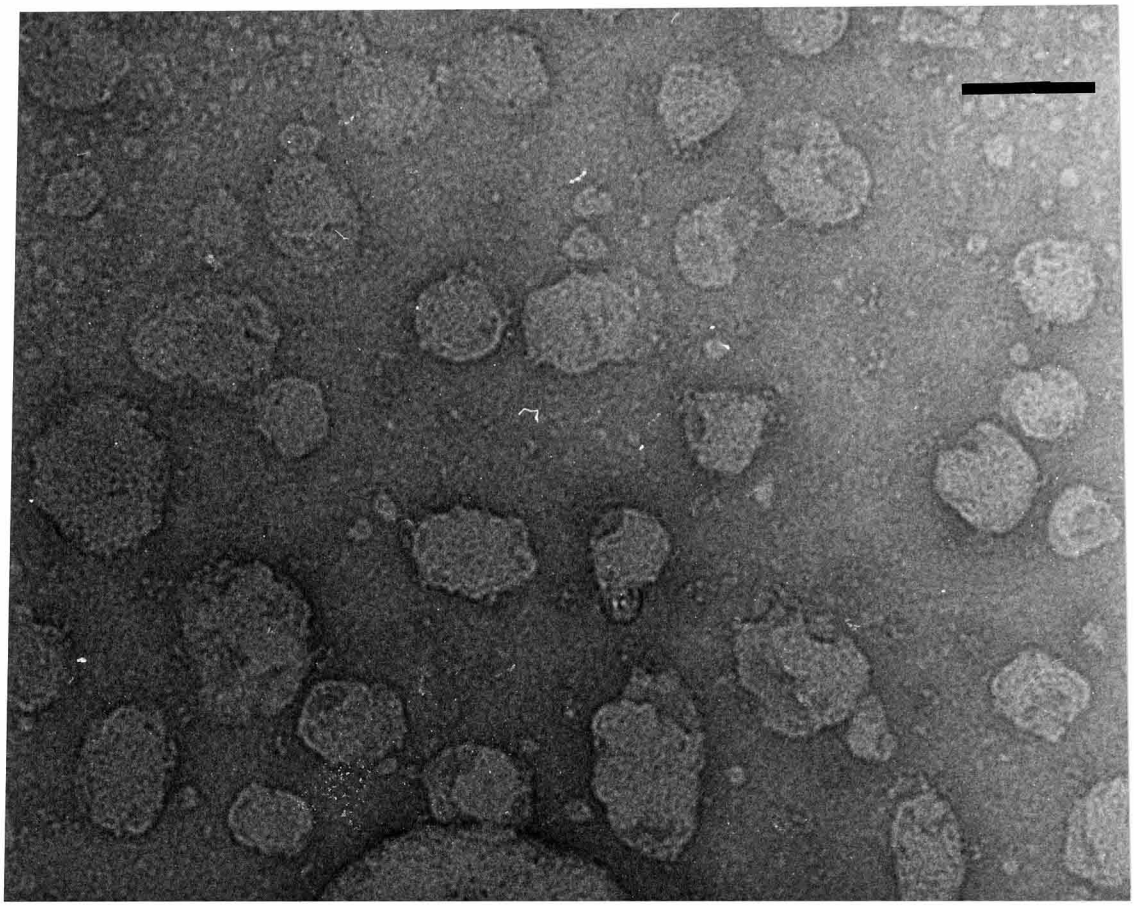

FIGURE 10. Electron micrograph of the reconstituted proteoliposomes. 0.1 mg/ml SR vesicles were reconstituted with 50 mg/ml $C_{12}E_9$ and 20 mg/ml asolectin as described in FIGURE 3. The proteoliposomes were negatively stained with 1 % uranium acetate as described in "EXPERIMENTAL PROCEDURES". Bar indicated 100 nm.

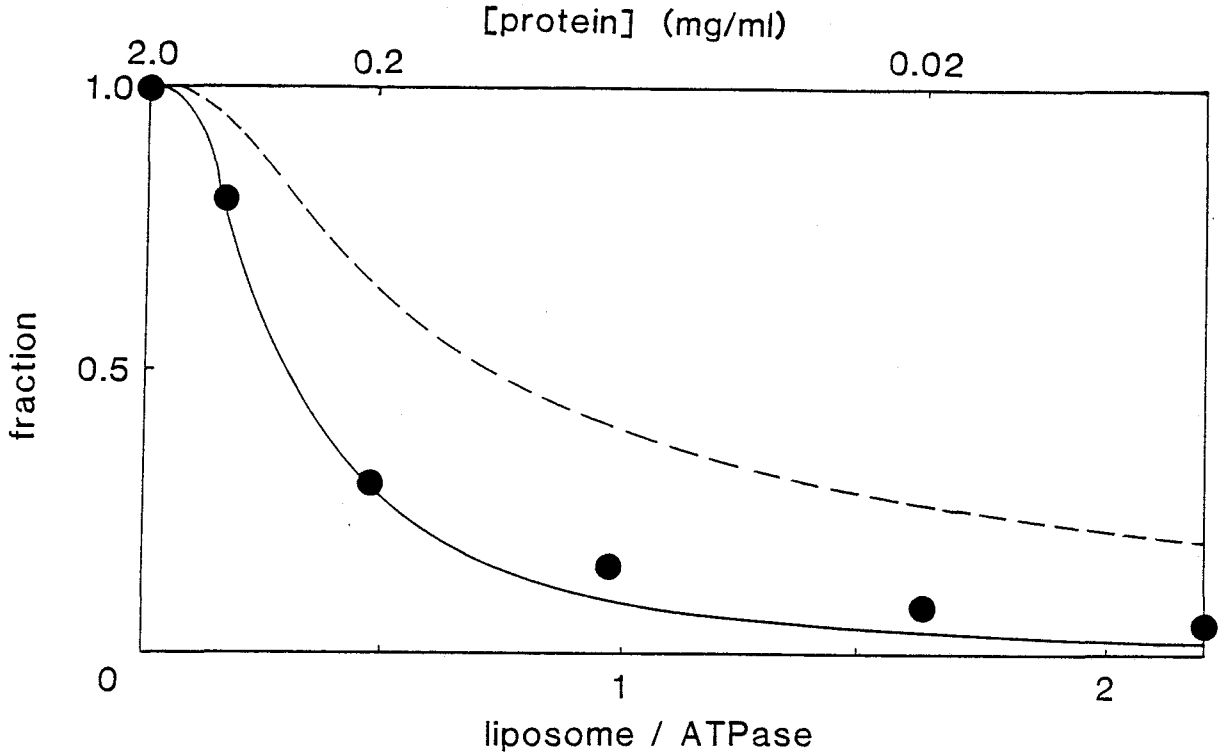

FIGURE 11. Dependence of the Ca^{2+} storing capacity on the extent of lipid dilution. The Ca^{2+}-storing capacity (muximum amount of Ca^{2+} accumulated nmol/mg lipid) by each proteo-liposomes was measured by repeatedly added 25 μM $CaCl_2$ and 0.3 mM ATP as described in the legend to FIGURE 3. The capacity of the Ca^{2+} accumulated by the proteoliposomes reconstituted from 2 mg/ml SR protein and 20 mg/ml asolectin was assumed to be the total inner space(53.3 nmol Ca^{2+}/mg lipid) of the liposomes. The ratio (Ca^{2+} capacity of each proteoliposomes)/(total inner space) was assumed to be the fraction of active liposomes to total liposomes in number. It was plotted(●) against the number of unit liposome (ϕ=114 nm) per ATPase (bottom). The concentration of SR protein in the reconstitution medium was also shown (top). Solid or dashed lines were simulated assuming "dimer" or "monomer" for functional unit, respectively, as described in "EXPERIMENTAL PROCEDURES".

