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Remarkable stability of solubilized and delipidated sarcoplasmic reticulum Ca2+-ATPase with tightly bound fluoride and magnesium against detergent-induced denaturation

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Remarkable Stability of Solubilized and Delipidated Sarcoplasmic Reticulum Ca^{2+}-ATPase with Tightly Bound Fluoride and Magnesium against Detergent-induced Denaturation

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RUNNING TITLE

Stabilization of Solubilized SR Ca^{2+}-ATPase by F^- and Mg^{2+}

SUMMARY

Conditions were developed in the absence of Ca^{2+} for purification, delipidation, and long-term stabilization of octaethylene glycol monododecyl ether ($C_{12}E_8$)-solubilized sarcoplasmic reticulum Ca^{2+}-ATPase with tightly bound Mg^{2+} and F^-, an analog for the phosphoenzyme intermediate without bound Ca^{2+}. The Ca^{2+}-ATPase activity to monitor denaturation was assessed after treatment with 20 mM Ca^{2+} to release tightly bound Mg^{2+}/F^-. The purification and delipidation was successfully achieved with Reactive Red-agarose affinity chromatography. The solubilized Mg^{2+}/F^--bound Ca^{2+}-ATPase was very rapidly denatured at pH 8, but was perfectly stabilized at pH 6 against denaturation for over 20 days at 4 ºC even without exogenously added phospholipid and at a high $C_{12}E_8$/enzyme weight ratio (10:1). The activity was not restored unless the enzyme was treated with 20 mM Ca^{2+}, showing that tightly bound Mg^{2+}/F^- was not released during the long-term incubation. The perfect stability was attained with or without 0.1 mM dithiothreitol, but inactivation occurred with a half-life of 10 days in the presence of 1 mM dithiothreitol possibly due to reduction of a specific disulfide bond(s). The remarkable stability is likely conferred by intimate gathering of cytoplasmic domains of Ca^{2+}-ATPase molecule induced by tight binding of Mg^{2+}/F^-. The present study thus reveals an essential property of the $Mg^{2+}/F^-/Ca^{2+}$-ATPase complex, which will likely provide clues to understanding structure of the Ca^{2+}-released form of phosphoenzyme intermediate at an atomic level.

Sarcoplasmic reticulum (SR)[1] Ca^{2+}-ATPase is a 110 kDa-membrane protein and a representative member of P-type ion transporting ATPases. It catalyzes Ca^{2+} transport coupled with ATP hydrolysis (1, 2, and for recent reviews, see Refs. 3 and 4). According to the E_1/E_2 theory, the enzyme with bound Ca^{2+} (E_1) is autophosphorylated by MgATP to form ADP-sensitive phosphoenzyme (E_1P). This phosphorylation causes occlusion of the bound Ca^{2+} at the transport sites, and the subsequent conformational transition to ADP-insensitive form (E_2P) releases Ca^{2+} into the lumen. Finally, dephosphorylation takes place and returns the enzyme into unphosphorylated and Ca^{2+}-unbound form (E_2). E_2P can also be formed from P_i in the presence of Mg^{2+} and absence of Ca^{2+} by reversal of its hydrolysis.

Recently, three-dimensional structure of Ca^{2+}-ATPase with bound Ca^{2+} was solved by X-ray crystallography at an atomic level (5). The enzyme has 3 cytoplasmic domains (A, N, and P), which are widely separated in the crystal structure with bound Ca^{2+}. The modeling with a low resolution map of tubular crystals formed in the presence of decavanadate revealed that 3 cytoplasmic domains gather to form a single headpiece in the tubular crystals (5), although the state of the enzyme in the tubular crystals was ambiguous. Our previous limited and systematic proteolysis experiments showed (6, 7) that the decavanadate-bound state is very similar to E_2P, and that E_2P is the intermediate having the most compactly organized headpiece. The results further indicated that a large motion of A domain, i.e. rotation by ~90°, and its strong association with P and N domains most likely occur during the E_1P to E_2P transition, and suggested that the stabilization energy provided by intimate contacts between all 3 cytoplasmic domains in E_2P will provide energy for moving transmembrane helices and release the bound Ca^{2+} ions. For understanding mechanism of energy coupling in the Ca^{2+} transport, it is essential to reveal the structure of this key intermediate, E_2P, at an atomic level.

The stability of the enzyme is a prerequisite for crystallization; the conditions required for stability and crystallization necessarily overlap. For formation of three-dimensional crystals of the Ca^{2+}-bound Ca^{2+}-ATPase, Ca^{2+} at very high concentrations (10-20 mM) is an essential factor for stabilization against detergent-induced denaturation (8). Ca^{2+} bound at the transport sites likely contribute to stabilization of the transmembrane helices and thus, at least in part, to resistance against denaturation. On the other hand, two very serious problems impeding structural studies of E_2P are its inherent nature as a transient intermediate (being hydrolyzed within seconds) and the fact that the enzyme without bound Ca^{2+} is usually very rapidly denatured by solubilization with detergents (8-11) including $C_{12}E_8$ that is likely most suitable for (8, 11) and was actually employed for (5) structural studies of the Ca^{2+}-bound enzyme. These problems should be overcome.

