Independence of Two Conformations of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Molecules in Hydrolyzing Acetyl Phosphate

A TWO-PAIR MODEL OF THE ATPase STRUCTURAL UNIT*  

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The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase molecules have been shown to exist in two conformations (A and B) that result from intermolecular interaction of ATPase molecules (Nakamura, J., and Tajima, G. (1995) J. Biol. Chem. 270, 17350–17354). The A form binds two calcium ions noncooperatively, whereas the B form binds the calcium ions cooperatively. Here, we examined the independence of these two forms in the calcium-activated hydrolysis of acetyl phosphate (AcP) under asynchronous and synchronous conditions of their hydrolysis of AcP due to calcium that was bound to each of the forms, indicating the independence of the two forms in hydrolyzing AcP. Taking into account the monomer-dimer transition of the ATPase molecules on the sarcoplasmic reticulum membrane accompanying \(E_1\)–\(E_2\) transition of the molecules (Dux, L., Taylor, K. A., Ting-Beall, H. P., and Martonosi, A. (1985) J. Biol. Chem. 260, 11730–11743), the two types of molecules seem to independently carry out such monomer-dimer transition of the same type of molecules. Two pairs, each consisting of the same type of molecules, are suggested to be the structural unit of the ATPase molecules.

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\(^{*}\) The abbreviations used are: SR, sarcoplasmic reticulum; AcP, acetyl phosphate; AcPase, acetyl phosphatase; PIPES, pipperazine-\(N,N'\)-bis(2-ethanesulfonic acid).

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Based on the monomer-dimer transition of the ATPase molecules on the SR membrane (9), the structural unit of the molecules is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**
The procedures for isolation of the SR from the skeletal muscle of rabbit were the same as those described in a previous paper (14). Membranous Ca$^{2+}$-ATPase was purified from the SR by washing the SR with sodium deoxycholate in the same manner as reported previously (15) in a 1:5 ratio of sodium deoxycholate to the reticulum protein. The maximum calcium binding capacity of the ATPase preparation was 9.0–10.7 nmol/mg of protein as estimated by Scatchard analysis of the calcium titration curve of the calcium-binding sites in the preparation, and the maximum level of the preparation that was phosphorylated with ATP was 4.2–4.6 nmol/mg of protein obtained in 0.1 mM ATP, 0.1 mM CaCl$_2$, and 5 mM MgCl$_2$ at pH 7.0. The purified ATPase preparation exhibited the same characteristics of calcium binding as those of the ATPase preparation (7) (see Figs. 1 and 2) that was purified by solubilization of the sarcoplasmic reticulum with sodium deoxycholate and by reformation of the membranous ATPase by removal of the detergent (data not shown).

**Assays**

AcP Hydrolysis—The total AcP hydrolysis activity of the ATPase was carried out in 20 mM PIPES buffer solution (pH 6.23 or 7.40) containing 0.1 or 0.5 mg of ATPase protein/ml, 0.12 mM KCl, 5 mM MgCl$_2$, 5 mM AcP, and 0.01–1000 µM Ca$^{2+}$ at 5 or 25°C. To obtain reproducible values of the activities at these pH values and temperatures, the reaction times in which the reactions were linear and the protein concentrations of the ATPase were set at 3 (at pH 7.40) or 5 (at pH 6.23) h and 0.5 mg of protein/ml at 5°C and at 30 (at pH 7.40) or 60 (at pH 6.23) min and 0.1 mg of protein/ml at 25°C, respectively, based on the following observations: (i) The AcPase activities at pH 6.23 were about half of those at pH 7.40. (ii) The activities at 5°C were one-thirtieth to one-fortieth of those at 25°C. (iii) No inactivation of the activity was observed when the ATPase was preincubated in the absence of AcP for 2 and 6 h at 5°C and at 25°C, respectively, which is different from the case (8) of the detergent-solubilized ATPase, which was gradually inactivated during the preincubation. The calcium-independent activity of AcP hydrolysis was determined under identical conditions, except that 5 mM EGTA was added without the addition of calcium. The calcium-dependent activity of AcP hydrolysis (Ca$^{2+}$-AcPase activity) was obtained by subtracting the calcium-independent activity from the total activity. The amount of remaining AcP was determined according to the method of Lipmann and Tuttle (16). Before reading the absorbance at 505 nm of the brown complex formed with iron(III), the ATPase protein suspended in the medium for the assay of AcP was removed by centrifugation (1000 × g for 5 min).

