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Susceptibility Test of Two Ca^{2+}-ATPase Conformers to Denaturants and Polyols to Outline Their Structural Difference

Aya Kotake · Genichi Tajima · Yuusuke Maruyama · Jun Nakamura · Chikara Sato

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Abstract To determine the effect of denaturants [guanidine hydrochloride (GdnHCl) and urea] and polyols [with various molecular masses (62.1–600)] on calcium binding at the two hypothesized conformers (A and B forms) of the chemically equivalent sarcoplasmic reticulum Ca^{2+}-ATPase, which bind two calcium ions in different manners, we examined the effect of these reagents on the calcium dependence of ATP-supported phosphorylation of the ATPase molecules and of their calcium-activated, acetyl phosphatate hydrolytic activity. (1) GdnHCl (~0.05 M) and urea (~0.5 M) increased the apparent calcium affinity ($K_{0.5}$) of 2–6 μM of noncooperative binding [Hill coefficient (n_H) ~ 1] of the A form to 10–40 μM. (2) The employed polyols transformed the binding of the A form into cooperative binding (n_H ~ 2), accompanying the approach of its $K_{0.5}$ value to that ($K_{0.5}$ = 0.04–0.2 μM) of the cooperative binding (n_H ~ 2) of the B form; the transition concentration (0.025–2 M) of the polyols, above which such transformation occurs, was in inverse relation to their molecular mass. (3) The binding of the B form was resistant to these denaturants and polyols. Based on these data, a structural model of the two forms, calcium-binding domains of which are loosely and compactly folded, is presented.

Keywords Sarcoplasmic reticulum · Ca^{2+}-ATPase · Calcium binding · Conformation · Denaturant · Polyol

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Introduction

The Ca^{2+}-ATPase (SERCA1a) of the sarcoplasmic reticulum (SR) from adult fast-twitch skeletal muscle is a calcium pump in the SR (Inesi et al. 1990; MacLennan et al. 1997); calcium binding to the two calcium transport sites of the ATPase triggers the phosphorylation of the enzyme with ATP, which drives its transport reaction. Structural studies suggest that ATPase molecules are in close contact in the SR membrane (Scales and Inesi 1976; Vanderkooi et al. 1977: Franzini-Armstrong and Ferguson 1985). Monomeric ATPase, however, has been shown to be a functional unit of the calcium pump (Andersen et al. 1982; Andersen 1989). On the other hand, in the SR membrane de Meis and Hasselbach (1971) and Ogawa and Ebashi (1973) earlier observed the heterogeneity of the ATPase molecules with regard to calcium dependence of their calcium transport activities, which are supported by acetyl phosphate (AcP). Dupont (1982) also observed the existence of two types of calcium binding, respectively, with and without the induction of the change in the intrinsic fluorescence intensity of the molecules. These heterogeneous behaviors of the molecules have been suggested to be a manifestation of the existence of two conformational variants (A and B forms) of the chemically equivalent ATPase molecules (Nakamura and Furukohri 1994; Nakamura 1994), these hypothesized forms being aggregated at a ratio of 1:1 in

the SR membrane. This model of two ATPase conformers shows their calcium binding to be as follows: the A and B forms noncooperatively ($n_H \sim 1$) and cooperatively ($n_H \sim 2$) bind two calcium ions, respectively, with pH-independent and -dependent affinities for calcium. The nonequivalence of the membranous ATPase molecules has been shown to be canceled by solubilization of the molecules with detergents (Nakamura and Tajima 1995). The crystal structures of the monomeric ATPase have been determined (Toyoshima et al. 2000; Toyoshima and Inesi 2004; Olesen et al. 2007). However, no information on the structural difference between the two ATPase conformers has been obtained.

In this study, an attempt was made to outline their conformational difference by examining the susceptibility of their calcium binding to guanidine hydrochloride (GdnHCl), urea and polyols [ethylene glycol, glycerol, triethylene glycol, sorbitol, sucrose, trehalose and polyethylene glycol 600 (PEG 600)]. GdnHCl and urea are well known as denaturants. On the other hand, polyols are known to stabilize protein structures, and their protein-unfolding effects are also known (Timasheff 1993). These employed denaturants and polyols downgrade and upgrade the calcium binding of the A form without significant effect on the binding of the B form. Based on the data, the mechanism of the observed polyol effect on the A form is discussed, and a structural model of the two conformers is presented. The correspondence of the structural model to the ATPase crystal structures is also discussed.