The Ca^{2+} storing capacity of the proteoliposomes decreased from about 53.3 to 3.5 nmol/mg phospholipid with the decrease in the SR protein/lipid ratio. As shown in Fig. 11, the ratio of the number of active liposomes, which means the measured Ca^{2+} capacity, to that of total liposomes, which means the total inner space for Ca^{2+} accumulation, decreased as a function of lipid dilution. The data could be fitted with the theoretical curve which was obtained by assuming that Ca^{2+} transport can be catalyzed by dimeric form of the ATPase, than with that obtained for monomeric form, under following assumption. The number of the Ca^{2+}-ATPase incorpolated in one liposome varied from about 67 to 0.5 at the SR protein range from 2 to 0.015 mg/ml, if we assume that the monomeric ATPase were incorporated at random into lipid bilayer of sphere liposomes which have an uniform diameter of 114 nm, and that the lipid area is 60 A^2/lipid(25). The assumed value of the diameter was well agreed to the value obtained from observation in electron microscopy or colomn chromatography.

DISCUSSION

In the present study, I have provided a fine procedure for reconstituting an active Ca^{2+} transport from the monomeric Ca^{2+}-ATPase solubilized with excess $C_{12}E_9$. In PART I, it was shown that EP hydrolysis in intact SR vesicles was strongly inhibited by high concentration of Ca^{2+} inside the vesicle with K_{Ca} about 5 mM (26). In reconstituting Ca^{2+} transport from monomeric Ca^{2+}-ATPase, it has been one of the most important problems that Ca^{2+}-ATPase can be solubilized into monomer with retention of full enzymatic activity in high concentration of $C_{12}E_8$ or $C_{12}E_9$ without addition of Ca^{2+}. This problem has been solved by the finding that the enzymatic activity of the $C_{12}E_8$- or $C_{12}E_9$-solubilized ATPase could be retained for a long time in the presence of phospholipid instead of high concentration of Ca^{2+}(Fig. 2). It is unkown whether or not the mechanism by which the lipid stabilizes the soluble ATPase is the same as that by which Ca^{2+} does. It is unlikely that phospholipid stimulates the formation of oligomeric ATPase under the conditions in which SR ATPases were solubilized by excess concentration of $C_{12}E_8$ over that of asolectin. As shown in Fig. 2a, the Ca^{2+}-ATPase was still able to interact with phospholipid even in the presence of much higher concentration of $C_{12}E_8$ than that of phospholipid. This suggests that the Ca^{2+}-ATPase might have a specific region to which phospholipids bind in preference to the detergent. It may be possible that the specific region, which is sensitive to the stability of the enzyme, of the Ca^{2+}-ATPase will be exposed to detergent or phospholipid without high concentration of Ca^{2+}.

Under the condition as described in Fig. 2a, phospholipids of asolectin would bind to the region by replacing with $C_{12}E_8$ and prevent the denaturation of the enzyme to stabilize the molecular structure. Since it has been reported that the Ca^{2+}-ATPase was unstable when it was solubilized with other detergents such as deoxycholate (27) or Triton X-100 (28) at low concentration of Ca^{2+}, the instability of the Ca^{2+}-ATPase would not be due to chemical effects specific of $C_{12}E_8$ or $C_{12}E_9$. As the lipid stabilized the detergent solubilized monomeric ATPase, it is suggested that lipid-protein interaction is essential for the stability of the ATPase than protein-protein interaction.

As shown in Fig. 8, both the rates of Ca^{2+} transport and ATP hydrolysis by the proteoliposomes increased with the increase in the concentration of asolectin in the reconstitution medium, and reached the maximum level at about 60 mg/ml asolectin. These activities were twice higher than those of intact SR vesicles. On the other hand, the amount of EP formed by the proteoliposomes at steady state was reduced to half of the intact SR (Fig. 9) probablly due to the random orientation of the Ca^{2+}-ATPase molecules in the reconstituted membrane (see below). It is possible that soybean asolectin contained slight amount of specific lipids which can directly interact with the Ca^{2+}-ATPase to enhance the reaction cycle of the Ca^{2+}-ATPase. Another possibility is that oligomer formation by the ATPase regulates the rates of some steps of the enzyme reaction to be reduced.

The method for the reconstitution of Ca^{2+} transport described here is useful to investigate the detailed processes of the membrane organization by varing the incubation time with

Bio-Beads. As shown in Fig. 3, the concentration of $C_{12}E_9$ in the reconstitution medium decreased with the incubation time with Bio-Beads and reached the minimum level in an hour. The Ca^{2+} transport activity increased in correspondance to the time-course of the detergent removal, and reached the maximum activity at one hour. On the other hand, oxalate impermeable vesicles were formed within 30 min after the start of incubation with Bio-Beads, although 20 % of $C_{12}E_9$ still remained in the reconstitution medium (Fig. 5). One of the possible explanation for the time difference between the vesicle formation and the complete restoration of Ca^{2+} transport activity is the time required for rearrangement of ATPase molecules in the membrane to form the functional structure for Ca^{2+} transport.

The method of reconstitution of the active proteoliposomes from monomeric ATPase is further available to elucidate whether or not the Ca^{2+}-pump ATPase exists in the reconstituted membrane in an oligomeric form. I examined the effect of lipid phase dilution of the reconstituted membrane upon the fluorescence energy transfer from a fluorescence donor to acceptor which are located on the separate molecules of the ATPase (29). The intensity of the fluorescence energy transfer was not reduced by the lipid dilution up to 30-fold excess of the SR lipid. Under these conditions average distance between the ATPase molecules in the lipid bilayer will be 5-6 times greater than that in intact SR. This suggests that Ca^{2+}-ATPase exists in the reconstituted membrane as an oligomer when the small dilution of the lipid phase is performed.

It is still unknown whether the minimal functional unit of

the Ca^{2+} transport by the Ca^{2+}-ATPase in intact SR membrane is an oligomer (11,12,30) or a monomer (31,32). In my reconstitution method, the Ca^{2+}-ATPase could be extremely diluted with phospholipid probably into the minimum form in the membranous state. Therefore, it would be useful in elucidating the relationship between function and quarternary structure of the enzyme. In Fig. 11, the decrease in Ca^{2+} uptake capacity of proteoliposome could be fitted to "dimeric assumption curve" as the ratio of ATPase/liposome decreased to below 1 by large dilution of the ATPases with the liposomes. If most of the ATPases are dispersed as monomeric form into different proteoliposomes, it could be concluded that the monomeric Ca^{2+}-ATPase can not transport Ca^{2+}. However, since EP levels and coupling stoiochiometry (Ca/ATP) were retained constantly even in the range of extensive dilution in the lipid phase (Figs. 8 and 9), it seemed that the monomeric ATPase can transport Ca^{2+}. The 0.5 EP per available protein could be explained by that the dimer is consist of a catalytic subunit and a regulatory subunit and that each function is retained in monomeric ATPase in membranous state. It would be also possible that kinetical parameters of the reaction cycle might limit the maximum level of EP at steady state. In the present study, these possibilities has not yet been experimentaly distinguished. It is even possibile that the Ca^{2+}-ATPase exists in both intact SR membrane and reconstitued proteoliposomes as a dimer which could also be consist of a catalytic and regulatory subunits. In the presence of high concentration of the detergent, the dimer dissociated into subunits with retension of each function. When the detergent is

removed, the subunits readily form the dimer again. The observation that EP level at steady state is half of the available ATPase protein either in the membranous or solubilized form might reflect the possibility. The clarification of these possibilities will take further experiments.