It was previously demonstrated in kinetic and binding studies (12-15) that F^- and Mg^{2+} simultaneously bind

tightly at the phosphorylation site in the absence of Ca^{2+} to form a transition-state analog for E_2P formation from P_i, and that this $Mg^{2+}/F^-/E_2$ complex in membrane has an extremely long half-life and does not decompose at least for several days unless a high concentration of Ca^{2+} is present. The enzyme with bound Mg^{2+}/F^- was shown to be stable even against thermal inactivation (13). We further demonstrated by the systematic and limited proteolysis (6, 7) that the cytoplasmic domain organization of the enzyme with tightly bound Mg^{2+}/F^- is actually the same as that of E_2P formed from P_i, being very compact and completely resistant to all proteinase K, V8 protease, and trypsin at T2 site. The $Mg^{2+}/F^-/E_2$ complex is thus analogous to E_2P in its structure.

In the present study, taking advantage of the tight binding nature of the $Mg^{2+}/F^-/E_2$ complex, we explored effects of solubilization, purification, and delipidation of this complex on stability against denaturation. We report here that the $C_{12}E_8$-solubilized $Mg^{2+}/F^-/E_2$ complex is remarkably stable against denaturation, and can be purified and delipidated by affinity column chromatography, and that the $Mg^{2+}/F^-/E_2$ complex thus obtained is perfectly stable for long-term incubation in $C_{12}E_8$ in the absence of Ca^{2+} even without exogenously added phospholipid under defined conditions that are suitable for structural studies at an atomic level.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles and Treatment with Mg^{2+}/F^- — SR vesicles were prepared from rabbit leg white muscle and stored at –80 ºC as described previously (16). The content of phosphorylation site determined with $^{32}P_i$ according to Barrabin *et al.* (17) was 5.0 ± 0.2 nmol/mg of vesicle protein (n = 6). Treatment of the vesicles with F^- in the presence of Mg^{2+} was performed as described previously (15): SR vesicles (2 mg/ml protein) were incubated with 1 mM KF at 25 ºC for 3 hours in a medium containing 10 mM $MgCl_2$, 0.1 M KCl, 1 mM EGTA, 20% (v/v) Me_2SO, 20% (v/v) glycerol, and 40 mM imidazole/HCl (pH 7.5). Unbound F^- and Mg^{2+} was removed by centrifugation as described (15). This treatment resulted in tight binding of the stoichiometric amounts of F^- and Mg^{2+} (two F^- and one Mg^{2+} per Ca^{2+}-ATPase molecule) at the catalytic site and complete suppression of the Ca^{2+}-dependent ATPase activity, as described in detail in our previous studies (14, 15).

Ca^{2+}-induced Release of Tightly Bound F^- and Mg^{2+} — The Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- in vesicles or in solubilized form was treated with 20 mM Ca^{2+} in 100 mM Tris/HCl (pH 7.4) for 1 hour at 25 ºC, as described previously (14). This treatment resulted in entire release of the bound F^- and Mg^{2+}, and therefore complete restoration of the Ca^{2+}-ATPase activity.

Ca^{2+}-ATPase Activity — The total ATPase activity was determined at 25 ºC in a medium containing 10 μg/ml protein, 5 mg/ml $C_{12}E_8$, 5 mM [γ-^{32}P]ATP, 7 mM $MgCl_2$, 0.1 M KCl, 0.5 mM $CaCl_2$, 0.4 mM EGTA (0.11 mM free Ca^{2+}), and 50 mM MOPS/Tris (pH 7.0). The Ca^{2+}-ATPase activity was obtained by subtracting the Ca^{2+}-independent ATPase activity, which was determined in the presence of 5 mM EGTA without added $CaCl_2$, otherwise as above. The tightly bound Mg^{2+}/F^- were released immediately before the ATPase assay as described above, by mixing samples with an equal volume of 40 mM $CaCl_2$ in 200 mM Tris/HCl (pH 7.4).

