ADP-insensitive phosphoenzyme intermediate of sarcoplasmic reticulum Ca^{2+}-ATPase has a compact conformation resistant to proteinase K, V8 protease and trypsin

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Abstract Sarcoplasmic reticulum Ca^{2+}-ATPase was digested with proteinase K, V8 protease and trypsin in the absence of Ca^{2+}. Unphosphorylated enzyme was rapidly degraded. In contrast, ADP-insensitive phosphoenzyme formed with P_i and phosphorylated state analogues produced by the binding of F^- or orthovanadate, were almost completely resistant to the proteolysis except for tryptic cleavage at the T1 site (Arg^{505}). The results indicate that the phosphoenzyme and its analogues have a very compact form in the cytoplasmic region, being consistent with large domain motions (gathering of three cytoplasmic domains). Results further show that the structure of the enzyme with bound decavanadate is very similar to ADP-insensitive phosphoenzyme. Thapsigargin did not affect the changes in digestion time course induced by the formation of the phosphorylated state analogues.

Key words: Ca^{2+}-ATPase; P-type ion transporting ATPase; Ca^{2+} pump; Sarcoplasmic reticulum; Phosphorylated intermediate; Proteolysis

1. Introduction

Sarcoplasmic reticulum (SR) 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]. During the catalytic cycle, the enzyme forms four major intermediates, E_2 and E_1 (non-phosphorylated states), and E_1P and E_2P, which are ADP-sensitive phosphoenzyme and ADP-insensitive phosphoenzyme, respectively (for recent reviews, see [3,4]).

Limited proteolysis has been used extensively with P-type ATPases to explore the domain structure and the effects of ions to be transported. With SR Ca^{2+}-ATPase, trypsin was first used successfully to detect the conformational changes due to Ca^{2+} binding and phosphorylation [5,6]; it was later found that proteinase K (prtK) may be more useful in identifying conformational changes induced by Ca^{2+} binding [7] and that prtK can isolate a large cytoplasmic domain [8]. Nevertheless, limited proteolysis has never been employed systematically to study the conformations of the enzyme in various states. For example, it is known for more than 15 years that native SR vesicles can form tubular crystals in the presence of decavanadate and absence of Ca^{2+} [9], but the functional state of the enzyme in the tubular crystals is yet to be established. This is because decavanadate solution always contains orthovanadate [10], which is considered to be a phosphate analogue and its binding presumably fixes the enzyme in the 'E_2P' state; decavanadate is a large anion and perturbs the reaction cycle in a way different from orthovanadate [9,11–13]. The situation is even more complicated by the use of thapsigargin (TG), a very potent inhibitor, to improve the crystallinity, because TG is reported to interfere with the enzyme phosphorylation [14,15] and may put the enzyme in a different conformation.

We now know where the sites for proteolysis are located in the primary [16] and the three-dimensional [17] structure of the enzyme (Fig. 1). The comparison of the atomic model for the Ca^{2+}-bound state with a low-resolution map of tubular crystals with TG showed large domain motions between these two states [17], although the state of the enzyme in the tubular crystals was ambiguous. The phosphorylated form is proposed to be the most compact form in which all the cytoplasmic domains gather to form a single headpiece [17] (Fig. 1). Then, the digestion patterns are expected to be different. However, the experiments with which we can test this expectation are very limited (only with trypsin) and not interpreted as such [6,18]. Rather, the differences were attributed to the conformational difference between E_1 and E_2 states, not between E_2 and E_2P. This is because the E_2P state was not recognized as a conformational state distinct from E_2.

Therefore, we conducted systematic proteolysis experiments including the conditions used for making tubular crystals, paying particular attention to the potential E_1–E_2 equilibrium. The results are very clear and consistent with large domain motions in the cytoplasmic region.