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PART III

TRYPSIN DIGESTION OF SARCOPLASMIC RETICULUM Ca^{2+}-ATPase IN THE PRESENCE OF NON-IONIC DETERGENTS

ABSTRACT

Sarcoplasmic reticulum(SR) isolated from skeletal muscle was solubilized in the nonionic detergents, octaethyleneglycol mono-n-dodecylether($C_{12}E_8$) or polyoxyethylene(9)lauryl ether($C_{12}E_9$)and digested with trypsin at 35 °C. Column chromatography of the trypsin cleaved SR in the presence of the detergents showed that the amount of this 110 K dalton ATPase polypeptide chain decreased with increasing digestion time, and that lower molecular weight peptides were concurrently produced.

Both the activities of Ca^{2+}-dependent ATP hydrolysis and formation of the phosphorylated intermediate(EP) were reduced below 25% of the initial activities after the 60 min digestion. The capacity for 2'(3')-O-(2,4,6-trinitrophenyl)-ATP(TNP-ATP) binding to the ATPase was reduced slowly by the tryptic digestion, and about 70% of the capacity still remained after the 60 min digestion.

When the tryptic digest of the solubilized SR was passed through a molecular sieve column(TSK G3000SW) in the presence of 1 μM TNP-ATP, the cacpacity for TNP-ATP binding was found to be associated with the low molecular weight peptides derived from the ATPase protein. But no TNP-ATP binding capacity was observed when SR had been treated with fluorescein isothiocyanate(FITC) prior to the trypsin digestion.

INTRODUCTION

The Ca^{2+},Mg^{2+}-dependent ATPase of the SR membrane serves not only as an energy transducer but also as a Ca^{2+}-translocator across the membrane. This enzyme is composed of a single polypeptide chain of about 110 K dalton which contains one phosphorylation site, one high affinity nucleotide-binding site, and two Ca^{2+}-binding sites.

Proteolysis of the Ca^{2+},Mg^{2+}-dependent ATPase, in combination with studies on ligand binding and chemical modification can provide information concerning the topology of the enzyme molecule. Many studies on the tryptic digestion of the SR ATPase have been reported (1-9). However, even when the ATPase protein is extensively digested with trypsin, the cleaved ATPase is not dissociated into subfragments in soluble form, existsing instead as cohesive units in a medium containing detergents other than SDS. Recently I observed that, when SR ATPase was digested in the presence of $C_{12}E_8$ (10) or $C_{12}E_9$, the ATPase protein was cleaved into small peptides with only slight reduction of the TNP-ATP binding capacity. In order to isolate the tryptic fragments which retain the capacity for TNP-ATP binding, the cleaved ATPase was separated into low molecular weight peptides by molecular sieve column chromatography in the presence of the detergent.

EXPERIMENTAL PROCEDURES

SR Preparation-- SR was isolated from rabbit dorsal muscle as already described (11). The isolated SR was suspended in a medium containing 0.1 M KCl, 5 mM Tris-maleate (pH 7.0), and 20% glycerol. The SR suspension was divided into 0.5 ml aliquots which were quickly frozen in liquid nitrogen and stored at -80 °C.

Trypsin Digestion-- SR membrane (1-2.5 mg protein/ml) were solubilized by 50 mg/ml $C_{12}E_8$ or $C_{12}E_9$ in the standard medium containing 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 M KCl or NaCl, 20% glycerol, and 20 mM TES (pH 7.5). Digestion was carried out at 35 °C in the presence of 0.01-0.025 mg/ml trypsin. The reaction was terminated at various intervals of incubation by addition of 0.05 mg/ml trypsin inhibitor.

Column Chromatography-- A 0.1-ml sample of the trypsin digested SR suspension was applied to TSK-G3000SW column which had been equilibrated with the standard medium containing 2 mg/ml $C_{12}E_8$ or 10 mg/ml $C_{12}E_9$. Elution profile of SR-protein was obtained using a high performance liquid chromatography (HPLC, TSK-803D) which was connected to a UV monitor (TSK UV-8 II).

Measurements of Activities-- Ca^{2+} dependent ATP hydrolysis was measured under the following conditions; 0.2 mg/ml protein of SR, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 M KCl, 20% glycerol, and 40 mM Tris-maleate (pH 6.5) at 20 °C. The reaction was started by the addition of 1 mM ATP to the mixture and stopped at various times by the addition of 5% trichloroacetic acid. The amount of P_i liberated from ATP was determined as described elsewhere (12). SR

ATPase was phosphorylated by 50 μM[γ-^{32}P]ATP in the standard medium at 0 °C for 30 sec. The amount of EP was measured as described previously (12).

TNP-ATP binding to SR protein--- Titration of the TNP-ATP binding site of SR-ATPase was performed according to Watanabe and Inesi (13) and Dupont et al. (14) in the standard medium containing 2 mg/ml $C_{12}E_8$. The concentration of TNP-ATP in the medium was varied from 0 to 6 μM by succesive addition of 2 μl of 0.5 mM TNP-ATP to 3 ml of the reaction mixture. Fluorescence intensities of TNP-ATP in the presence and absence of 0.2 mg/ml SR protein were measured by using Hitachi fluorometer (model MPF-4).

TNP-ATP binding to the ATPase was also measured at 23 C by the method of Hummel and Dreyer (15) with slight modifications; 0.1 ml of $C_{12}E_9$-solubilized SR was passed through a HPLC column (TSK G3000SW) shich had been equilibrated with the standard medium containing 10 mg/ml $C_{12}E_9$ and 1 μM TNP-ATP. Elution patterns of SR protein and TNP-ATP absorbance were simultaneously detected with TSK UV monitor and Hitachi spectrophotometer (model 200), respectively. The concentration of TNP-ATP was determined spectrophotmetrically using the extinction coefficient 17,000 $M^{-1}cm^{-1}$ at 405 nm under the conditions described above.

Materials-- $C_{12}E_8$ and $C_{12}E_9$ were purchased from Nikko Chemical Co., and the former was recrystalized once in hexane. TES was purchased from Dojindo Co. TNP-ATP was gift from Dr. T. Watanabe of National Cardiovascular Center Research Institute. FITC, soybean trypsin, and trypsin inhibitor were purchased from Sigma Chemical Co. All other chemicals were of reagent grade.

RESULTS

Dissociation of Trypsin-digested ATPase in the Presence of $C_{12}E_8$-- I varied the conditions for proteolysis of SR-ATPase in an attempt to dissociate the cleaved ATPase protein into small peptides in soluble form. I tested the effect of the nonionic detergent, $C_{12}E_8$ or $C_{12}E_9$ on the trypsin digestion of SR membrane. In the experiment shown in Fig. 1, 1 mg/ml SR protein was solubilized with 50 mg/ml $C_{12}E_8$ in the presence of 5mM $CaCl_2$, 20% glycerol at pH 7.5.