Purification and Delipidation of Solubilized Ca^{2+}-ATPase with Tightly Bound Mg^{2+}/F^- by Reactive Red-agarose Affinity Chromatography — The $C_{12}E_8$-solubilized Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- was purified in the absence of divalent cations by the use of Reactive Red 120-agarose (type 3000-CL, Sigma) with modifications of the method employed for the Ca^{2+}-bound Ca^{2+}-ATPase (18, 19): The Mg^{2+}/F^--treated vesicles (2 mg protein) were solubilized at 4 ºC in 0.5 ml of a medium containing 10 mg/ml $C_{12}E_8$, 20% (v/v) glycerol, 20 mM MES/NaOH (pH 6.0), and 1 mM EDTA. After centrifugation at 150,000 × g, 4 ºC for 15 min, the supernatant was applied to the Reactive Red column (bed volume, 0.2 ml) preequilibrated with the column buffer (1 mg/ml $C_{12}E_8$, 20% (v/v) glycerol, 20 mM MES/NaOH (pH 6.0), and 1 mM EDTA) at 4 ºC. The column was washed with 1.0 ml of the column buffer, and then the Ca^{2+}-ATPase was eluted with the column buffer containing

5 mM ADP. The Ca^{2+}-ATPase thus purified and delipidated was concentrated and washed to remove ADP repeatedly by centrifugal filter device Microcon YM-30 (Millipore Corp.) at 14,000 × g, 4 ºC with a buffer containing 20% (v/v) glycerol, 0.1 M KCl, 3 mM $MgCl_2$, 2 μg/ml BHT, 3 mM NaN_3, 1 mM EGTA, and 20 mM MES/NaOH (pH 6.0). The concentration of ADP remaining in the sample was estimated by absorbance at 259 nm in the filtrate, and that of $C_{12}E_8$ was determined according to Garewal (20). The phospholipid content was determined by extracting phospholipids with chloroform/methanol (2:1 v/v) and then digesting with perchloric acid, as described previously (21).

Size Exclusion HPLC of $C_{12}E_8$-solubilized Ca^{2+}-ATPase ―SR vesicles untreated or treated with Mg^{2+}/F^- were solubilized with $C_{12}E_8$ as described above. After centrifugation, the supernatant was subjected to size exclusion HPLC at 25 ºC by the use of TSKgel G3000SW column (0.75 × 30 cm, Tosoh, Japan) at a flow rate of 0.5 ml/min with a buffer containing 1 mg/ml $C_{12}E_8$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 20 mM MOPS/Tris (pH 7.0). The absorbance of eluted protein was monitored at 280 nm.

Miscellaneous ― Protein concentrations were determined by the method of Lowry *et al.* (22) with bovine serum albumin as a standard. $C_{12}E_8$ was obtained from Nikko Chemicals (Tokyo, Japan). [γ-^{32}P]ATP was purchased from NEN Life Science Products. Phosphatidylcholine (from frozen egg yolk) was purchased from Sigma. All the chemicals were of the highest purity available. Data were analyzed by non-linear regression using the program Origin (Microcal Software, Northampton, MA).

RESULTS AND DISCUSSION

Stability of Ca^{2+}-ATPase in $C_{12}E_8$-solubilized SR Vesicles at 25 ºC — Mg^{2+}/F^--treated or untreated SR vesicles were incubated for various periods with $C_{12}E_8$ at a high detergent/protein weight ratio (10:1) and pH 6.0, 25 ºC in the presence of 20% (v/v) glycerol. The vesicles were then treated with 20 mM Ca^{2+} for 1 hour, and the Ca^{2+}-ATPase activity was determined (Fig. 1). In the control experiments with vesicles untreated with Mg^{2+}/F^-, the activity was rapidly decreased and completely abolished in about 24 hours when Ca^{2+} was absent during the $C_{12}E_8$-incubation, while almost no loss in the activity was observed when 20 mM Ca^{2+} was present. The results are consistent with the previously observed protection of the Ca^{2+}-ATPase by high concentrations of Ca^{2+} against detergent-induced denaturation (8). On the other hand, the Ca^{2+}-ATPase of the Mg^{2+}/F^--treated vesicles was strongly resistant to denaturation in the absence of Ca^{2+}, showing only small loss of the activity in 48 hours. It should also be noted that the Mg^{2+}/F^--treated vesicles showed no Ca^{2+}-ATPase activity unless they were treated with 20 mM Ca^{2+} immediately before the ATPase assay to release the tightly bound Mg^{2+}/F^-.

In another set of experiments, we confirmed that the Ca^{2+}-ATPase in Mg^{2+}/F^--treated vesicles as well as in untreated vesicles was completely solubilized with $C_{12}E_8$ under conditions in the above experiments, and thus full Ca^{2+}-ATPase activity of the solubilized vesicles was recovered in the supernatant after centrifugation at 150,000 × g, 15 min (data not shown). Full activity was actually recovered in the supernatant even at a $C_{12}E_8$/protein weight ratio as low as 2.