2. Materials and methods

2.1. Preparation of SR vesicles and treatment with F^-

SR vesicles were prepared from rabbit skeletal muscle as described

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Abbreviations: SR, sarcoplasmic reticulum; E_1P, ADP-sensitive phosphoenzyme; E_2P, ADP-insensitive phosphoenzyme; prtK, proteinase K; TG, thapsigargin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid

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previously [19]. The content of phosphorylation sites determined with $^{32}P_i$ according to Barrabin et al. [20] was 5.0 ± 0.2 nmol/mg of vesicle protein ($n = 6$). The Ca^{2+}-dependent ATPase activity determined at 25°C as described previously [19] was 2.51 ± 0.05 μmol/min/mg of vesicle protein ($n = 3$). SR vesicles were treated with F^- in the presence of Mg^{2+} for 4.5 h under the conditions described previously [21], in which the stoichiometric amounts of F^- and Mg^{2+} (two F^- and one Mg^{2+} per Ca^{2+}-ATPase molecule) tightly bind to the catalytic site of the enzyme. Unbound F^- was removed as described [21]. The Ca^{2+}-dependent ATPase activity was completely suppressed by this treatment. The activity was entirely restored by incubating them in a buffer containing 20 mM Ca^{2+} at 25°C for 30 min [22] (see also Section 2.4).

2.2. Proteolysis of SR vesicles

The untreated or Mg^{2+}/F^--treated SR vesicles were digested with prtK, V8 protease or trypsin under the conditions described below. The concentration (mg/ml) of vesicle protein, that of the protease, and temperature were 1, 0.03 and 25°C for the prtK proteolysis, 0.3, 0.065 and 37°C for the V8 proteolysis, and 1, 0.01 and 25°C for the tryptic proteolysis, respectively, unless otherwise stated. For the V8 proteolysis, octaethylene glycol monododecyl ether at a non-solubilizing low concentration of 0.05 mg/ml was always included in the reaction mixture as described previously [23]. For examining the effects of Mg^{2+}/F^-, orthovanadate, phosphorylation with P_i, decavanadate and Ca^{2+}, the following buffers were used: (1) Mg^{2+}/F^-: 2 mM EGTA, 50 mM NaCl and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–Tris (pH 6.0). (2) Orthovanadate (±0.5 mM): 2 mM EGTA, 50 mM NaCl, 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–Tris (pH 7.0) and 5 mM $MgCl_2$. (3) P_i (±10 mM): 5 mM EGTA, 50 mM MES–Tris (pH 6.0) and 10 mM $MgCl_2$. (4) Decavanadate (±5 mM, the concentration used for formation of the tubular crystals [17]): 2 mM EGTA, 5 mM $MgCl_2$, 0.1 M KCl and 50 mM MOPS–Tris (pH 7.0). (5) Ca^{2+} (0.5 mM $CaCl_2$ with 0.4 mM EGTA or 2 mM EGTA): 50 mM NaCl and 50 mM MES–Tris (pH 6.0). When the effects of Mg^{2+} were also examined, $MgCl_2$ was replaced by 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

In the above experiments, the vesicles were preincubated for 10 min with or without orthovanadate, P_i, decavanadate or Ca^{2+}, and then the digestion was started by addition of the protease. In some experiments, TG was added to give 5 or 2 μM as indicated after the above preincubation and the vesicles were further incubated for 10 min before the start of the digestion. The tryptic digestion was terminated by adding three volumes of Laemmli sample buffer [24]. Those with prtK and with V8 were terminated by adding ice-cold trichloroacetic acid to give 2.5% (w/v) and diluting five times with a modified Laemmli sample buffer containing 3% (w/v) sodium dodecyl sulfate (SDS). 15 μl of each sample thus obtained was loaded on a 10.5% gel for SDS–PAGE according to Laemmli [24]. For better resolution of the peptides, 1 mM $CaCl_2$ was included in both the stacking and separating gels for the V8- and prtK-treated samples [23]. The gels were stained by Coomassie brilliant blue R-250, and subjected to densitometric analysis with a GT9500-GT95FLU flatbed scanner (Epson, Tokyo, Japan) and Scion Image software (Scion Corp., Frederick, MD, USA).

2.3. Steady state measurements of tryptophan fluorescence

The steady state intensity of the tryptophan fluorescence of SR vesicles was measured on a RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan), as described previously [19]. The excitation and emission wavelengths were set to 290.0 and 338.4 nm, respectively. When the effects of TG on fluorescence were examined, 1.2 μl of TG was added to 2.4 ml of a suspension of the Mg^{2+}/F^--treated or untreated vesicles (0.05 mg/ml). The steady state level of the fluorescence was obtained within 1 min after addition of TG.