The solubilized ATPase was incubated with 0.01 mg/ml trypsin at 35 °C. At various time intervals, 0.5 ml aliquots of the reaction mixture were pipetted into a medium containing 0.05 mg/mg trypsin inhibitor. A 0.1 ml sample was then applied to TSK-G3000SW column and eluted by the standard medium in the presence of 2 mg/ml $C_{12}E_8$. Elution profiles of the tryptic digest show that the amount of the major 110 K dalton ATPase protein decreased with increasing digestion time, and that peptides with a molecular weight lower than that of the ATPase were simultaneously produced. At 60 min after the start of tryptic digestion, almost all of the 110 K dalton ATPase polypetide chain was dissociated into small peptides. A significant amount of tryptic fragments was eluted as a large aggregate at the void volume of the column. The amount of the aggregate increased with the digestion time as seen in the figure. When SR was digested with trypsin in the absence of $C_{12}E_8$ and then dissolved in the detergent, the cleaved ATPase

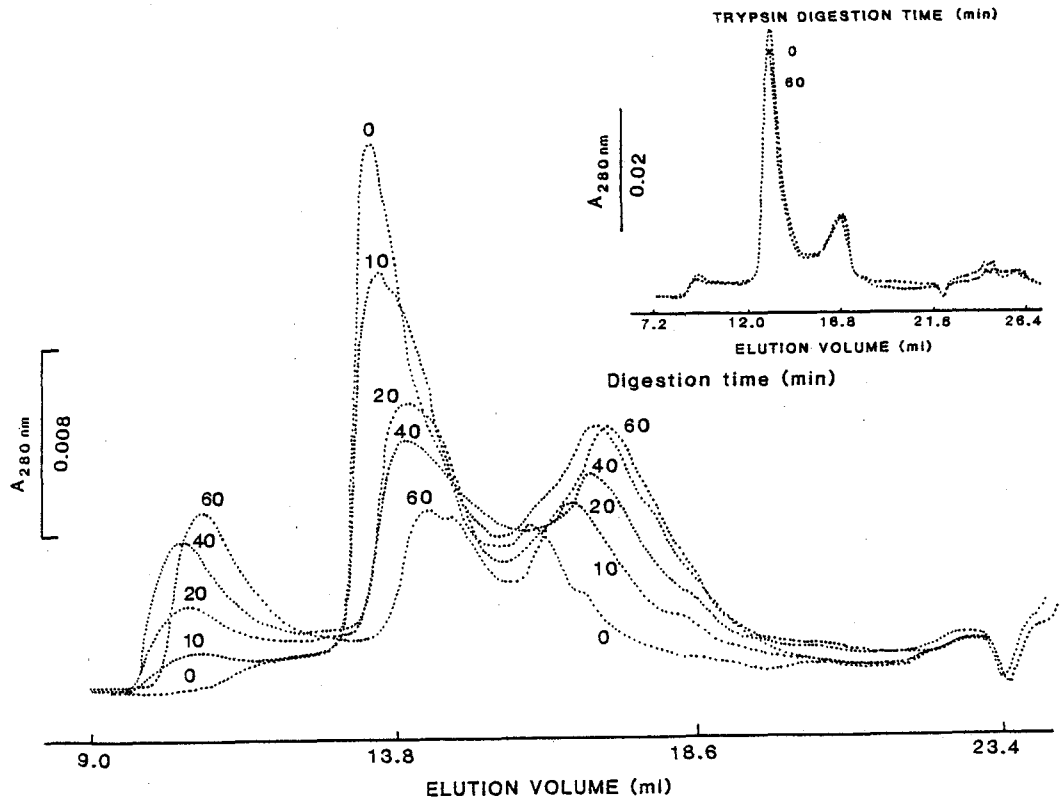

Fig. 1. Column chromatography of SR protein digested by trypsin in the presence of $C_{12}E_8$.

Trypsin(0.01 mg/ml) was added to 1 mg/ml of SR protein which was solubilized by 50 mg/ml $C_{12}E_8$ in the presence of 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 M NaCl, 20% glycerol and 20 mM TES(pH 7.5). At 0, 10, 20, 40, and 60 min afer the start of incubation, 1-ml portions of the reaction mixture were withdrawn and mixed with 0.05 mg/ml of trypsin inhibitor. A 0.1-ml sample of the resulting mixture was applied to TSK-G3000SW column and eluted with the solution described above except that 2 mg/ml $C_{12}E_8$ was used instead of 50 mg/ml. Inset: One mg/ml SR was digested with 0.01 mg/ml trypsin for 0 and 60 min in the same solution as described above except that $C_{12}E_8$ was absent. After digestion SR membranes were dissolved in 50 mg/ml $C_{12}E_8$. Separation of SR protein by HPLC was performed as described above.

protein was not separated into small peptides on chromatography under the same conditions described above(Fig. 1. inset).

Figure 2 shows the HPLC pattern of the tryptic digest of the FITC-labelled SR protein. The absorbance of FITC at 510 nm was mainly associated with the ATPase protein, but minor incorporation of FITC into some proteins other than the ATPase occurred. About 0.8 mol of FITC was incorporated into one mol of ATPase under these conditions. The absorbance of FITC at the position of the ATPase protein decreased with an increase in the digestion time, and new peaks of the FITC absorbance emerged around the position corresponding to the tryptic subfragments of the ATPase protein. It should be noticed that no FITC was incorporated into the aggregated protein which emerged at the void volume of the column. SDS-polyacrylamide gel electrophoresis showed that the aggregated protein was mixture of proteins with molecular weight lower than 14 k (Data not shown). Since the amount of these small proteins increase with the decrease in the ATPase protein during the tryptic digestion, it is possible that these proteins were liberated as the tryptic fragments from the Ca^{2+}-ATPase.

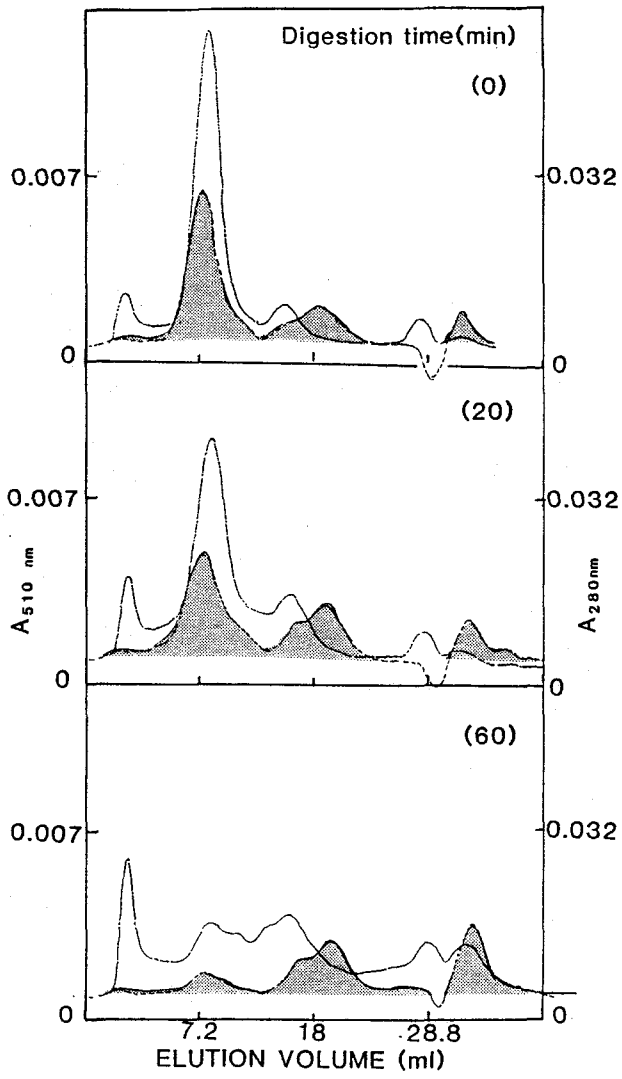

Fig. 2. Elution profiles of protein(open area) and FITC absorbance(shaded area) accompanying the passage of trypsin digested FITC-labelled SR through G3000SW column.