Ca^{2+}-ATPase in Solubilized Mg^{2+}/F^--treated Vesicles Is Completely Stable at pH 6 and 4 °C for 14 Days — In order to develop conditions under which the Ca^{2+}-ATPase in the solubilized Mg^{2+}/F^--treated vesicles is perfectly stable against denaturation for long term, incubation in $C_{12}E_8$ in the absence of Ca^{2+} was performed at different pH values and at 25 and 4 °C (Fig. 2). At 25 °C, the enzyme was very rapidly inactivated at pH 8.0 but it was maximally stabilized when pH was reduced to 6.0 (Fig. 2*A*). The stabilization by lowering pH is remarkable; more than 200-fold decrease in the inactivation rate with lowering pH from 8.0 to 6.0. It was previously reported (8, 11) that the $C_{12}E_8$-solubilized Ca^{2+}-ATPase (without bound Mg^{2+}/F^-) is more stable at pH 6 than at pH 8 both in the presence and absence of Ca^{2+}, but the inactivation rates differ by only about 3-fold.

We then examined the long-term stability at the optimum pH at 25 and 4 ºC, and found that the Ca^{2+}-ATPase was completely protected at 4 ºC against inactivation with no loss of the activity even after 14 days (Fig. 2*B*). The Mg^{2+}/F^--treated vesicles showed no Ca^{2+}-ATPase activity unless they were treated with 20 mM Ca^{2+} immediately before the ATPase assay, thus tightly bound Mg^{2+}/F^- were not released during the long-term

incubation. We also examined possible effects of phosphatidylcholine, because it was previously reported (8) that the decreased activity of the $C_{12}E_8$-incubated Ca^{2+}-ATPase with bound Ca^{2+} was partially restored by addition of phospholipids. In the present experiments, phosphatidylcholine in large excess over protein (100:1 weight ratio) was added to the $C_{12}E_8$-incubated samples, and then the tightly bound Mg^{2+}/F^- were released and the Ca^{2+}-ATPase activity was determined. Although the activity was somewhat higher in the presence of phosphatidylcholine, the inactivation rates obtained at 25 ºC with and without addition of phosphatidylcholine were essentially the same, thus no restoration of the lost activity was observed.

Purification and Delipidation of Solubilized Ca^{2+}-ATPase with Tightly Bound Mg^{2+}/F^- — For formation of three-dimensional crystals of the Ca^{2+}-ATPase and its structural studies, it is essential to purify the enzyme as solubilized and delipidated form and to develop conditions in which the enzyme thus purified is perfectly stable during long-term incubation without being denatured. For $Mg^{2+}/F^-/E_2$ complex, Ca^{2+} should be eliminated from the systems in order to avoid release of the tightly bound Mg^{2+}/F^-. In the following experiments, we established methods and conditions for fulfilling these requirements by using Reactive Red-agarose affinity column, which has been successfully employed for the Ca^{2+}-bound Ca^{2+}-ATPase (18, 19). Since it was previously shown (14, 15) that the nucleotide binding pocket of Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- is still available for high affinity binding of metal-free ATP (but not to ATP complexed with Mg^{2+}), we performed the chromatography in the absence of divalent cations (Fig. 3).

In Fig. 3, Mg^{2+}/F^--treated vesicles were solubilized with $C_{12}E_8$, and applied to the Reactive Red-agarose column. A large portion of the Ca^{2+}-ATPase was bound to the column, and approximately 65% of the total Ca^{2+}-ATPase activity was recovered with 5 mM ADP in *fractions 10-17*. The specific Ca^{2+}-ATPase activity in these fractions (6.7 ± 0.4 μmol/mg/min) was approximately 1.7 times higher than the activity of the solubilized vesicles (*cf.* Figs. 1 and 2*B*) (note that the Ca^{2+}-ATPase activity in Fig. 3 was expressed as μmol/min/ml of fraction). None of these fractions exhibited Ca^{2+}-ATPase activity unless they were treated with 20 mM Ca^{2+}, and thus the tightly bound Mg^{2+}/F^- in the $Mg^{2+}/F^-/E_2$ complex was not released during the chromatography. On the SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R-250 after electrophoresis (23), only a single band at 110 kDa corresponding to the Ca^{2+}-ATPase chain was found without any other bands for these fractions (data not shown). The lipid content was reduced by this chromatography from 84 mol of phospholipids/mol of Ca^{2+}-ATPase in SR vesicles to 4 mol of phospholipids/mol of Ca^{2+}-ATPase in the purified form. The phospholipid content in this purified enzyme is similar to or lower than that of other delipidated enzyme preparations reported (8, 19). The rest of the Ca^{2+}-ATPase was eluted nonspecifically upon addition of 2 M NaCl

with some other proteins.

The Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- thus delipidated and eluted with ADP was washed repeatedly by a centrifugal filter device to remove ADP and to concentrate the enzyme. After washing, the concentration of ADP in the samples was lower than 10 μM and was much lower than that of the Ca^{2+}-ATPase (approximately 100 μM). The concentration of $C_{12}E_8$ in the samples was approximately 10 mg/ml. The Ca^{2+}-ATPase activity of thus purified enzyme (determined after release of tightly bound Mg^{2+}/F^- by the Ca^{2+} treatment) was 7.1 ± 0.2 μmol/mg/min (n = 8) (see the activity at incubation-time zero in Fig. 4) and approximately 1.8 times higher than the activity of the solubilized Mg^{2+}/F^--treated vesicles (determined after release of tightly bound Mg^{2+}/F^-) (*cf.* Figs. 1 and 2*B*). In a separate set of experiments as a control, we also purified Ca^{2+}-ATPase from SR vesicles untreated with Mg^{2+}/F^- according to original procedure (18,19) using the affinity column in the presence of Ca^{2+}, which protect the enzyme against detergent-induced denaturation. The Ca^{2+}-ATPase activity of the enzyme thus purified was 6.9 ± 0.6 μmol/mg/min (n = 7). Therefore, the activity of the purified enzyme with tightly bound Mg^{2+}/F^- (determined after release of Mg^{2+}/F^-) was essentially the same as that expected for optimally prepared enzyme.

Perfect Stability of Purified and Delipidated Ca^{2+}-ATPase with Tightly Bound Mg^{2+}/F^- — The purified Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- (1 mg/ml) was then incubated in 10 mg/ml $C_{12}E_8$ at pH 6.0 in the absence of Ca^{2+} at 25 or 4 °C with or without exogenously added phosphatidylcholine under the conditions thus adaptable to formation of three-dimensional crystals of the enzyme. After incubation for various periods, the samples were treated with 20 mM Ca^{2+} and the Ca^{2+}-ATPase activity was determined (Fig. 4). When incubated at 25 °C, the activity decreased with a rate similar to that for the solubilized Mg^{2+}/F^--treated vesicles at 25 °C (*cf.* Fig. 2). When incubated at 4 °C, the Ca^{2+}-ATPase was perfectly stable over 20 days with no loss of the activity. Addition of phospholipid was not required for the stabilization. The enzyme showed no Ca^{2+}-ATPase activity unless it was treated with 20 mM Ca^{2+} before the ATPase assay, therefore, the tightly bound Mg^{2+}/F^- was not released during the incubation. The fact that the $Mg^{2+}/F^-/E_2$ complex is thus perfectly stable at the very high $C_{12}E_8$/enzyme ratio without exogenously added phospholipids, would be highly advantageous for formation of three dimensional crystals in the effort to search an appropriate amount of $C_{12}E_8$ (which would generally be much lower than that explored in this study) and a suitable type and amount of exogenous phospholipids (19).

We found also that the Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- was completely stable at 4 ºC during the incubation both with and without 0.1 mM DTT and that, however, the Ca^{2+}-ATPase activity decreased in the presence of 1 mM DTT with a half-life of about 10 days (data not shown). It is possible that a specific disulfide

bond(s) of the enzyme, of which reduction results in a loss of the Ca^{2+}-ATPase activity (due to blocking of the E_1P to E_2P transition) (24), was reduced by DTT at the higher concentration during the long-term incubation.

For comparison, the Mg^{2+}/F^--free Ca^{2+}-ATPase was purified in the absence of Ca^{2+} from SR vesicles untreated with Mg^{2+}/F^-, as the Mg^{2+}/F^--bound enzyme. More than half of the Ca^{2+}-ATPase activity was lost during this purification process that took about 2 hours at 4 ºC, and the remaining activity was completely lost within a few hours when incubated as in Fig. 4 with $C_{12}E_8$ at 4 ºC (data not shown). Thus, protection of the purified and delipidated Ca^{2+}-ATPase by tightly bound Mg^{2+}/F^- against denaturation is remarkable.

Stabilization of Ca^{2+}-ATPase with Tightly Bound Mg^{2+}/F^- by Formation of Compactly Organized Conformation — It was previously suggested (8) that detergent-solubilized Ca^{2+}-ATPase with bound Ca^{2+} is stabilized against denaturation by formation of its oligomeric form. In the present study, we thus performed size exclusion HPLC as described under "Experimental Procedures" and observed that $C_{12}E_8$-solubilized Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- eluted predominantly at the position corresponding to its monomeric form but not to possible dimeric or higher oligomeric form (data not shown). We previously demonstrated (6, 7; see Introduction), on the other hand, that 3 cytoplasmic domains of the Ca^{2+}-ATPase molecule gather to form a very compactly organized single headpiece by tight binding of Mg^{2+}/F^-. The observed remarkable stability of the solubilized and delipidated enzyme with tightly bound Mg^{2+}/F^- is therefore probably attained by intimate contacts between all 3 cytoplasmic domains. F^- is capable of forming strong hydrogen bonds with hydrogen-bond donors owing to the highest electronegativity of fluoride among all the elements. Hydrogen bonding of F^- with residues at or near the phosphorylation site in the $Mg^{2+}/F^-/E_2$ complex likely contributes at least in part to stabilization of such compactly organized domain structure.