Calcium Binding—Calcium binding was performed according to the rapid filtration method (17) at 0°C as described previously (18). A Millipore HA filter (0.45-µm pore size) was used. The ATPase preparation (0.2 mg of protein/ml) was preincubated in 20 mM PIPES buffer solution (pH 6.23 or 7.40) containing 0.12 mM KCl and 5 mM MgCl$_2$,

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**Fig. 1.** Calcium binding in the absence of AcP at pH 7.40 and 0°C (7). A, calcium titration curves of the two types of ATPase molecules. □, total binding to the A and B forms; △, rapid binding to the A form, which is in $E_1/E_2$ dependent on pH and pre-exists in $E_1$ at this alkaline pH; ▼, slow binding to the B form, which pre-exists in $E_2$ independent of pH (see “Results and Discussion” for details). B, Hill plots of the total (□), rapid (▲), and slow (▼) binding.

**Fig. 2.** Calcium binding in the absence of AcP at pH 6.23 and 0°C (7). A, total calcium titration curves of the two types of ATPase molecules. Calcium binding to the A form (×), which is in a state of $E_1/E_2$ dependent on pH and pre-exists in $E_2$ at this acidic pH, was obtained by subtracting the binding to the B form (---), which pre-exists in $E_2$ independent of pH, from the total binding (□). Calcium binding to the B form was simulated by using parameters of $n_H = 2.0$ and $K_{0.5} = 5.3$ µM, obtained on the basis of the observations of the calcium-dependent change in fluorescence intensity of the molecule and maximum binding capacity = half (4.9 nmol/mg of protein) of the capacity (9.8 nmol/mg of protein) of the observed total binding (cf. “Discussion” in Ref. 7). B, Hill plots of the total binding.
without the addition of CaCl₂. Aliquots (1 ml) of the ATPase suspension were placed on the filter. After removal of calcium that was bound to the ATPase by washing it on the filter with EGTA, calcium binding to the calcium-unbound ATPase was initiated by washing the ATPase on the filter with binding solution containing various concentrations of ⁴⁵Ca²⁺ and having the same pH buffer as that used in the preincubation of the ATPase, as described previously (19). The association constants for EGTA-calcium at the employed pH values of 6.23 and 7.40 were 6.31 × 10⁴ and 1.38 × 10⁷ M⁻¹, respectively (20). In the presence of 5 mM AcP, calcium accumulation in the ATPase membrane vesicles was observed. After the calcium that was bound to the ATPase preparation reached equilibrium in the absence of AcP, the preparation further took up calcium at a rate of 2–4 nmol/mg of protein/min when AcP was added. The calcium that was taken up with the help of AcP was completely released by the addition of the calcium ionophore A23187 (data not shown). Therefore, the amount of calcium that was bound to the ATPase in the presence of AcP was obtained by subtracting the amount of the accumulated calcium from the amount of total calcium that was taken up by the membrane vesicles.