Materials and Methods

Preparation

The SR was prepared from rabbit skeletal muscle (Nakamura et al. 1977). The SR mainly consists of two regions of terminal cisternae and longitudinal elements, respectively, with and without a "feet" structure (Chu et al. 1988). No contamination of the SR preparation with the vesicles having "feet" was detected by electron microscopic observations of the SR preparation (data not shown). The preparation, therefore, seemed to be predominantly composed of vesicles derived from the longitudinal SR. Membranous Ca^{2+}-ATPase was partially purified from the SR preparation by washing with sodium deoxycholate at a 1:5 ratio of detergent to SR protein (Nakamura 1983); the peripheral proteins on the SR membrane (MacLennan and Wong 1971; Ostwald and MacLennan 1974; Michalak et al. 1980) were washed out to reduce the possibility of an intermolecular interaction between the peripheral protein(s) and the ATPase molecule. It was stored with 0.3 M sucrose at −80 °C and treated with 2 μM calcium ionophore, A23187, before use. The density of the ATPase polypeptide in the ATPase preparation was estimated to be ~8.5 nmol/mg of preparation protein (Nakamura et al. 2002). The maximum level of the phosphorylated ATPase (EP) in the preparation was 3.7–4.8 (average 4.2, $n = 6$) nmol/mg of protein obtained with 0.1 mM $CaCl_2$ and 0.1 mM ATP.

Assays

Specific calcium binding to the calcium transport sites of the ATPase molecules was estimated by examining the calcium dependence of their phosphorylation (with 10 μM ATP) and AcP hydrolytic activity (Ca^{2+}-AcPase activity, with 5 mM AcP), which are activated by the specific binding, based on the following possibility and observations: (1) a denaturant and/or polyol, as was used here, may induce some nonspecific calcium binding, which cannot trigger the phosphorylation; (2) 10 μM ATP (Nakamura et al. 2002) and 5 mM AcP (Nakamura and Tajima 1997) do not significantly affect calcium binding of the ATPase molecules.

The ATPase preparation was pretreated with a denaturant or polyol before addition of the preparation to the assay medium; the preparation (2.0 mg of protein/ml) was preincubated with a 20 mM Tris–maleate buffer solution (pH 7.40) containing 0.12 M KCl and various concentrations of a denaturant or polyol, contained in the assay medium, at 0 °C overnight, unless otherwise indicated. The steady-state level of the phosphorylation was assayed at pH 7.40 and 0 °C (Nakamura et al. 2002). In the pre-steady-state assay of the calcium-induced phosphorylation, a rapid filtration method (Nakamura and Furukohri 1994) was employed. Calcium concentrations, which were at 50 μM and less, were adjusted by the addition of 50 μM Ca^{2+} and 0–400 μM EGTA. The association constant for EGTA-calcium was taken as 1.335×10^7 M^{-1} (Harafuji and Ogawa 1980).

Ca^{2+}-AcPase activity was assayed in a 40 mM Tris–maleate (pH 7.40) buffer solution containing 0.01–50 μM Ca^{2+} and various concentrations of polyol at 25 °C (Nakamura and Tajima 1997).

Results

Effects of Denaturants on Calcium Dependence of Phosphorylation

In the absence of GdnHCl and urea, Hill plots of the calcium dependence (Fig. 1a) of the total, steady-state phosphorylation of the Ca^{2+}-ATPase molecules, which was composed of the A and B forms, exhibited a biphasic