Orthovanadate, a phosphate analog, binds at the phosphorylation site in the presence of Mg^{2+} and absence of Ca^{2+} (25-27), and produces a compactly organized conformation as Mg^{2+}/F^- does (6). On the other hand, the Ca^{2+}-ATPase in the presence of Mg^{2+}/vanadate and absence of Ca^{2+} was reported to be rapidly denatured by $C_{12}E_8$ (8). This is probably due to the equilibrium between the vanadate-bound and vanadate-free states, considering that irreversible denaturation proceeds if the population of vanadate-free state exists at all. It is clear that formation of tightly bound complex with an extremely long-life (*i.e.* $Mg^{2+}/F^-/E_2$), being almost irreversible unless Ca^{2+} is present, is one of essential factors for conferring resistance against detergent-induced denaturation.

It was very recently shown by glutaraldehyde cross-linking experiments (28) that thapsigargin stabilizes the Ca^{2+}-ATPase without bound Ca^{2+} against detergent-induced denaturation. Thapsigargin binds very tightly to a specific site on the transmembrane (or stalk) helices of the enzyme in E_2 state (29-34) or on the luminal loops (35).

The stabilization with thapsigargin is likely due to locking of the transmembrane helices in an immobilized state (28). On the other hand, the stabilizing force for the $Mg^{2+}/F^{-}/E_2$ complex is in the cytoplasmic domains and fixes the enzyme in the state analogous to E_2P. Thapsigargin is not required for this stabilization. Thus, the $Mg^{2+}/F^{-}/E_2$ complex will likely provide clues to understanding not only the structure of cytoplasmic domains in E_2P but also the structure of transmembrane helices and luminal loops in this Ca^{2+}-released form, and even possible dynamic movements of the helices and loops occurring in this form in future studies.

It was also recently reported (36,37) that acetyl phosphate- or P_i-phosphorylated FITC-labeled Ca^{2+}-ATPase of Ca^{2+}-loaded SR vesicles is stabilized by thapsigargin and decavanadate or by dodecylmaltoside in the absence of Ca^{2+}, and forms two-dimensional arrays on the membrane of Ca^{2+}-loaded vesicles in the presence of thapsigargin and decavanadate and absence of detergent and Ca^{2+} (37). The enzyme in these arrays was reported as should probably be classified as an E_1P-like form (36,37). In our present study, again, none of thapsigargin, decavanadate, and Ca^{2+} (and FITC-labeling) is required for the stabilization of the enzyme with tightly bound Mg^{2+}/F^{-} against detergent-induced denaturation. We think that utilizing the Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^{-} is a very simple approach to obtain three-dimensional crystals and structural information on the E_2P state.

In summary, we demonstrated here that the $C_{12}E_8$-solubilized, delipidated, and purified Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^{-} is perfectly stable and resistant to detergent-induced denaturation for long-term incubation in the absence of Ca^{2+} under defined conditions even in a very high concentration of $C_{12}E_8$ without exogenously added phospholipids. The property of $Mg^{2+}/F^{-}/E_2$ complex offers a great advantage for formation of three-dimensional crystals and for structural studies at an atomic level.

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FOOTNOTES

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[1]The abbreviations used are: SR, sarcoplasmic reticulum; E_1P, ADP-sensitive phophoenzyme; E_2P, ADP-insensitive phosphoenzyme; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EPPS, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid; $C_{12}E_8$, octaethylene glycol monododecyl ether; DTT, dithiothreitol; BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography; FITC, fluorescein 5-isothiocyanate.

FIGURE LEGENDS

FIG. 1. **Stability of Ca^{2+}-ATPase in $C_{12}E_8$-solubilized SR vesicles.** SR vesicles treated (○,●) or untreated (Δ,▲) with Mg^{2+}/F^- (1 mg/ml protein) were solubilized with 10 mg/ml $C_{12}E_8$ in 20% (v/v) glycerol, 0.1 M KCl, 3 mM $MgCl_2$, 1 mM DTT, 2 μg/ml BHT, 3 mM NaN_3, and 20 mM MES/NaOH (pH 6.0) in the presence of 20 mM $CaCl_2$ (▲) or 1 mM EGTA without added $CaCl_2$ (○,●,Δ), and incubated at 25 ºC for various periods. At the indicated times, aliquots were taken and treated with (○,Δ,▲) or without (●) 20 mM Ca^{2+} for 1 hour, and then Ca^{2+}-ATPase activity was determined, as described under "Experimental Procedures." Solid lines show least-squares fits of a single exponential to the time course, in which the first-order inactivation rate constants ($hour^{-1}$) were 0.005 for the Mg^{2+}/F^--treated vesicles (○) and 0.159 for the untreated vesicles (Δ).