1.5 mg/ml SR was incubated for 30 min at 20 °C with 10 μM FITC in the presence of 0.1 M KCl, 0.3 M sucrose, 0.1 mM EGTA, and 10 mM Tris-Cl (pH 8.8). SR membranes were washed by centrifugation to remove free FITC in 20 volume of the standard medium. SR membranes were resuspended in the standard medium and incubated with trypsin in the presence of 50 mg/ml $C_{12}E_9$ for the indicated time. Column chromatography was performed as described in Fig. 1 except that 10 mg/ml $C_{12}E_9$ was used instead of 2 mg/ml $C_{12}E_8$.

Effects of Tryptic Digestion on the Enzymatic Activities of Detergent-solubilized ATPase-- It is well established that the membrane-bound ATPase of SR is initially cleaved by trypsin into 50 and 45 K dalton fragments with retention of calcium uptake and overall Ca^{2+}-dependent ATP hydrolysis, and that further cleavage of the 50 K dalton fragment produces 20 and 30 K dalton fragments with loss of the calcium transport activity, but without loss of the ATP-hydrolysis and EP-formation activities (4,7,8).

I measured the activities of ATP hydrolysis, EP formation, and nucleotide binding to the Ca^{2+},Mg^{2+}-dependent ATPase as a function of incubation time with trypsin in the presence of $C_{12}E_8$ at 35 °C (Fig. 3). Both the activities of Ca^{2+}-dependent ATPase and EP formation were decreased by the trypsin digestion in the presence of $C_{12}E_8$ with a half life of approximately 15 and 30 min, respectively, and more than 70% of both initial activities were lost in 60 min after the start of digestion. The inhibition of ATPase activity by tryptic digestion at 35 °C was increased with increasing the concentration of detergent and the maximum inhibition was observed at 48 mg/ml of $C_{12}E_8$ (Fig. 4).

Tryptic digestion of the ATPase in the presence of $C_{12}E_8$ was markedly affected by the reaction temperature. As shown in Fig. 5, the activity of EP formation was not reduced when the detergent-solubilized SR was digested with trypsin below 25 °C.

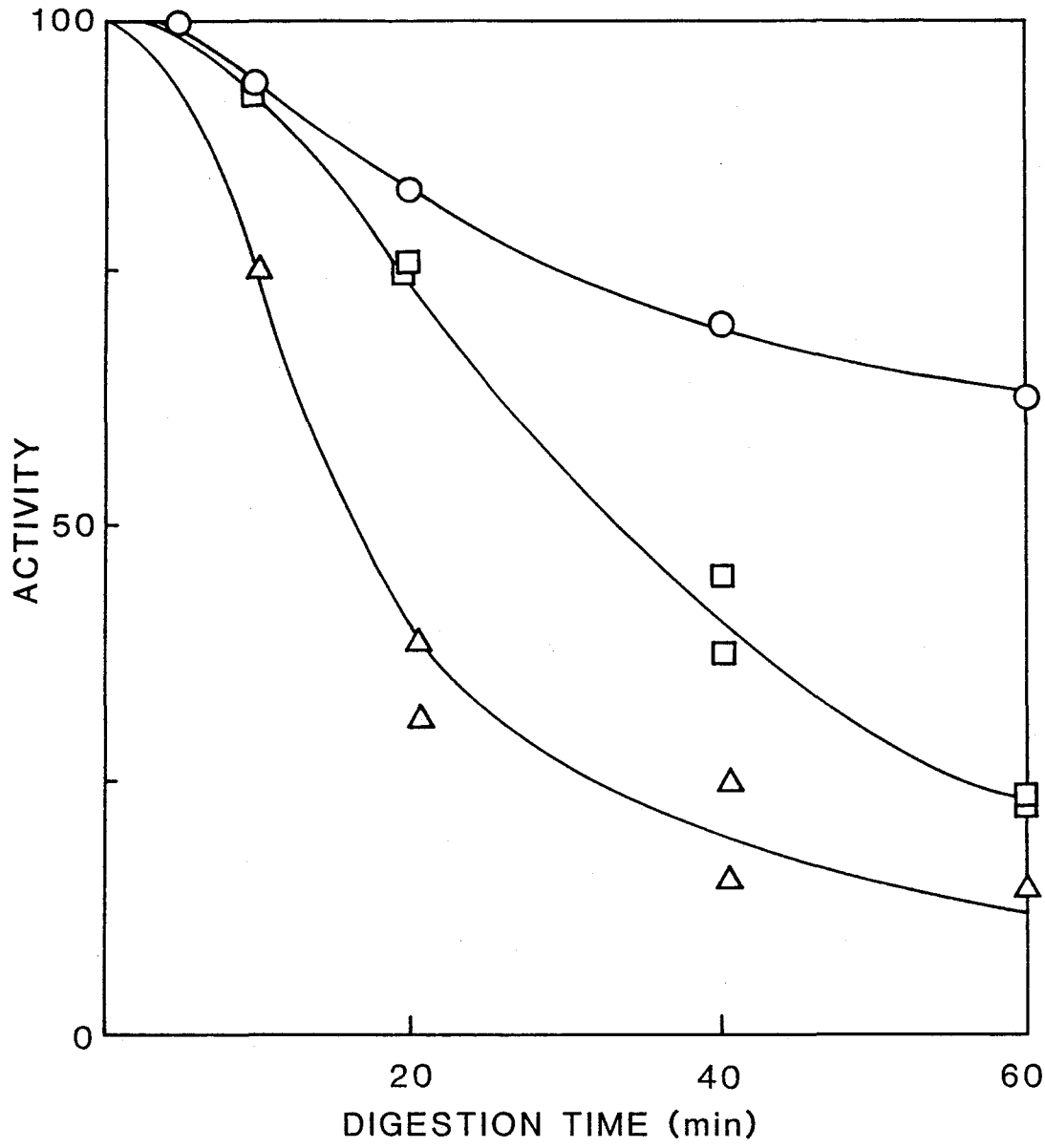

Fig. 3. Effects of tryptic cleavage on the enzymatic activities of Ca^{2+},Mg^{2+}-ATPase.

SR(1 mg/ml) was digested with trypsin in the presence of $C_{12}E_8$ as described in Fig. 1. At 0, 5, 10, 20, 40, and 60 min, digestion was stopped by adding trypsin inhibitor. The activities of ATP hydrolysis(△), EP formation(□), and TNP-ATP binding(○) were measured as described in "EXPERIMENTAL PROCEDURES".