2.4. Restoration of ATPase activity by removal of Mg^{2+}/F^-

After preincubation of the Mg^{2+}/F^--treated or untreated vesicles (1 mg/ml) with or without 5 μM TG under the conditions used for the proteolysis, the vesicles were washed twice by centrifugation and incubated with 20 mM Ca^{2+} to remove the tightly bound F^- and Mg^{2+} [22], and the Ca^{2+}-dependent ATPase activities of the vesicles were determined as described above. When preincubated without TG, the activity of the Mg^{2+}/F^--treated vesicles was entirely restored by the incubation with Ca^{2+} to the level of the untreated vesicles. On the other hand, when preincubated with TG, no activity was observed with either the Mg^{2+}/F^--treated or untreated vesicles.

2.5. Miscellaneous methods

V8 protease, prtK and trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) were obtained from Sigma. Orthovanadate and decavanadate were prepared according to Varga et al. [25]. Protein concentrations were determined by the method of Lowry et al. [26] with bovine serum albumin as a standard. Data were analyzed by non-linear regression using the program Origin (Microcal Software, Northampton, MA, USA).

3. Results

Proteolysis of SR Ca^{2+}-ATPase was carried out using prtK, V8 protease and trypsin to examine the effects of P_i, Mg^{2+}/F^-, orthovanadate, decavanadate, Mg^{2+} and TG. The pH for examining the effects of P_i and Mg^{2+}/F^- was chosen so that potential equilibrium between E_1 and E_2 was most shifted to E_2 (i.e. pH 6.0) [27]. The effects of Ca^{2+} binding (i.e. formation of Ca_2E_1 from E_2) were also examined at pH 6.0. Typical digestion patterns are shown in Fig. 2. The degradation of the 110 kDa ATPase chain measured by densitometric scan of the SDS gels was well approximated by a first-order reaction kinetics (Fig. 2B). All the major digestion products in the prtK, V8 and trypsin proteolysis were found to be consistent with those published previously [5–7,18,23] and therefore only rel-

Table 1
Effects of Mg^{2+}/F^-, orthovanadate, P_i, decavanadate and Ca^{2+} on the degradation rate of SR Ca^{2+}-ATPase

Protease	Primary cleavage sites	Relative rate constant (%)					
		no ligands	Ca^{2+}	Mg^{2+}/F^-	orthovanadate	P_i	decavanadate
Proteinase K	L119(↓), T242(↑), M733/A746	100	113	2	1 (75)	22 (74)	0
V8 protease	E231(↑), E715(↓)	100	107	1	0 (206)	24 (113)	0
Trypsin (T1)	R505	100	116	447	87 (101)	103 (209)	57
Trypsin (T2)	R198(↑)	100	211	4	0 (103)	18 (56)	0

The Mg^{2+}/F^--treated vesicles, and the untreated vesicles preincubated with the ligand (Ca^{2+}, orthovanadate, P_i or decavanadate) and Mg^{2+} were digested with prtK, V8 and trypsin as described in Section 2. The first-order rate constants for the degradation of the 110 kDa Ca^{2+}-ATPase polypeptide chain were obtained by the least-squares fit as in Fig. 2. In the tryptic digestion, the decay rates for the band consisting of the A (N-terminal to Arg^{505}) and B (Ala^{506} to C-terminal) fragments were also determined (T2). The rates were normalized to that obtained with the untreated vesicles in the absence of the ligands under otherwise identical conditions (no ligands) (100%). The numbers in parentheses show the degradation rates in the presence of the ligand but in the absence of Mg^{2+}; they were normalized to the rates in the absence of both the ligand and Mg^{2+} (100%). The primary cleavage sites [7,16,23] were indicated at their C-terminal residues: (↑) and (↓) indicate the increase and decrease, respectively, in the cleavage rate upon addition of Ca^{2+}, i.e. upon conformational change from E_2 to Ca_2E_1. The results obtained with orthovanadate at 0.1 mM were almost the same as those listed above obtained at 0.5 mM (data not shown).