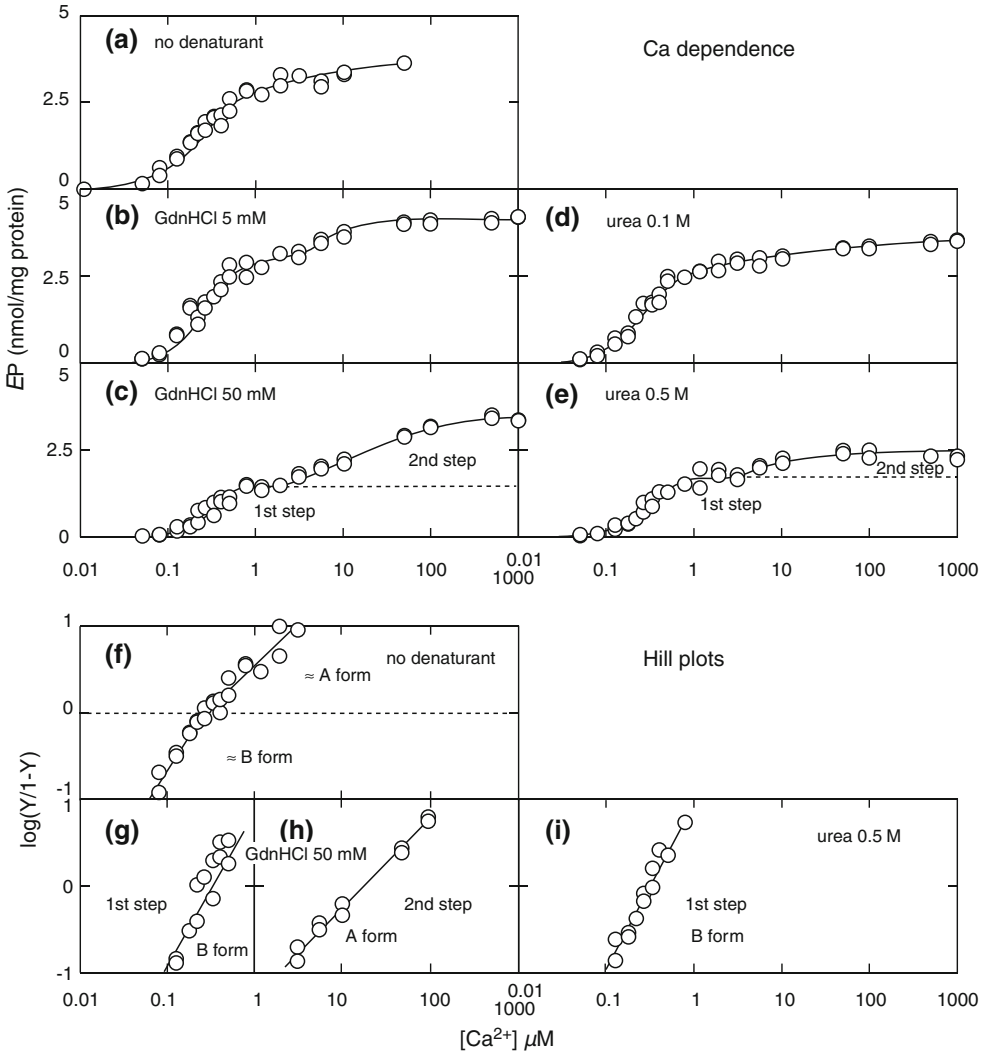

Fig. 1 Calcium dependence of phosphorylation in the presence and absence of GdnHCl and urea. **a** Calcium dependence of total phosphorylation of the A and B forms in the absence of GdnHCl and urea and **f** its Hill plots. *Y* is the ratio of EP at each calcium concentration to the maximum EP (3.6 nmol/mg of protein). **b** Calcium dependence of phosphorylation in the presence of 5 mM GdnHCl. **c** Calcium dependence of phosphorylation in the presence of 50 mM GdnHCl and Hill plots of the calcium dependences of the first (**g**) and second (**h**) steps of the phosphorylation. *Y* is the ratio of EP in the first and second steps at each calcium concentration to their maximum EP (1.5 and 2.0 nmol/mg of protein, respectively). **d** Calcium dependence of phosphorylation in the presence of 0.1 M urea. **e** Calcium dependence of phosphorylation in the presence of 0.5 M urea and **i** Hill plots of the calcium dependence of the first step of phosphorylation. *Y* is the ratio of EP in the first step at each calcium concentration to the maximum EP (1.8 nmol/mg of protein)

profile (Fig. 1f) with slopes of ~1.8 and ~1.0, respectively, at an employed pH of 7.40; Hill plots of the calcium dependence of the phosphorylations of the A and B forms have been shown to exhibit their profiles with Hill coefficient (n_H) values of about 1.1 and 2.0, respectively, and with $K_{0.5}$ values of about 5 and 0.08–0.1 μM (Nakamura et al. 2002). The two lines of the Hill plots ($K_{0.5}$ ~ 0.3 μM) of the total phosphorylation intersect near zero on the ordinate, showing that the A and B forms have almost the same level (1.7–2.0 nmol of EP/mg of protein) of maximum phosphorylation.

In the presence of 50–60 mM GdnHCl (Fig. 1c), the total phosphorylation exhibited a two-step type of calcium dependence. The first and second steps of the phosphorylation were distinctly separated and reached their peak levels, respectively, at 0.8–2.0 μM and 0.5–1.0 mM Ca^{2+}. The first step showed almost the same calcium-dependent profile (n_H ~ 1.9 and $K_{0.5}$ ~ 0.3 μM) (Fig. 1g) as that (n_H ~ 2.0, $K_{0.5}$ ~ 0.2 μM) of the phosphorylation of the B form without the denaturants, mentioned above. Next, the second step of the phosphorylation was obtained by subtracting the maximum level of the first step from the total phosphorylation. The calcium-dependent profile (Fig. 1h) of the second step showed the same n_H value of ~1 as that of the phosphorylation of the A form without the denaturants. However, its $K_{0.5}$ value (20–40 μM) was higher than that (~5 μM) without the denaturants. The ratio of the maximum level (1.5–2.2 nmol EP/mg of protein) of the first step to that (1.3–2.2 nmol EP/mg of protein) of the second step was about 1:1, and their maximum levels were near those (1.7–2.0 nmol EP/mg of protein) of the A and B forms in the absence of denaturants (see Fig. 1f). At a low concentration (5–25 mM) of GdnHCl (Fig. 1b), the first step was not clearly distinguished from the second step. At more than 60 mM of GdnHCl, the observed values of the phosphorylation were scattered, though its calcium dependence roughly exhibited a two-step pattern.