FIG. 2. **Effects of pH and temperature on stability of $C_{12}E_8$-solubilized Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^-.** *A*, Mg^{2+}/F^--treated vesicles were solubilized and incubated with $C_{12}E_8$ at 25 ºC and at different pH values with 20 mM buffer (MES/NaOH (pH 5.5-6.5), MOPS/Tris (pH 7.0), or EPPS/NaOH (pH 8.0)), otherwise as described in the legend to Fig. 1. After incubation for various periods, the samples were treated with 20 mM Ca^{2+} and the Ca^{2+}-ATPase activity was determined. The first-order inactivation rate constants shown on the ordinate as reciprocal numbers were obtained by least-square fits of a single exponential as shown by solid lines in the *inset* for pH 6.0 (●), 7.0 (○), and 8.0 (▲). *B*, long-term stability of Ca^{2+}-ATPase in $C_{12}E_8$-solubilized Mg^{2+}/F^--treated vesicles was examined at 25 (○,Δ,□) or 4 (●,▲,■) ºC at pH 6.0, otherwise as in *A*. At the indicated times, the samples were treated with (○,Δ,●,▲) or without (□,■) 20 mM Ca^{2+} in the absence (○,●,□,■) or presence (Δ,▲) of 100 mg/ml phosphatidylcholine, and the Ca^{2+}-ATPase activity was determined. First-order inactivation rate constants (day^{-1}) at 25 ºC obtained by least-squares fits of a single exponential (solid lines) in *B* were 0.11 without phosphatidylcholine (○) and 0.12 with phosphatidylcholine (Δ).

FIG. 3. **Purification and delipidation of Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- by Reactive Red-agarose column.** The Mg^{2+}/F^--treated SR vesicles were solubilized with $C_{12}E_8$ and applied to Reactive Red 120-agarose column, as described under "Experimental Procedures." Proteins were eluted with 5 mM ADP or 2 M NaCl as indicated, and fractions of 0.1 ml each were collected. Protein concentration (hatched bar) and Ca^{2+}-ATPase activity (μmol/min/ml of fraction) with (○) or without (●) release of the tightly bound Mg^{2+}/F^- by the Ca^{2+}-treatment were determined.

FIG. 4. **Stability of purified and delipidated Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^-.** The purified and delipidated Ca^{2+}-ATPase with tightly bound Mg^{2+}/F^- in *fractions 10-14* in Fig. 3 was washed to remove ADP as described under "Experimental Procedures." The Ca^{2+}-ATPase (1 mg/ml) was then incubated in a medium containing 10 mg/ml $C_{12}E_8$, 20% (v/v) glycerol, 0.1 M KCl, 3 mM $MgCl_2$, 2 μg/ml BHT, 3 mM NaN_3, 1 mM EGTA, and 20 mM MES/NaOH (pH 6.0) in the absence (○,□,Δ,◊) or presence (●,■,▲,♦) of 1 mg/ml phosphatidylcholine at 25 (Δ,▲,◊,♦) or 4 (○,●,□,■) ºC. At the indicated times, the Ca^{2+}-ATPase activity was determined with (○,●,Δ,▲) or without (□,■,◊,♦) release of the tightly bound Mg^{2+}/F^- by the Ca^{2+}-treatment. The first-order inactivation rate constants (day^{-1}) obtained at 25 ºC by least-squares fits of a single exponential to the time course (solid lines) were 0.136 without phosphatidylcholine (Δ) and 0.128 with phosphatidylcholine (▲).

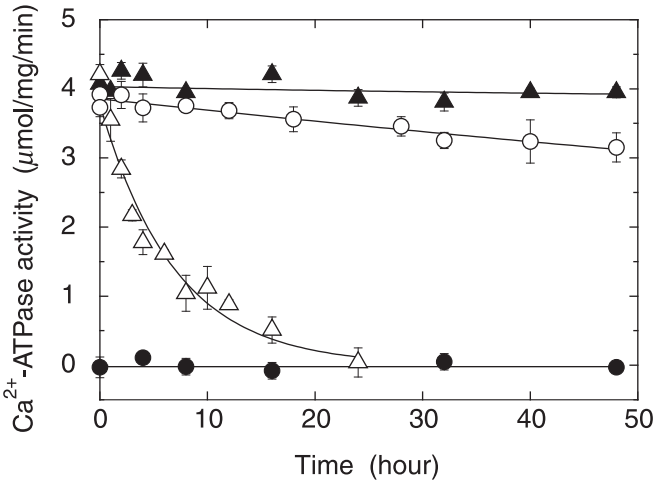
Ca2+-ATPase activity (μmol/mg/min)
Time (hour)
0
1
2
3
4
5
0
10
20
30
40
50