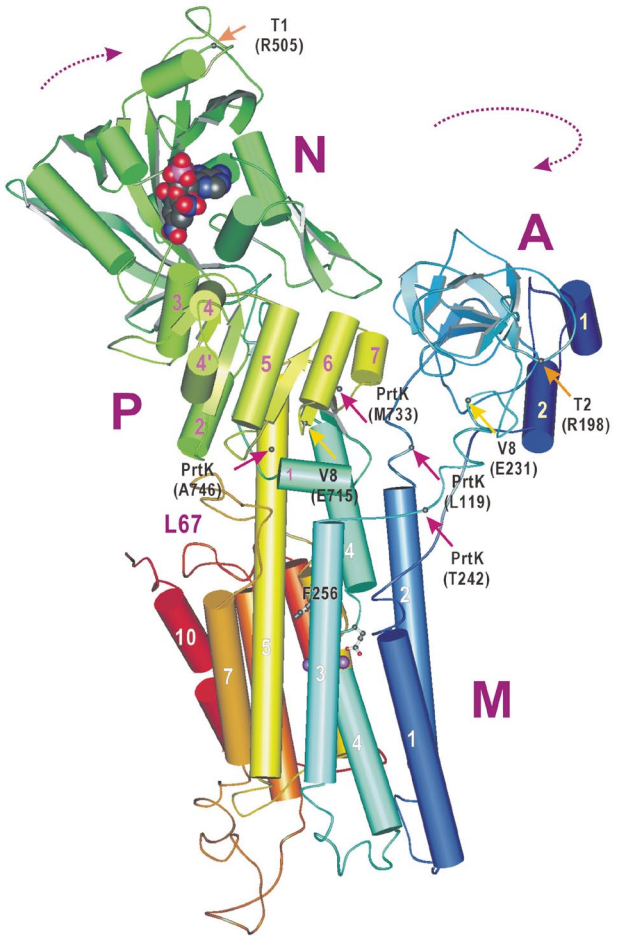

Fig. 1. Location of proteolytic cleavage sites on the Ca^{2+}-ATPase. The position of the main digestion sites by prtK [7], V8 [23] and trypsin [16] are shown on the crystal structure of the Ca^{2+}-ATPase with bound Ca^{2+} (purple sphere) ([17]; PDP accession code 1EUL). The arrows in broken lines show the movements of A and N domains required to fit the atomic model to the map of tubular crystals [32].

ative rate constants are listed in Table 1. The effects of TG on the digestion rates are summarized in Table 2.

From Table 1, the first conclusion to be made is that the Ca^{2+}-ATPase assumes a very compact form in the phosphorylated (E_2P) state and its analogues. No degradation of the 110 kDa polypeptide chain of the Ca^{2+}-ATPase was observed with prtK and V8 for those regarded as stable phosphorylated state analogues, namely the complex with Mg^{2+}/F^- [21,22,28,29] and that with Mg^{2+}/orthovanadate [11–13]. With trypsin, digestion at the T1 site, which is located in the outermost loop of domain N (Fig. 1), occurred rapidly whereas the digestion at T2 in domain A was blocked nearly completely. When no Mg^{2+} was present, orthovanadate showed no ability to make the Ca^{2+}-ATPase resistant to protease attacks, consistent with the absolute requirement of Mg^{2+} for orthovanadate binding [13,30]. The digestions actually proceeded even more rapidly with V8 if Mg^{2+} was absent.

Proteolysis of the Ca^{2+}-ATPase phosphorylated with P_i and Mg^{2+} was not straightforward because the phosphorylation is in equilibrium and it is impossible to make 100% phosphorylated ATPase without a high concentration of Me_2SO [31]. In fact, the blocking of digestion was evident but not as complete as for the stable analogues (Table 1). With trypsin, almost a complete block of cleavage at the T2 site was observed in the presence of 30% (v/v) Me_2SO (data not shown), consistent with previous reports [6].

The second conclusion to be made is that the Ca^{2+}-ATPase treated with a decavanadate solution also assumes a very compact form, because it was completely resistant to prtK and V8 attacks and to trypsin at the T2 site. Hence, it is clear that the structure of Ca^{2+}-ATPase must be different from the untreated (E_2) form; it will be similar but not entirely identical to that with Mg^{2+}/orthovanadate or Mg^{2+}/F^- (or E_2P), because tryptic digestion at the T1 site was also substantially reduced (Table 1).