The calcium-dependent profile of the total phosphorylation was also converted by 0.4–0.5 M urea from a biphasic to a two-step type (Fig. 1e). The first step showed almost the same calcium-dependent profile (with $n_H \sim 2.0$ and $K_{0.5} \sim 0.3$ μM) (Fig. 1i) as that of the phosphorylation of the B form without the denaturants. The maximum phosphorylation level (~1.8 nmol EP/mg of protein) of the first step was also close to that (1.7–2.0 nmol EP/mg of protein) (see Fig. 1f) of the B form without the denaturants. It is, therefore, thought that the first step is predominantly of the B form. Considering this, the second step with 0.4–0.5 M urea should be predominantly of the A form. The maximum level (~0.5 nmol EP/mg of protein) of the second step with $K_{0.5} = 10$–50 μM, however, was significantly lower than that (1.7–2.0 nmol EP/mg of protein) (see Fig. 1f) of the A form without the denaturants; the observed values in the second step were too small to subject these values to Hill analysis. At more than 0.5 M of urea, a two-step type of calcium dependence was also observed, though the maximum levels in the first and second steps were decreased. At 0.1 M urea (Fig. 1d), the Hill plots of calcium dependence of the total phosphorylation had a main linear phase (slope ~ 1.8), followed by a minor downward deviation from the linear line at a high concentration of Ca^{2+} (data not shown).

Effects of Polyols on Calcium Dependence of Phosphorylation and Ca^{2+}-AcPase Activity

The observed calcium-dependent profiles of the total steady-state phosphorylation of the A and B forms were classified into five types, with glycerol concentrations varying from 0 to 6.79 M glycerol: (1) a biphasic type (Hill slopes of ~1.7 and 0.8–1.0, $K_{0.5} \sim 0.3$ μM) (Fig. 2a, f) at 0.041–0.082 M glycerol, (2) a mesophasic type [linear Hill plots (slope ~ 1.7, $K_{0.5} \sim 0.2$ μM) with small downward deviation at a high concentration of Ca^{2+}] (Fig. 2b, g) at 0.17 M glycerol, (3) a monophasic type ($n_H \sim 1.9$, $K_{0.5} \sim 0.2$ μM) (Fig. 2c, h) at 0.17–0.68 M glycerol, (4) a two-step type (first step with $K_{0.5} = 0.2$–0.5 μM and its peak at 0.4–2.0 μM Ca^{2+}, second step with $K_{0.5} =$ 20–100 μM and its peak at 5–1,000 μM Ca^{2+}) (Fig. 2d) at 1.13–4.07 M glycerol, and (5) a hill type [a slope (ranging from 0.17 μM to 1 mM Ca^{2+}) with a $K_{0.5}$ of 20–200 μM] (Fig. 2e) at 4.07–6.79 M glycerol. Such a monophasic type ($n_H = 1.7$–2.1, $K_{0.5} = 0.09$–0.5 μM), as mentioned above, was also observed at 0.1–1.31 M sucrose and 0.05–0.1 M PEG 600 (data not shown). At 0.3–0.6 M PEG 600, the profile was a hill type, like that observed at 4.07–6.79 M glycerol.

Similar to the calcium-dependent profile of the total phosphorylation, the profile of the total Ca^{2+}-AcPase activity was progressively changed by ethylene glycol,

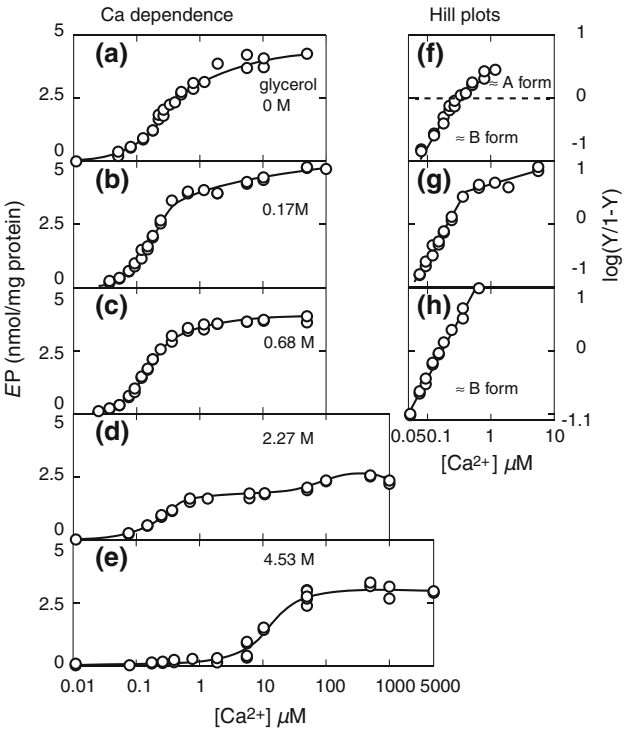

Fig. 2 Calcium dependence of steady-state phosphorylation at 0–4.53 M glycerol. **a** Calcium dependence of total phosphorylation of the A and B forms in the absence of glycerol and **f** its Hill plots. *Y* is the ratio of EP at each calcium concentration to the maximum EP (4.1 nmol/mg of protein). **b** Calcium dependence of phosphorylation at 0.17 M glycerol and **g** its Hill plots. *Y* is the ratio of EP at each calcium concentration to the maximum EP (4.7 nmol/mg of protein). **c** Calcium dependence of phosphorylation at 0.68 M glycerol and **h** its Hill plots. *Y* is the ratio of EP at each calcium concentration to the maximum EP (3.6 nmol/mg of protein). **d** Calcium dependence of phosphorylation at 2.72 M glycerol. **e** Calcium dependence of phosphorylation at 4.53 M glycerol

glycerol, triethylene glycol, sorbitol, sucrose, trehalose and PEG 600 from a biphasic type to mesophasic, monophasic and two-step types but not to a hill type, depending on their concentrations; a $K_{0.5}$ value (3–6 μM) of the second step in the two-step type was lower than that (20–100 μM) in the case of the phosphorylation. The transition concentration (0.025–2 M) of the polyols, above which the calcium-dependent profile was transformed into a monophasic type, almost linearly decreased with increasing molecular mass of the polyols (62.1–600) (Fig. 3).