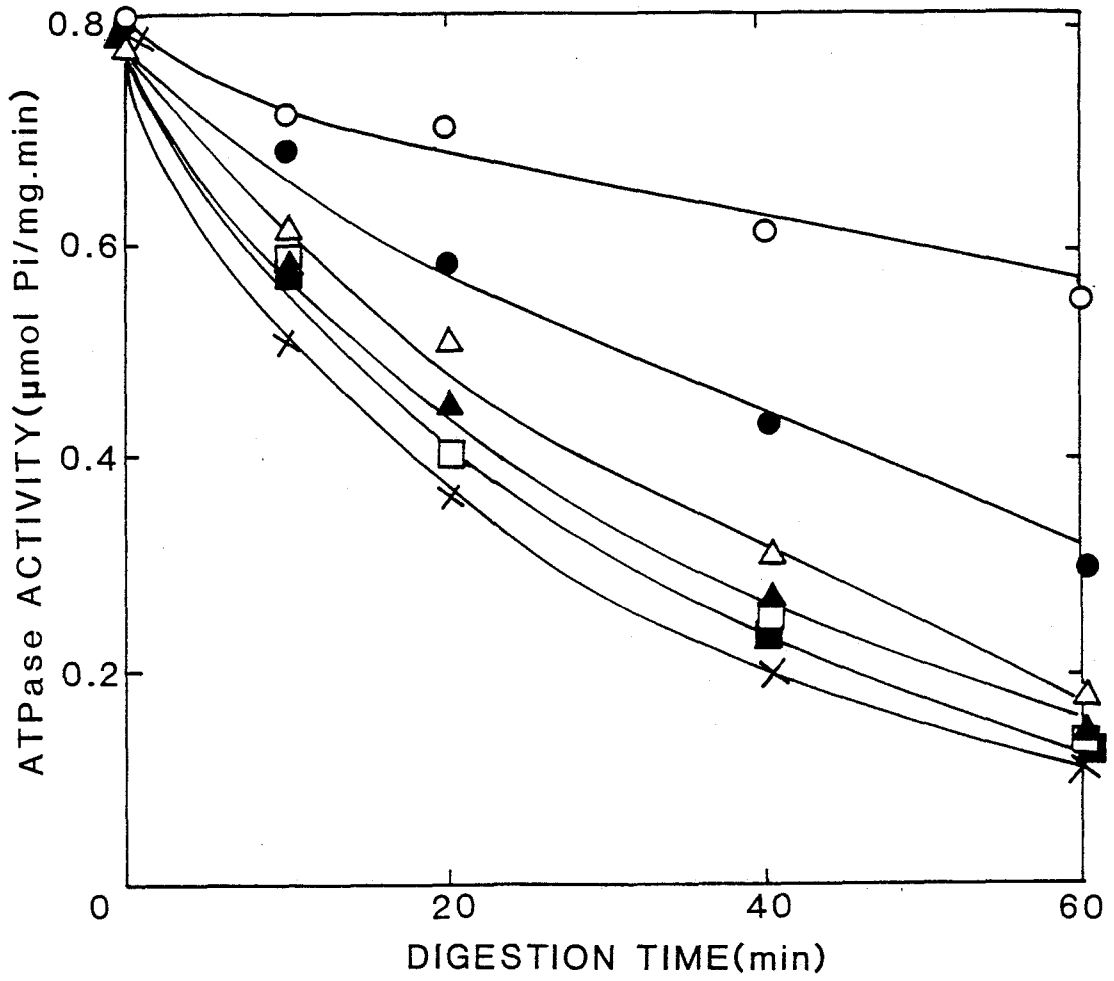

Fig. 4. Effects of $C_{12}E_8$ concentration of the ATPase activity

SR(1 mg/ml) was solubilized in 0(○), 0.3(●), 1.2(△), 4.8(▲), 12(□), 24(■), and 48(X) mg/ml $C_{12}E_8$, and digested with 0.01 mg/ml trypsin at 35 °C for the indicated incubation time.

ATPase activity was measured as described in "EXPERIMENTAL PROCEDURES".

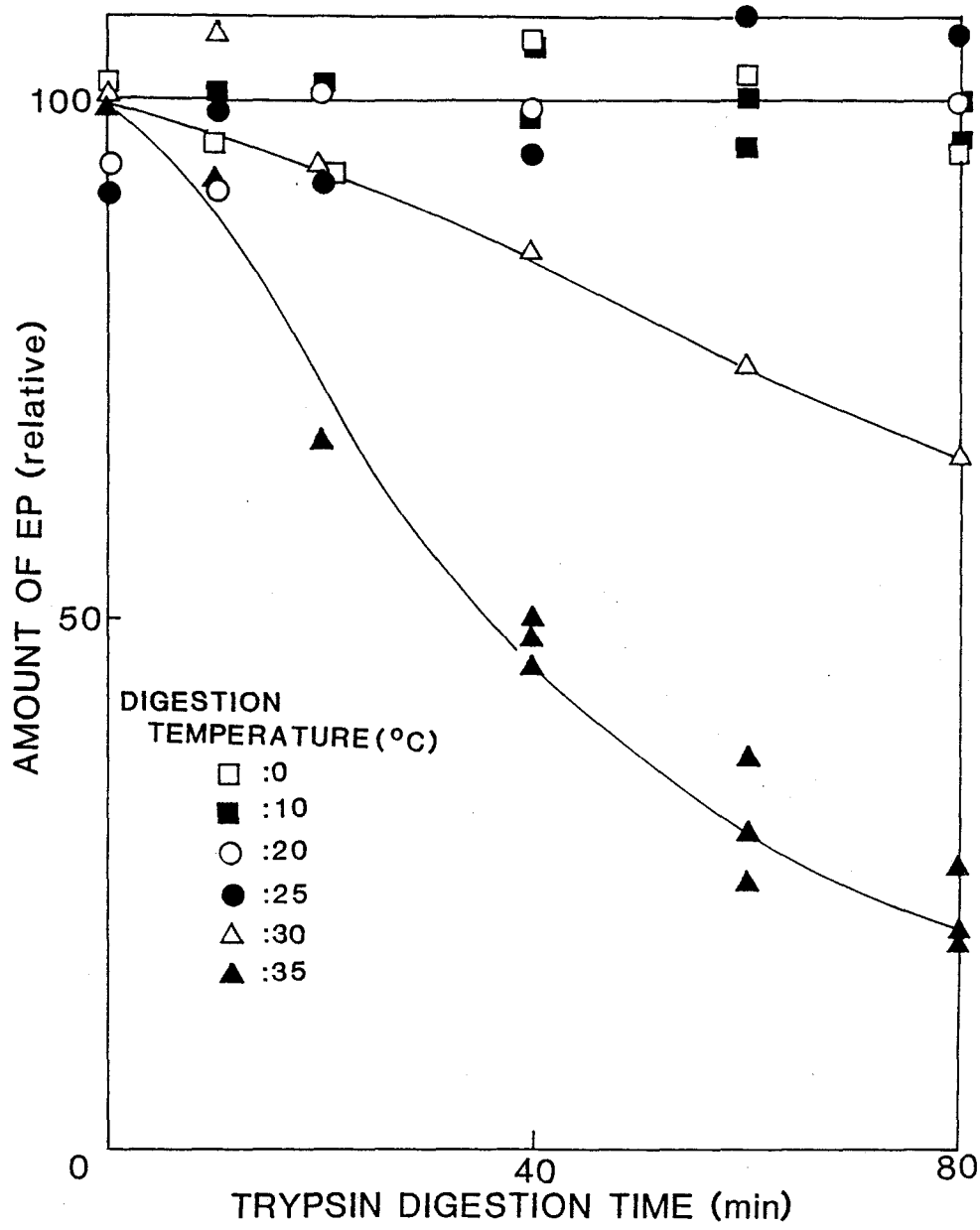

Fig. 5. Temperature dependence of the inhibitory effect of tryptic digestion on EP-formation.

SR was digested with trypsin under the conditions as described in Fig. 2 at 0(□), 10(■), 20(○), 25(●), 30(△), and 35 ℃(▲). EP was measured as described in "EXPERIMENTAL PROCEDURES".