Ca^{2+} binding to the Ca^{2+}-ATPase (i.e. formation of Ca_2E_1 from E_2) increased appreciably the digestion rate at the T2 site (Table 1), in harmony with previous observations [6]. The degradation rates of the 110 kDa Ca^{2+}-ATPase polypeptide chain with prtK and with V8 were only slightly increased by Ca^{2+}, although the relative speeds of formation of different digestion products changed, consistent with previous studies [7,23].

The effects of TG on proteolysis were examined subsequently and summarized in Table 2. Of main interest here was if TG could reverse the conformational changes induced by phosphorylation. The answer was clear: TG hardly affected the resistance to proteolytic attacks. Even with the untreated Ca^{2+}-ATPase in the absence of ligands, the effects of TG on the digestion rates were also rather small. The rates somewhat depended on the buffer conditions used, and those listed in Table 2 (no ligands) were intermediate ones. In all the cases examined, virtually no protection was observed with trypsin at the T1 site and maximum protection was observed with V8.

Table 2
Effects of TG on the degradation rate of SR Ca^{2+}-ATPase

Protease	Relative rate constants (%)			
	no ligands	Mg^{2+}/F^-	orthovanadate	decavanadate
Proteinase K	72/100	0/2	0/1	0/0
V8 protease	52/100	1/1	0/0	0/0
Trypsin (T1)	101/100	139/447	80/87	33/57
Trypsin (T2)	60/100	0/4	0/0	0/0

The first-order rate constants for the degradation of the Ca^{2+}-ATPase of the Mg^{2+}/F^--treated vesicles and the untreated vesicles (1 mg/ml for prtK and trypsin proteolysis, and 0.3 mg/ml for V8 proteolysis) preincubated with the ligand and Mg^{2+} were obtained in the presence of TG (5 μM for prtK and trypsin proteolysis, and 2 μM for V8 proteolysis) (before the slash (/)) or in its absence (after the slash; the values are the same as in Table 1) under otherwise the same conditions as described in Table 1. The rates obtained were normalized to that obtained with the untreated vesicles in the absence of the ligands and TG (100%). The buffer conditions used for 'no ligands' were the same as those used for 'decavanadate'.