The assay media for the AcP hydrolysis and the phosphorylation were contaminated by 2–5 mM of sucrose from the stock of sucrose (0.3 M)–suspended ATPase preparation. Removal of the contaminating sucrose did not affect the biphasic calcium-dependent profile of the Ca^{2+}-AcPase activity (data not shown). On the other hand, the SR preparation without sucrose was earlier observed to exhibit such a biphasic profile of calcium transport activity, supported by AcP (de Meis and Hasselbach 1971; Ogawa and Ebashi 1973). The present and previous observations

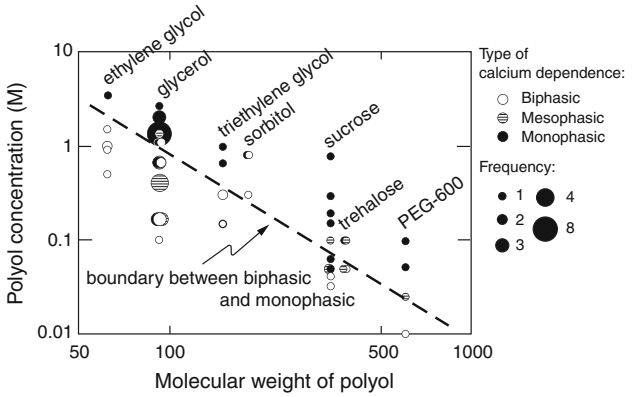

Fig. 3 Distribution analysis of types of calcium dependence of Ca^{2+}-AcPase activity in the presence of polyols. Type [biphasic (*unfilled circle*), mesophasic (*circle with horizontal line*) or monophasic (*filled circle*)] of calcium dependence observed at the employed concentration of each polyol and its frequency were plotted in a double-logarithmic figure of polyol concentration (M) against molecular weight of polyol. Frequency is represented by the *size* of the *circle*. *Broken line* roughly shows the boundary between biphasic and monophasic types, being the transition concentrations of the polyols, above which the biphasic type was transformed into the monophasic type

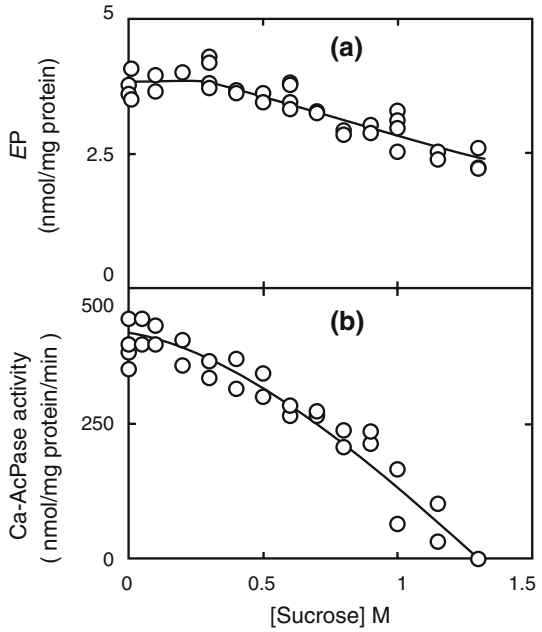

Fig. 4 Sucrose dependence of maximum levels of total steady-state phosphorylation of the A and B forms (**a**) and of the total Ca^{2+}-AcPase activity of the two forms (**b**). Phosphorylation and AcPase activity were assayed in the presence of 50 μM Ca^{2+}. Before the start of these assays, the ATPase preparation was preincubated with an assay medium containing 0–1.31 (**a**) and 0–2.0 (**b**) M sucrose at 0 °C overnight

suggest that the biphasic calcium-dependent profile of AcPase activity is the native profile of the ATPase molecules.

The two-step type of calcium dependence of the phosphorylation (see Fig. 2d) which was observed at 1.13–4.07 M glycerol, seems to be near the two-step types (Fig. 1c, e) which were observed at about 0.05 GdnHCl and 0.5 M urea. On the other hand, it was observed that the concentration (1.13–4.07 M) of glycerol for the two-step type is higher than that (0.7–0.68 M) for the monophasic type and that the concentration (4.07–6.79 M) of glycerol for the hill type is ever higher than that for the two-step type (Fig. 2c–e). Such a hill type, as observed at 4.07–6.79 M glycerol, was also observed at a higher concentration (0.3–0.6 M) of PEG 600 than that (0.05–0.1 M) for the monophasic type (data not shown). It is, therefore, thought that the observed two-step and hill types result from the unfolding of the ATPase protein by these neutral solutes. An unfolding effect of glycerol and PEG 1000 has been found (George 1986; Lee and Lee 1987).