The capacity for TNP-ATP binding to the ATPase was only slightly reduced and about 70% of the capacity seemed to be retained even when the cleaved ATPase protein was dissociated into low molecular weight segments in the presence of $C_{12}E_8$ (see Fig.3). As a supplementary experiment, to test whether or not TNP-ATP binds to the $C_{12}E_8$-solubilized ATPase with high affinity as well as to the membrane bound ATPase, I performed titration of the nucleotide-binding site of the enzyme over the range of TNP-ATP concentrations from 0 to 6 μM, in the presence of $C_{12}E_8$ at 10-fold higher concentration than that of SR protein (Fig. 6). Fluorescence intensity increased when TNP-ATP was added to the reaction mixture even in the absence of SR. This increase in the fluorescence intensity is due to the presence of $C_{12}E_8$ in the mixture. Therefore I had to measure the difference of fluorescence intensity between in the presence and absence of SR at different concentration of TNP-ATP. As shown in Fig. 6, the difference in the fluorescence intensity increased linealy with increase in the concentration ot TNP-ATP, and reached the maximum level around 2 μM TNP-ATP. The titration curve indicates that about one mol of TNP-ATP binds with high affinity to one mol of the ATPase polypeptide chain assuming that one mg/ml SR protein contains 8-9 nmols of ATP binding site. This value agrees with those obtained with intact SR-ATPase (13,14,16).

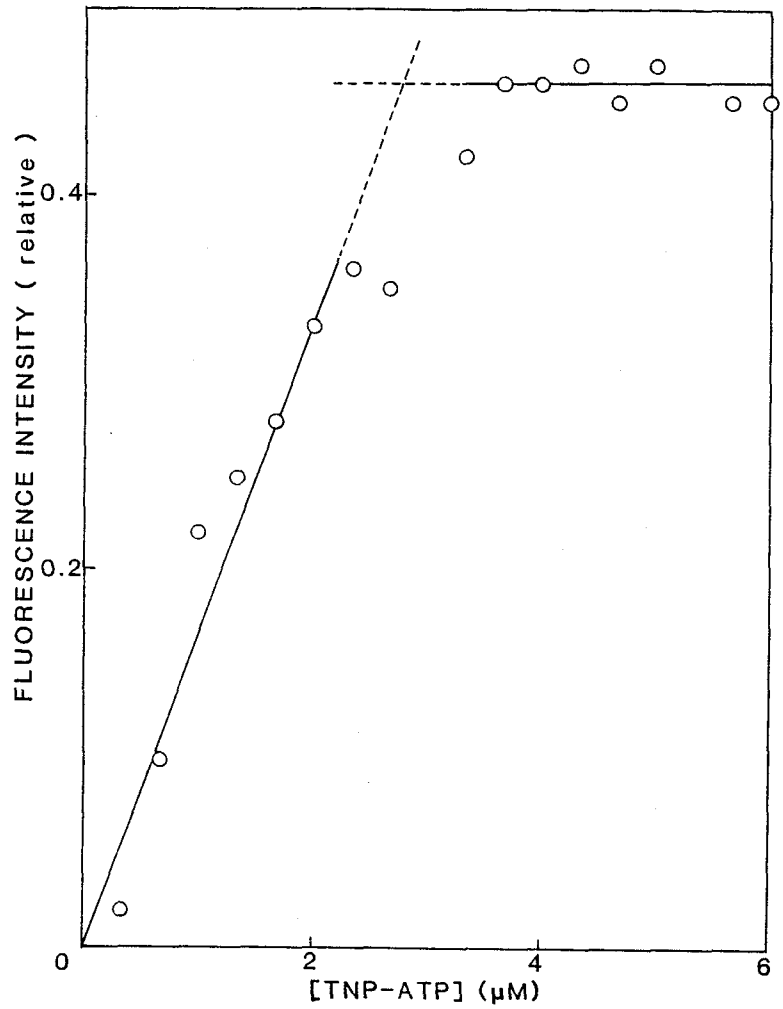

Fig. 6. TNP-ATP concentration dependence on TNP-ATP binding to SR protein in the presence of $C_{12}E_8$.

2-μl portion of 0.5 mM TNP-ATP was added in increments to a cuvette in the presence or absence of 0.2 mg/ml of SR protein in the standard medium containing 2 mg/ml $C_{12}E_8$. Excitation and emission wavelengths were 440 and 520-560 nm, respectively. The fluorescence enhancement of TNP-ATP by the addition of SR protein was plotted as a function of TNP-ATP concentration.

The binding of TNP-ATP to the $C_{12}E_8$-solubilized ATPase was also measured under equilibrium condition by using the column chromatography method described by Hummel and Dreyer (15). SR protein of 2.5 mg/ml solubilized in 50 mg/ml of $C_{12}E_9$ was applied to a TSK G3000SW column equilibrated in the standard medium containing 10 mg/ml $C_{12}E_9$ and 1.0 μM TNP-ATP. Separation of the undigested SR protein by HPLC (Fig. 7, curve a) shows that the major peak of TNP-ATP absorbance at 405 nm is tightly associated with the protein absorbance of the ATPase protein.
About 0.5 mol TNP-ATP was estimated to be bound per mol of the intact ATPase under the conditions tested. On the other hand, when SR was digested with trypsin in the presence of $C_{12}E_9$ for 1 h at 35°C, the major peak of 405 nm absorbance lowered markedly and a new peak emerged around the position corresponding to the tryptic subragments of the ATPase (Fig. 7, curve b). There observed some differences in the elusion profiles of tryptic digest of SR protein between in Fig. 1 and Fig. 7. These differences may be caused by the difference in the detergent used. In addition, absorbances at 405 nm widly varied at the elution volume greater than 20 ml. However, since both the trypsin digested and the undigested SR samples showed a similar elution pattern of $A_{405\ nm}$, the variation in the absorbance observed around the elution volume greater than 20 ml can be regarded as the base line which is independent of the TNP-ATP binding to SR protein.

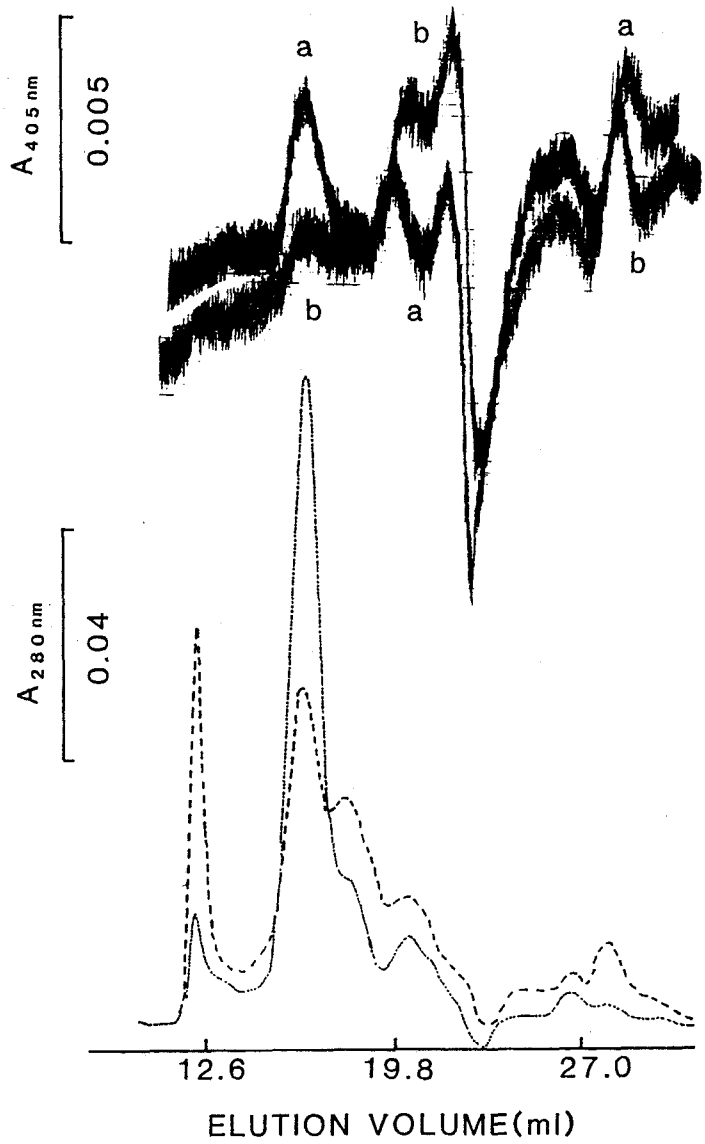

Fig. 7. Elution profiles of SR protein and TNP-ATP absorbance accompanying the passage of trypsin digest of solubilized SR through G3000SW column.