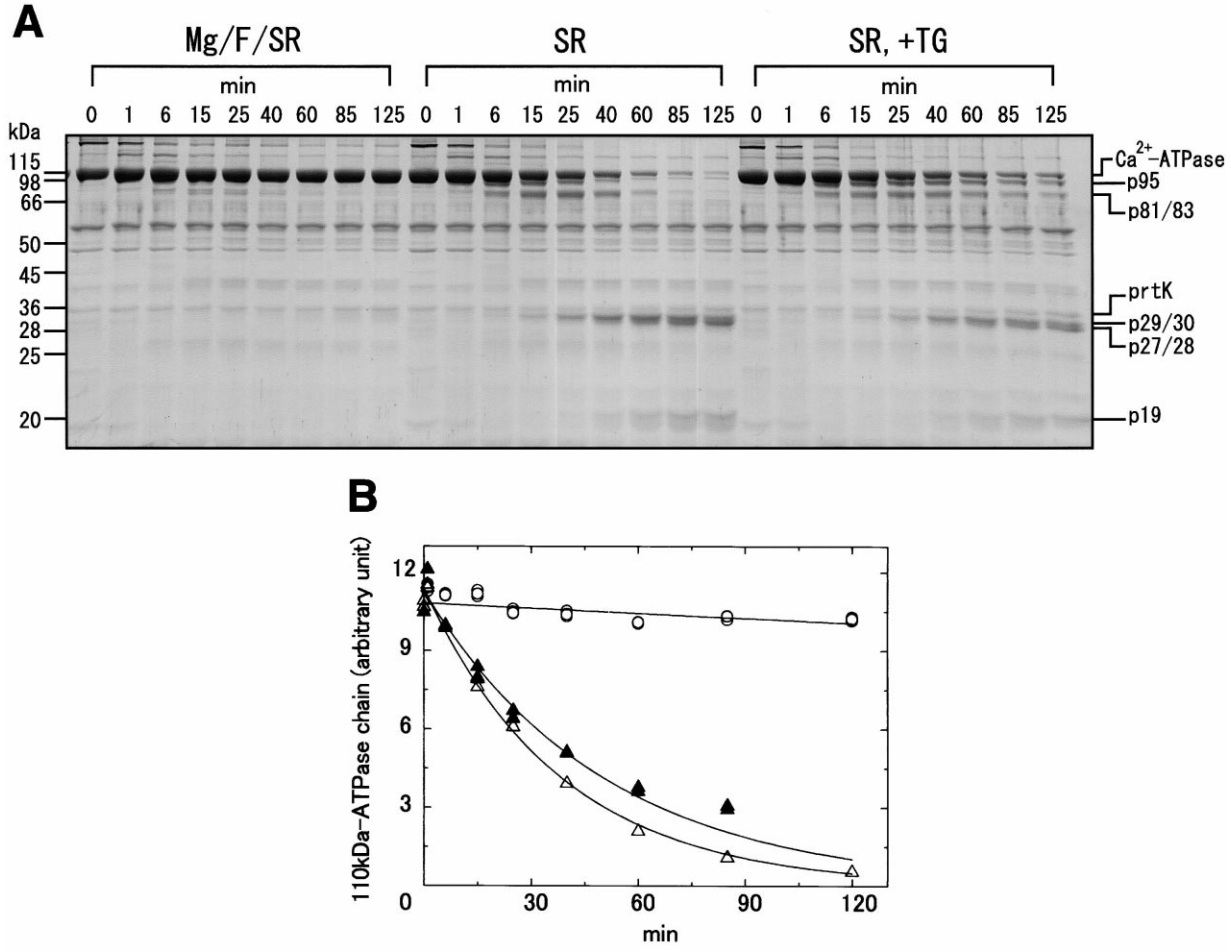

Fig. 2. Effects of binding of F^- to the phosphorylation site and those of TG on the prtK digestion of SR Ca^{2+}-ATPase. The SR vesicles treated with F^- and Mg^{2+} (Mg/F/SR) and untreated vesicles (SR) (0.3 mg/ml) were digested for various periods with prtK (0.01 mg/ml) in the absence or presence (+TG) of 2 μM TG in a medium containing 2 mM EGTA, 50 mM NaCl and 50 mM MES–Tris (pH 6.0). A: SDS gels of the digests. The positions of the Ca^{2+}-ATPase and its proteolytic fragments, and those of the molecular mass markers are indicated in the right and left margins, respectively. B: The time courses for the amounts of 110 kDa Ca^{2+}-ATPase polypeptide chain with the Mg^{2+}/F^--treated vesicles (○) and with the untreated vesicles (△, ▲) in the absence (○, △) or presence (▲) of TG. Solid lines in (B) show least-square fits of a single exponential to the time course, in which the first-order rate constants (h^{-1}) were 0.037 for the Mg^{2+}/F^--treated vesicles, 1.6 for the untreated vesicles, and 1.2 for the untreated vesicles in the presence of TG.

Then the question to be asked was whether TG actually binds to the Ca^{2+}-ATPase under the conditions used. Qualitatively the answer was clear because the Mg^{2+}/F^--bound Ca^{2+}-ATPase incubated with TG did not restore the ATPase activity after adding 20 mM Ca^{2+} to remove the tightly bound F^- and Mg^{2+} [22] (see Section 2.4). Also, TG dramatically improved the crystallinity of two-dimensional arrays of the Mg^{2+}/F^--bound Ca^{2+}-ATPase (Toyoshima, unpublished observations). Furthermore, to examine the effect of TG quantitatively, we measured the changes in intrinsic fluorescence from tryptophan residues in the Ca^{2+}-ATPase under the same conditions as for the proteolysis, because TG reduces the tryptophan fluorescence [14]. TG (at saturating 1 μM) indeed reduced the fluorescence with the Mg^{2+}/F^--treated vesicles, although the decrease was slightly smaller (1.4%) than that with untreated vesicles (1.9%). It is known that TG binds to decavanadate-treated vesicles because the unit cell dimensions of induced crystalline arrays were clearly different depending on the presence of TG [32]. These results show that TG certainly binds to the Ca^{2+}-ATPase in phosphorylated state analogues and that it does not alter gross conformations of cytoplasmic domains but does affect the conformation of the transmembrane segment.