RESULTS AND DISCUSSION

We previously showed that two different conformations (A and B) of chemically equivalent Ca²⁺-ATPase molecules exist in the SR membrane at a ratio of 1:1 (6). They independently bind two calcium ions (7): the A form binds the ions noncooperatively, whereas the B form binds the ions cooperatively (Figs. 1 and 2). Recently, however, these two forms have been shown to result from intermolecular interaction of the ATPase molecules (8). Here, we therefore further examined the independence of the two forms in transporting calcium by hydrolytic coupling of a substrate as an energy source. To examine this point, we compared the calcium dependence of the total hydrolysis activity of the two forms with calcium binding to the forms in the absence of the substrate. The comparison was carried out to elucidate whether these forms independently hydrolyze the substrate due to calcium that is bound to each of the forms. For this comparison, it is necessary to use a substrate that reacts with the ATPase molecules to transport calcium across the SR membrane and that does not have any regulatory effect, other than that as a substrate for the molecules, on their respective calcium binding and hydrolysis. AcP has been shown to support calcium transport (11) and to have no regulatory effect on the turnover of AcP hydrolysis (12, 13). However, it is not known whether AcP affects calcium binding. In Fig. 3, we therefore first examined calcium binding in the absence and presence of 5 mM AcP at pH 7.40 and 0 °C. As shown), therefore, the amount of calcium that was bound to the ATPase in the presence of AcP was obtained by subtracting the amount of the accumulated calcium from the amount of total calcium that was taken up by the membrane vesicles.
Binding dependent on pH (7): at pH 7.40, the A form is in different enzyme states before calcium binding. On the other hand, the two forms of the ATPase molecules have been shown to be in different enzyme states before calcium binding, which is composed of the AcPase activities of the two forms, was examined by varying the calcium concentration and temperature at the mentioned pH. At 25 °C, Hill plots of the total AcPase activity as a function of calcium concentration were biphasic (Fig. 4B). The two lines of the plots, the slopes of which were −0.9 and 1.7, respectively, intersected near the zero point of the ordinate, suggesting the existence of two different types of AcP hydrolysis reaction with the same level of maximum activities. The calcium concentration for the half-maximum activity ($K_{0.5}$) was −0.2 μM. In a previous paper (7), we showed that at pH 7.40 and 0 °C, the A form, which pre-exists in $E_1$, noncooperatively (Hill value ($n_H$) = 1) binds calcium with an apparent calcium affinity (calcium concentration for the half-maximum binding ($K_{0.5}$)) of −2.0 μM, whereas the B form, which pre-exists in $E_2$, cooperatively ($n_H$ = 2) binds calcium with a $K_{0.5}$ of −0.2 μM (Fig. 1, A and B). Hill plots of the total binding, which is composed of the binding of the two forms at a ratio of 1:1, were biphasic, with slopes of −0.8 and 1.8. The two lines of the Hill plots also intersected near the zero point of the ordinate. The $K_{0.5}$ of the total binding was −0.4 μM. This profile of the total binding is very close to that of the total Ca$^{2+}$-AcPase activity at 25 °C, which is shown in Fig. 4. The profile of the total binding at 0 °C was not affected by increasing the temperature to 25 °C (data not shown). At 25 °C, calcium has been found to be rapidly bound to the ATPase molecules at the same millisecond rate irrespective of their enzyme states ($E_1$ and $E_2$) before calcium binding (21). These results therefore suggest that at a temperature of 25 °C, the two forms equally and independently hydrolyze AcP due to calcium that is bound to each of the forms in a different manner. It was previously shown that at 0 °C, the A form, which pre-exists in $E_1$, apparently rapidly ($t < 2$ s) binds calcium, whereas the B form, which pre-exists in $E_2$, slowly ($t > 2$ s) binds calcium (7). Based on the above discussion of independent AcP hydrolysis by the two forms.
forms, it is therefore likely that at a lower temperature, the A form, which rapidly binds calcium, more rapidly turns over its hydrolysis cycle than the B form, which slowly binds calcium. This possibility was examined in Fig. 5A, in which the calcium dependence of the total AcPase activity was studied at 5 °C. Almost all of the Hill plots of the total activity were monophasic, with a slope of 0.9–1.3, although a small deviation from the linear line was observed (Fig. 5B). The slope (0.9–1.3) of the main portion in the Hill plots is near the \( n_h \) value (1) of the binding to the A form, which pre-exists in \( E_1 \), whereas the slope (>2) of the minor portion is rather near the \( n_h \) value (2) of the binding to the B form, which pre-exists in \( E_2 \) (cf. Fig. 1B). These results support the above discussion of independent AcPase hydrolysis by the two forms at pH 7.40, where the forms asynchronously turn over their hydrolysis cycles.