SR(2.5 mg/ml protein) was dissolved in 50 mg/ml $C_{12}E_9$ and digested with 0.025 mg/ml of trypsin at 35°C for 60 min. A 0.1-ml portion of the SR suspension was applied to a column which had been equilibrated with the standard medium containing 10 mg/ml $C_{12}E_9$ in the presence of 1 μM TNP-ATP. Upper panel, absorbance of TNP-ATP at 405 nm; Curve a, control; Curve b, digested. Lower panel, protein absorbance at 280 nm. ·····, control; ----, digested.

In Fig. 8, SR had been pretreated with 10 μM FITC to avoid the nucleotide binding activity of the SR-ATPase. The FITC labelled SR was then digested with trypsin in the presence of $C_{12}E_9$ at 35°C for 0, 20 and 60 min, and separation of tryptic fragments of SR protein by HPLC was performed under a similar condintion to that described in Fig. 7. It was observed that the amount of TNP-ATP bound to the ATPase protein in the control SR was decreased with increasing the digestion time was longer, while the the amount of TNP-ATP bound to the tryptic subfragments was increased. On the other hand, when SR was previously labelled with FITC, the biding of TNP-ATP to the major ATPase protein or to the tryptic subfragments was no longer observed (Figs. 8. a and b). In addition, the wide variation of absorbance at 405 nm observed around the elution volume greater than 20 ml was essentially unaffected by the modification of SR with FITC.

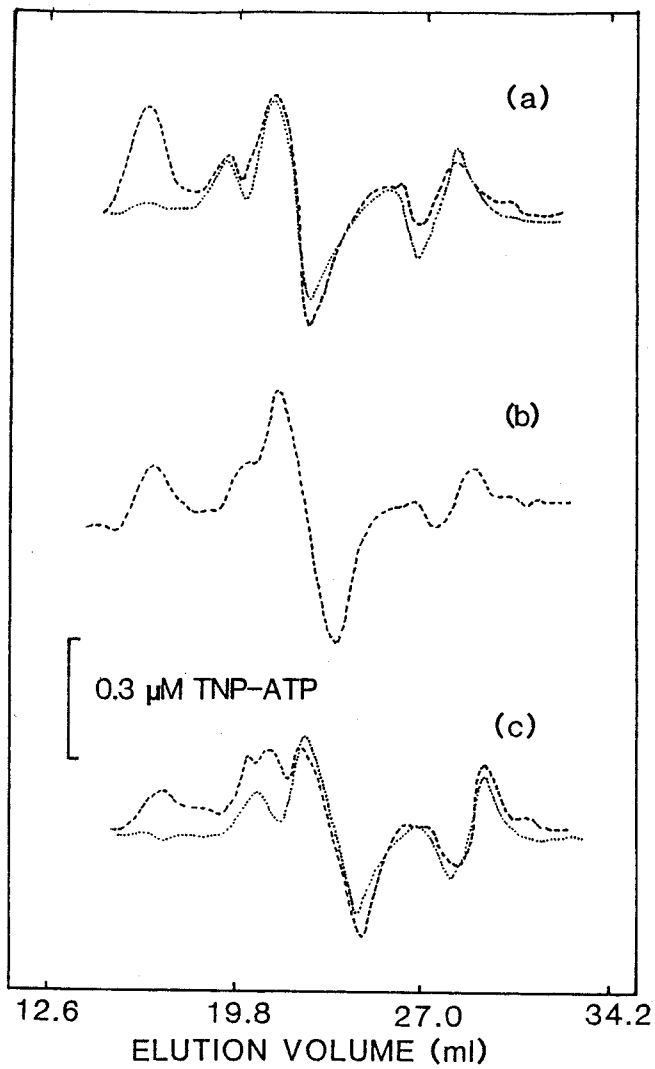

FIG. 8. Elution profiles of TNP-ATP absorbance accompanying the passage of trypsin digest of solubilized SR through G3000SW column.

SR was labelled with FITC under the same condition described in the "EXPERIMENTAL PROCEDURS". 1.5 mg/ml of intact SR (-----) and FITC-labelled SR (·····) were digested with 0.015 mg/ml trypsin in the presence of 50 mg/ml $C_{12}E_9$ for 0(a), 40(b), and 60(c) min at 35 °C. 0.1 ml aliquats of these tryptic digests were applied to the HPLC column which had been equilibrated with the standard medium containing 10 mg/ml $C_{12}E_9$ and 1 μM TNP-ATP. Separation of SR protein by HPLC was performed at 23 °C under the similar conditions as described in Fig. 7, and elution profiles of TNP-ATP absorbance at 405 mn were measured.

DISCUSSION

When membrane-bound ATPase is cleaved with trypsin, the tryptic fragments can be dissociated only after denaturation of the ATPase protein in SDS (1-3). The present work demonstrates that when the Ca^{2+},Mg^{2+} -dependent ATPase of SR is digested by trypsin at 35 C in the presence of $C_{12}E_8$, the cleaved ATPase protein is dissociated into low molecular weight peptides in a soluble form.

As shown in Fig. 2, TNP-ATP binding capacity was only slightly reduced even after extensive cleavage of the Ca^{2+}-ATPase protein into small peptides. This raise the possibility that the structure of the nucleotide binding site is protected from degradation by trypsin and that some low molecular weight fragments can be separated by column chromatography in enzymatically active form. Data from Figs. 7 and 8 suggest the possibility of isolation and characterization of the TNP-ATP binding site with retention of the enzymatic activity.

The reason why the cleaved ATPase is dissociable in the presence of $C_{12}E_8$ or $C_{12}E_9$ at 35 °C is not clear. Analysis of the digestion pattern of the $C_{12}E_8$-solubilized ATPase using SDS-polyacrylamide gel electrophoresis indicates that the 45 K dalton tryptic fragment is degraded in the early phase of the incubation (data not shown), whereas membrane-bound ATPase is resistant to further cleavage of the 45 K dalton fragment by trypsin. The tryptic fragments may be structurally dependent on each other through the 45 K dalton core protein, and the interaction may be blocked when the 45 K fragment is attacked by

trypsin in the presence of the detergent. The rapid inactivation of ATP hydrolysis and EP formation by tryptic digestion appears to be caused by the disruption of the interaction between the tryptic fragments in the presence of the detergent.

Recently Andersen and Jorgensen (9) demonstrated that the SDS-polyacrylacide gel electrophoresis pattern of the tryptic degest of $C_{12}E_8$-solubilized ATAase was essentially the same as that of the memrbane-bound ATPase. The difference in results may be due to the fact that we digested the solubilized SR with trypsin at temperatures much higher than they used. As shown in Fig. 5, the capability of EP formation of the ATPase was not reduced when SR was digested with trypsin in the presence of $C_{12}E_8$ at a reaction temperature below 25°C.

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