In the presence of glycerol (0.17–2.04 M), sucrose (0.05–0.5 M) and PEG 600 (0.05–0.1 M), which changed the calcium-dependent profiles of the total phosphorylation and the total AcPase activity into a monophasic type, the maximum level of phosphorylation was maintained at more than 90 % of that (3.50–4.07 nmol EP/mg of protein) without additional polyols (see Fig. 4a). Although PEG 600 also did not significantly affect the maximum level of AcPase activity [351–444 nmol AcP/(mg protein min)], glycerol and sucrose decreased the activity level by about 30 % (see Fig. 4b).

Pre-Steady-State Analysis of Phosphorylation with Polyols

The effects of glycerol (data not shown), sucrose (Fig. 5) and PEG 600 (data not shown) on the equilibriums of the A and B forms between E_1 (high-affinity state of the enzyme for calcium) and E_2 (low-affinity state of the enzyme for calcium) before calcium binding were examined with the pre-steady-state analysis method (Nakamura and Furukohri 1994) of calcium-induced phosphorylation at 0 °C. Namely, at the alkaline pH of 7.40, employed here, the A form in E_1 rapidly binds calcium. The B form in E_2, however, apparently slowly binds calcium because of the slow transition of the form from E_2 to E_1 at low temperature. The two forms, which are preincubated with 10 μM ATP and without calcium, are, therefore, rapidly and slowly phosphorylated by the ATP, respectively, by the addition of calcium (see Fig. 5). In the presence of sucrose (≤1.31 M), these rapid and slow reactions were observed after the addition of 10.3 μM Ca^{2+}; at 10.3 μM Ca^{2+}, about 90 % of the calcium binding capacity of the ATPase molecules was saturated with calcium in the presence of 1.31, 0.1 and 0.68 M of sucrose, PEG 600 and glycerol, respectively, and lesser amounts of the three (see Fig. 2). For example, at 0.15 M sucrose, about half of the steady-state level of the phosphorylation was rapidly induced (<2 s, 1.7 nmol EP/mg of protein), which was followed by

the slow phosphorylation of the remaining half ($t_{1/2}$ = 3–7 s, 1.6 nmol EP/mg of protein) (Fig. 5a, b). Such rapid and slow phosphorylations at a ratio of about 1:1 were also observed in the presence of 0.05–0.1 M PEG 600 (data not shown). These results suggest that sucrose and PEG 600 do not significantly affect the enzyme states (E_1 and E_2) of the A and B forms before calcium binding. With 0.082 M and more of glycerol, the phosphorylation reaction exhibited a slow ($t_{1/2} \sim 2$ s), monophasic pattern without the rapid phase of the reaction (data not shown), though at −10 °C rapid and slow reactions of calcium binding have been observed with 4.08 M glycerol (Dupont 1982).

Calcium titration curves of the rapid and slow phosphorylations of the A and B forms were obtained in the presence of 0.5 M sucrose (Fig. 6). The calcium-dependent profiles of the rapid and slow phosphorylations were monophasic with the same n_H value of 2.0–2.2 but with the different $K_{0.5}$ values of 0.15–0.5 and 0.04–0.2 μM, respectively (Hill plots are not shown). The calcium dependence of the total phosphorylation, composed of the rapid and slow phosphorylations, exhibited a monophasic profile (n_H = 1.7–2.0, $K_{0.5}$ = 0.09–0.3 μM; Hill plots are not shown). In the presence of 0.1 M PEG 600 (data not shown), only slow phosphorylation ($t_{1/2} \sim 4$ s) was observed at 0.093 μM Ca^{2+}, while at 10.3 μM Ca^{2+} both rapid ($t < 2$ s) and slow ($t_{1/2} \sim 2$ s) phosphorylations were observed; at 0.093 μM Ca^{2+} the phosphorylation level was less than half of that at 10.3 μM Ca^{2+}.

Discussion

Structural Compactness of A and B Forms

Both GdnHCl (~0.05 M) and urea (~0.5 M) were observed to increase the apparent calcium affinity ($K_{0.5}$) of the A form with regard to its phosphorylation without a significant effect on that of the B form (Fig. 1), which is chemically equivalent to the A form; GdnHCl, not but urea, scarcely affected the phosphorylation capacities of the two forms with a ratio of about 1:1. These results show that calcium binding of the A form is more susceptible to these denaturants than that of the B form. In general, it is assumed that if the structure is more compact, it is more resistant to denaturants. The data, therefore, suggest that the structure of the calcium binding domain (Brandl et al. 1986) in the A form is less compact than that in the B form.