Figs. 6 and 7 show the calcium dependence of the AcPase activity at pH 6.23. At this acidic pH, as mentioned before, it is thought that the A and B forms synchronously turn over their cycle of AcP hydrolysis reaction because they are in the same enzyme states of \( E_2 \) before calcium binding. As shown in a previous report (7), at this acidic pH, the A form noncooperatively (\( n_h = 1 \)) binds calcium with a \( K_d \) of \( 7 \) \( \mu \)m, whereas the B form cooperatively (\( n_h = 2 \)) binds calcium with a \( K_d \) of \( 2-6 \) \( \mu \)m (see Fig. 2A). At the low temperature of 0 °C, both of the forms, which pre-exist in \( E_2 \), apparently slowly (\( t_1/2 > 2 \) s) bind calcium because of their slow transition to \( E_1 \). The total binding profile at this pH, which is composed of the binding to the two forms at a ratio of 1:1, was monophasic, with \( n_h = 1.3 \) and \( K_d = 5.8 \) \( \mu \)m (Fig. 2B). Such a profile was not affected by increasing the temperature to 25 °C (data not shown), although the ATPase molecules have been found to equally and rapidly bind calcium at a millisecond rate (21). At 25 °C, Hill plots of the calcium dependence of the total AcPase activity were monophasic, with \( n_h \) about 1.4 and \( K_d = 5.0 \) \( \mu \)m (Fig. 6B). The calcium-dependent profile (\( n_h = 1.4 \) and \( K_d = 3 \) \( \mu \)m) of the total AcPase activity at 5 °C (Fig. 7B) was almost the same as that at 25 °C. These profiles were the same as that of the total calcium binding (Fig. 2B), which is composed of the binding to the two forms at a ratio of 1:1. These results suggest that the two forms equally and independently hydrolyze AcP due to the calcium that is bound to each of the forms, even when the forms synchronously turn over their hydrolysis cycles.

These results demonstrate the independence of two forms of chemically equivalent Ca \(^{2+}\)-ATPase molecules in hydrolyzing AcP, irrespective of temperature and the synchronism of their hydrolysis reactions. On the other hand, if the two forms of the ATPase molecules were not independently operating in hydrolyzing AcP, either or both of the following results, which are contrasted with the results actually obtained, would be expected from the experiments carried out here. (i) The calcium-dependent profile of the total AcPase activity, which is composed of the activities of the two forms, would not correspond to that of the total calcium binding, which is composed of the calcium binding to the forms at a ratio of 1:1, under some condition(s) of pH and temperature except the condition of pH 7.40 and 5 °C. (ii) If 7.40, the calcium-dependent profile of the total AcPase activity would correspond to that of the total binding independent of temperature. Taking into account the monomer-dimer transition of the ATPase molecules on the SR membrane accompanying \( E_1 \)-\( E_2 \) transition of the molecules (9), the conclusion, mentioned above, indicates that the two types of ATPase molecules independently carry out such monomer-dimer transition of the same type of molecules, i.e. a structural unit model in which there are two different pairs of molecules, with each pair consisting of the same type of molecules and in which nonequivalence results from the intermolecular interaction of those molecules. The two-pair model is schematically represented in Fig. 8. The concept that at least two types of ATPase molecules are involved in the calcium transport mechanism first emerged from the studies by Froehlich and Taylor (22). Ikemoto et al. (23–25) reported the existence of two ATPase molecules that behave differently in sequential steps of the calcium transport reaction. Our results support these earlier reports.

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Fig. 8. Schematic representation of the monomer-dimer transition of the ATPase molecules in each of the two pairs of the molecules accompanying \( E_1 \)-\( E_2 \) transition. Open and hatched circles denote the two different types of molecules. Based on a recent report (26) that half of the ATPase molecules cannot be phosphorylated, the counterpart of each pair seems to be dormant.