4. Discussion

From the limited proteolysis experiments described here, we have obtained clear answers to the two questions asked. The first one was whether the Ca^{2+}-ATPase assumes a very compact form in the phosphorylated (E_2P) state. The answer is yes. The Ca^{2+}-ATPase becomes completely resistant to protease attacks except for that at the T1 site. No change at the T1 site was expected because it is located at the outermost loop of domain N (Fig. 1) and unlikely to be protected by domain motions. Particularly interesting is the block at Arg^{198} (T2 site for trypsin) and that at Leu^{119} (for prtK), because Arg^{198} is located at the outer surface of domain A and Leu^{119} is located at the interface between domains A and P. The block at these sites is consistent with the domain motions (gathering of three cytoplasmic domains) proposed by comparing the atomic model for the Ca^{2+}-bound state and the low-resolution map of tubular crystals induced by decavana-

date [17], and also consistent with the finding that Arg^{198} and His^{5} are located near the phosphorylation site in the Mg^{2+}/F^{-} complex [33,34].

This result is also consistent with the answer to the second question: whether the enzyme in the tubular crystals represents the E_2 or E_2P form. From the proteolysis experiments, the answer is that the state of the enzyme cannot be E_2 but very similar to E_2P, though it may not be exactly the same. The enzyme in E_2P in the decavanadate-induced crystals was not unexpected, because several vanadate species coexist at neutral pH due to rapid decomposition of decavanadate [10]. The block at Thr^{242} was also expected because the density map of the tubular crystal has a lobe of density at the expected position for Thr^{242} (Toyoshima and Nakasako, unpublished observations).

Historically, in terms of E_1/E_2 conformations, the enzyme phosphorylated with P_i without Ca^{2+} (E_2P) was regarded to assume a stabilized E_2 conformation. Therefore, the results of tryptic digestion with P_i or vanadate present were interpreted along this line and regarded as representing the conformational differences between E_1 and E_2 [6,18]. However, under the experimental conditions chosen in the present study, the enzyme must predominantly be in the E_2 state [27]. Actually, under these conditions, the change in the enzyme state from Ca_2E_1 to E_2 upon removal of Ca^{2+} appreciably reduced the digestion rate at the T2 site but not as completely as the formation of E_2P and its analogues from E_2 (Table 1). Furthermore, the change from Ca_2E_1 to E_2 had only small or almost no effects on the degradation rates of the 110 kDa Ca^{2+}-ATPase polypeptide chain with prtK and V8. Hence, we described here the differences in proteolysis in the absence and presence of P_i (and its analogues) as representing those between E_2 and E_2P.

It was shown previously [35] that the Ca^{2+}-ATPase preincubated with bidentate β,γ-CrATP, which is widely used to place the enzyme in the Ca^{2+}-occluded state [36], is almost completely resistant to prtK proteolysis. This suggests that the conformation in the cytoplasmic domains in the occluded state may be similar to E_2P and solicits further studies.

To clarify what TG does to the Ca^{2+}-ATPase needs careful study. TG has been postulated as a potent inhibitor that fixes the enzyme into the E_2 state [14,15]. We demonstrated here that TG does not alter significantly the susceptibility of the phosphorylated enzyme (E_2P analogues) to protease attacks. However, it should be noted that all these digestion sites are located in the cytoplasmic domain and TG did affect the conformation of transmembrane segments as demonstrated by tryptophan fluorescence. Because TG is thought to bind to the hydrophobic pocket formed by M3–M5 helices (near Phe^{256}; Fig. 1) [37], the change in the fluorescence will imply a reorganization of these helices. An alternative idea is that the transmembrane helices have a relatively large freedom (i.e. thermal movements) if no Ca^{2+} is present to stabilize a particular arrangement of these helices and TG fixes them in another arrangement. The smaller amount of fluorescence change with the Mg^{2+}/F^{-}-treated enzyme seems to suggest that TG can affect a smaller number of transmembrane helices because phosphorylation (binding of F^{-} to the phosphorylation site) has fixed some other transmembrane helices already. Some protection to the protease attacks, in particular to V8, was found with untreated vesicles in the absence of ligands (Table 2). This may simply reflect the closeness between the binding site (Phe^{256}) and the digestion sites; however, the protection at even the T2 site suggests that TG binding does cause a rearrangement of domain A or restricts its (large scale) domain motion (Fig. 1). We obviously need further studies to clarify the structural effects of TG.

At any rate, it seems clear that there is still a lot to be done using limited proteolysis for characterizing different states of ion transporting ATPases.

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