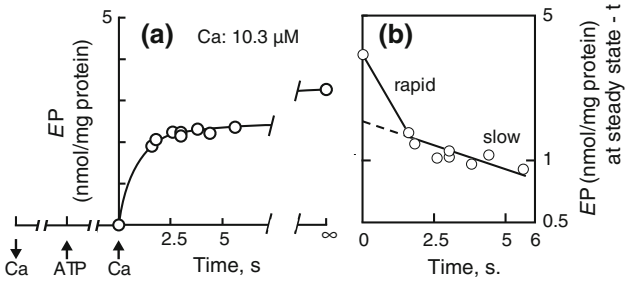

Fig. 5 The kinetics of calcium-induced phosphorylation in 0.15 M sucrose. Phosphorylation was induced by addition of 10.3 μM Ca^{2+}. Time course of phosphorylation (**a**) and semilogarithmic plots (**b**) of $(EP)_{steady\ state} - (EP)_t$ versus time after initiation of the reaction

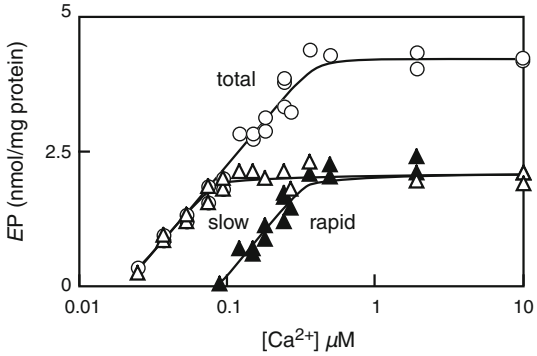

Fig. 6 Calcium dependence of rapid and slow phosphorylations in 0.5 M sucrose. Amounts of rapid (*filled triangle*) and slow (*unfilled triangle*) phosphorylations were determined by pre-steady-state analysis of calcium-induced phosphorylation at 0.025–10.3 μM of Ca^{2+}. Total phosphorylation (*unfilled circle*) is the sum of the slow and rapid phosphorylations

Polyol-Induced Transformation of A Form into B′ Form

All of the employed polyols (ethylene glycol, glycerol, triethylene glycol, sorbitol, sucrose, trehalose and PEG 600) converted the calcium-dependent profile of the total AcPase activity of the A and B forms from a biphasic into a monophasic type via a mesophasic type (Fig. 3). The biphasic profile of the total phosphorylation was also converted into a monophasic type by glycerol, sucrose and PEG 600 (see Fig. 2).

The monophasic, calcium-dependent profiles (n_H = 1.7–2.1, $K_{0.5}$ = 0.09–0.5 μM) of the total activity and the total phosphorylation (see Fig. 2c, h), transformed by the polyols, were close to that ($n_H \sim 1.1$, $K_{0.5}$ = 0.08–0.1 μM) of the phosphorylation of the B form but not to that ($n_H \sim 2$, $K_{0.5} \sim 5$ μM) of the A form (Nakamura et al. 2002). However, the pre-steady-state analysis of their phosphorylations with the transforming concentration of sucrose shows that the $K_{0.5}$ value (0.15–0.5 μM) of the rapid phosphorylation of the A form was slightly higher than that (0.04–0.2 μM) of the slow phosphorylation of the B form, though the n_H values of these phosphorylations were almost the same (2.0–2.2) (Fig. 6). In the presence of the transforming concentration (0.1 M) of PEG 600, only slow phosphorylation was observed at a low concentration of calcium, while at a high concentration of calcium both

rapid and slow phosphorylations were observed (Fig. 6a, b). This indicates that with PEG 600 the $K_{0.5}$ value of the slow phosphorylation is lower than that of the rapid phosphorylation. The results, mentioned above, show that the employed polyols upgrade calcium binding of the A form to its calcium affinity and its cooperativity in binding two calcium ions, suggesting transformation of the A form by polyols into a B-like form (B′) (Fig. 7).

Mechanism of A–B′ Transformation

Polyols have been shown to stabilize and/or unfold the structure of proteins (Timasheff 1993). As mentioned above, denaturants and polyols were observed to oppositely affect the calcium binding of the A form, degrading and upgrading, respectively (Fig. 7). It is, therefore, thought that the polyol-induced upgrade of the binding of the A form is a result of stabilization, but not of unfolding, of the structure of the A form by the polyols.

Sucrose (0.05–0.3 M) and PEG 600 (0.05–0.1 M), which induced A–B′ transformation, did not have a significant effect on the maximal level of the total steady-state phosphorylation of the A and B forms (see Fig. 4a) and on the 1:1 ratio of their phosphorylation levels (see Fig. 5b). This shows that the transforming concentration of these polyols transforms the structure of the calcium-binding domain of the A form without change in the calcium binding capacity of the form.

Some of the employed polyols (glycerol, sorbitol, sucrose and PEG 600) have been shown to stabilize globular proteins and to reduce their solubility with different mechanisms of the preferential interaction with the proteins (Timasheff 1993). However, the transition concentrations of polyols for A–B′ transformation were in inverse relation to their molecular mass (Fig. 3). The transformation, therefore, can be described according to the steric exclusion model (Schachman and Lauffer 1949; Timasheff 1993) as follows. The polyols are sterically excluded from the surface of the binding domain of the A form, resulting in a shell surrounding the surface of the domain, which cannot be further penetrated by the polyols but which is occupied by smaller water molecules. The consequence is an excess of water at the surface, leading to rectification by stabilization of the folding state of the binding domain. It is likely that the loose structure of the binding domain of the A form itself allows the polyols to interact with the domain in the same manner as in the steric exclusion model.