Figure 1

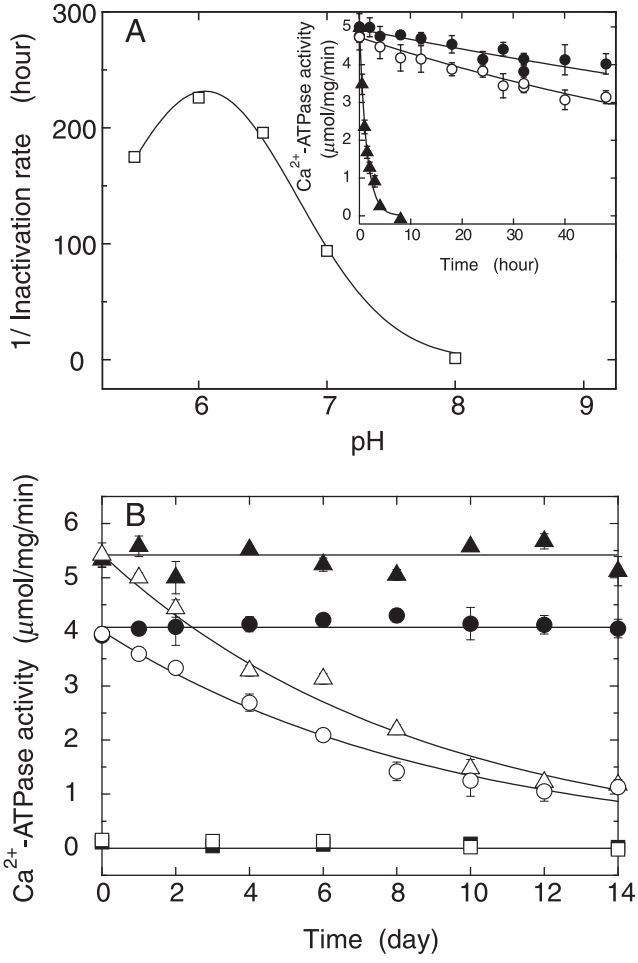
A
1/ Inactivation rate (hour)
pH
Ca2+-ATPase activity (μmol/mg/min)
Time (hour)
B
Ca2+-ATPase activity (μmol/mg/min)
Time (day)

Figure 2

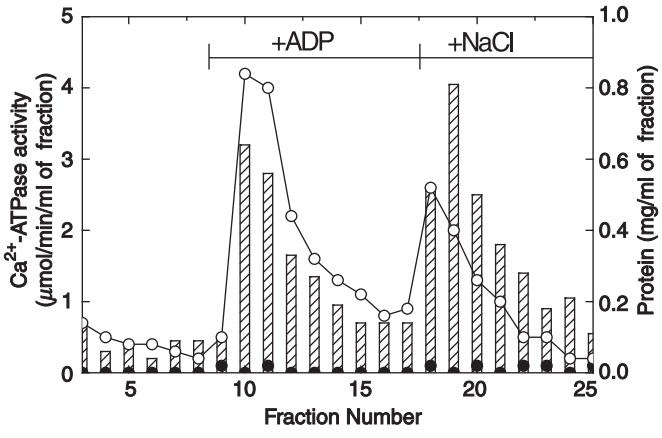
+ADP
+NaCl
Ca2+-ATPase activity (μmol/min/ml of fraction)
Protein (mg/ml of fraction)
Fraction Number
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
5
10
15
20
25

Figure 3

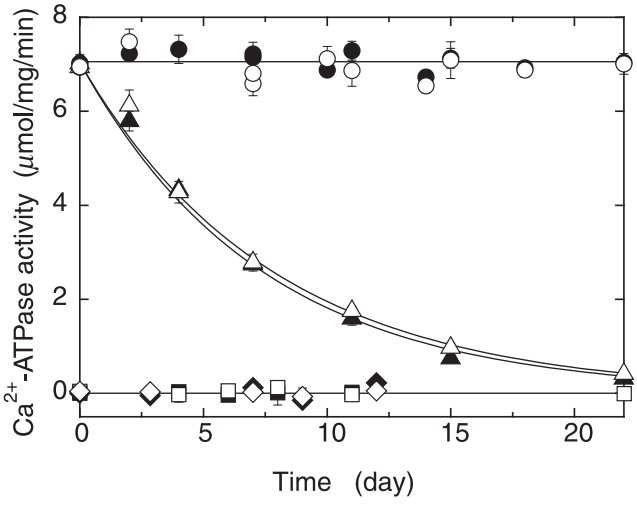

Figure 4