Structural Model of A and B Forms

The data showing the different susceptibility of the membranous A and B forms to denaturants and polyols suggest the energy diagram of the A, B′ and B forms as follows (Fig. 8a). Regarding the thermal energy (kT), the levels of activation energies of w_1, w_2, w_3 and w_4, respectively, for A → B′, B′ → A, B′ → B and B → B′ are on the order of $w_2 < w_1 < kT < w_3 < w_4$ in the presence of less than the transition concentration of polyol, where the existence of the B′ form is supposed. In the presence of a transforming concentration, the level of potential energy of the B′ form is lowered to less than that of the A form so that the order becomes $w_1 < w_2 < kT < w_3 < w_4$. In other words, the polypeptide of the B form is in a folding state with a

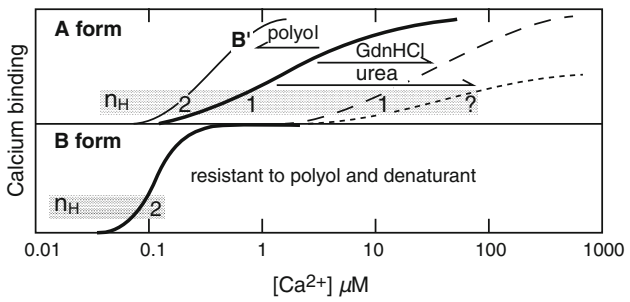

Fig. 7 Schematic representation of the effect of denaturants and polyols on calcium binding of the A and B forms. GdnHCl increases the $K_{0.5}$ value (2–6 μM) of the noncooperative calcium binding ($n_H \sim 1$) (*bold line*) of the A form to 20–40 μM (*broken line*). Urea induces a similar increase of the $K_{0.5}$ value of the A form with significant loss of its calcium binding capacity (*dotted line*). Polyols transform the noncooperative calcium binding of the A form into a cooperative binding ($n_H \sim 2$) (*thin line*), accompanying the approach of its $K_{0.5}$ value (2–6 μM) to that (0.04–0.2 μM) of the cooperative binding of the B form; the A form is transformed into a B-like (B′) form. The binding of the B form is resistant to these denaturants and polyols

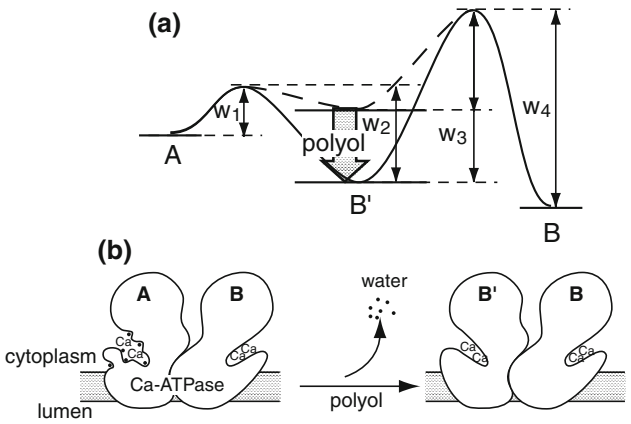

Fig. 8 Energy diagram of the A, B′ and B forms and their structural model. **a** Energy diagram. Regarding the thermal energy (kT), the levels of the activation energies of w_1, w_2, w_3 and w_4 are on the order of $w_2 < w_1 < kT < w_3 < w_4$ (*broken line*) in the presence of less than a transition concentration of polyol, where the existence of the B′ form is supposed. In the presence of a transforming concentration of polyol, the potential energy of the B′ form decreases to a level less than that of the A form (*solid line*). **b** Structural model

minimal level of potential energy in the SR membrane. Based on previous observations that the nonequivalence in the calcium binding of the two membranous forms is canceled by solubilization of the forms (Nakamura and Tajima 1995), the diagram further suggests a structural model of the A and B forms (Fig. 8b). The polypeptide of the A form folds as it interacts with the structure of the B form; the structure of the B form is a determinant of that of the A form and a barrier to the polyol-induced transformation of the A form into the B form. This structural model of the two forms complements the two-pair model of the ATPase structural unit (Nakamura and Tajima 1997). The atomic structures of ATPase have been solved by X-ray crystallography (Toyoshima et al. 2000; Toyoshima and Inesi 2004; Olesen et al. 2007). Considering the use of polyols (glycerol and/or various masses of PEG) in the crystallization of the enzyme, it is probable that the atomic structures correspond to the B form but not to the A form.

It is known that some proteins are chemically modified after their translation, resulting in versatility of their function. Referring to chemical modification, an asymmetric, intermolecular interaction, which produces two conformational variants of the chemically equivalent ATPase molecules with different levels of potential energy, is termed a physical modification of the ATPase proteins after their translation. Such a physical modification might often take place in the membrane system of a cell, where proteins are packed in an orderly way.

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