Substrate Regulation of Calcium Binding in Ca²⁺-ATPase Molecules of the Sarcoplasmic Reticulum

I. EFFECT OF ATP*

The effect of ATP on calcium binding of the Ca²⁺-ATPase of the sarcoplasmic reticulum has not been clarified. By comparing the calcium dependence of the ATPase activity and of phosphorylation of the ATPase molecules with that of calcium binding in the absence of ATP, we show the existence of two types of regulatory site of the enzyme molecules at which ATP binding variously improves the calcium binding performance of the molecules depending on the aggregation state of the molecules and pH; the two regulatory sites bind ATP at submillimolar (0.25 mM) and millimolar (5 mM) ATP, respectively. The results are discussed based on a model of two conformational variants (A and B forms) of the chemically equivalent ATPase molecules (Nakamura, J., and Furukohri, T. (1994) J. Biol. Chem. 269, 30818–30821). For example, in the sarcoplasmic reticulum membrane at pH 7.40, submillimolar ATP converted the calcium binding manner of the A form from noncooperative (Hill number \(n_H = 2\), \(K_{0.5} = 0.2\) μM), which was not affected by ATP, to cooperative (Hill number \(n_H = 0.5\) of \(-1\) to cooperative \(n_H = 2\)), concurrent with a decrease in the apparent calcium affinity \(K_{0.5}\) from 2–6 to 0.1–0.3 μM. The binding of the B form became almost the same as that of the A form \((n_H = 2, K_{0.5} = 0.2\) μM), which was not affected by ATP. Millimolar ATP further decreased the \(K_{0.5}\) of the cooperative binding of the two forms to \(-0.05\) μM. Regulation of the calcium binding performance by ATP is discussed in terms of monomeric and oligomeric pathway models.

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¶ The abbreviations used are: SR, sarcoplasmic reticulum; bis-tris propanone, 1,3-bis[(hydroxy)methyl]amino propane; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid).
Here, the calcium dependence of the Ca\textsuperscript{2+}-ATPase activity and of the phosphorylation of the molecules was compared with that of the kinetic calcium binding in the absence of ATP. The results show the existence of two types of regulatory site at which ATP binding changes the binding. The data on ATP regulation are discussed in terms of a model that never seems to come into sharper focus with regard to which conformers of the ATPase molecules are involved in the catalytic cycle of the ATPase, whether these conformers are on different subunits in an oligomer of the enzyme molecules, whether they convert one into the other, and so on.

EXPERIMENTAL PROCEDURES

Materials

The procedures for isolation of the SR from rabbit skeletal muscle were the same as those described in a previous study (31). Employing a modified method (32) of Meissner et al. (33), membranous Ca\textsuperscript{2+}-ATPase was purified from the SR by washing the SR with sodium deoxycholate at a 1.5 ratio of detergent to reticulum protein. The content of the ATPase protein in the purified ATPase preparation was estimated to be 93% by SDS-PAGE of the preparation, similar to the content in the preparations (32) that were obtained at a 1:4 to 1:3 ratio of detergent to membrane protein. Taking into consideration the molecular mass (110 kDa) of the Ca\textsuperscript{2+}-ATPase molecule (34), the purity (~93%) of the ATPase molecules in the enzyme preparation, and the number (one) of catalytic sites/ATPase polypeptide chain (15), the density of the catalytic sites in the preparation was estimated to be ~8.5 nmol/mg of preparation protein. The enzyme preparation was treated with 2 μM calcium ionophore A23187 before use. The maximum level of the preparation that was phosphorylated with ATP was 4.2–5.6 (average of 4.4; n = 17) nmol/mg of protein obtained in 0.05 mM ATP, and the activity of the detergent-solubilized enzyme was 8.5 nmol/mg of protein obtained in 0.05 mM ATP/mg of preparation (0.12 mM MgCl\textsubscript{2}, 0.1 mM MgCl\textsubscript{2}, and 0.1 mM CaCl\textsubscript{2} at pH 7.4 and 0 °C). In the preparation that was solubilized with the nonionic detergent C\textsubscript{12}E\textsubscript{8} (octaethylene glycol dodecyl ether), the maximum level of the preparation was estimated to be 8.5 nmol/mg of preparation protein.

Assays

Enzymatic Activities—The total activity of ATP hydrolysis, which is composed of calcium-independent and -dependent ATPase activities, was assayed in 20 mM Bistris propane (pH 6.23) or 40 mM Tris maleate (pH 7.40) buffer solution containing 0.05–0.05 mg/ml membranous enzyme protein, 0.12 mM KCl, 0.1 mM MgCl\textsubscript{2}, and 0.1 mM CaCl\textsubscript{2} at pH 7.4 and 0 °C. In the assay of the activity of the detergent-solubilized enzyme, the membranous enzyme (0.01 mg/ml enzyme protein) was directly solubilized in the assay medium containing 2.0 mg/ml C\textsubscript{12}E\textsubscript{8}. The reaction times for the assay of the ATPase activity of the membranous enzyme, in which the reactions were linear, were set at.

![Diagram](image-url)
ATP Regulation of Calcium Binding of Ca\textsuperscript{2+}-ATPase

90 s (at 0.01 mg/ml protein and 5 mM ATP) or 5 min (at 0.01 mg/ml protein and 0.25 mM ATP) at pH 6.23 and at 60 s (at 0.005 mg/ml protein and 10 \(\mu\)M ATP and at 0.01 mg/ml protein and 0.25 mM ATP) or 90 s (at 0.01 mg/ml protein and 1 mM ATP and at 0.05 mg/ml protein and 5 mM ATP) at pH 7.40. In the presence of the detergent, the reaction time was set at 55 s (at 0.1 mM ATP) or 60 s (at 0.25 mM ATP) at pH 7.40. At 10 \(\mu\)M to 0.25 mM ATP, the association constants for CaEGTA at pH 6.23 and 7.40 were taken as 6.311 \(\times\) 10\(^{-4}\) and 1,335 \(\times\) 10\(^{-3}\) \(\text{M}^{-1}\), respectively (35). At a higher ATP concentration of 5 mM, the association constants for CaEGTA at pH 6.23 and 7.40 were taken as 5.841 \(\times\) 10\(^{2}\) and 1.247 \(\times\) 10\(^{2}\) \(\text{M}^{-1}\), respectively (35). The association constants for CaATP and MgATP at pH 6.23 and 7.40 were taken as 3.097 \(\times\) 10\(^{-4}\) and 2.418 \(\times\) 10\(^{-5}\) \(\text{M}^{-1}\), respectively (36). The calcium-independent ATPase activity was assayed in the presence of 5 mM EGTA without the addition of calcium. The calcium-dependent ATPase activity was assayed in the presence of the total ATPase activity exhibited a biphasic profile of 5 mM EGTA without the addition of calcium. The calcium-dependent ATPase activity was assayed in the presence of 5 mM EGTA without the addition of calcium. ATP hydrolysis was measured by determining the amount of phosphate liberated from ATP. P, which was liberated from ATP at 10 \(\mu\)M, 0.1–0.25 mM, and 5 mM ATP, was measured using the methods of Chan et al. (37), Baginski et al. (38), and Bonting et al. (39), respectively.

**Phosphoenzyme**—In the steady-state study of the ATP-induced phosphorylation of the enzyme, the enzyme preparation (0.05 mg/ml protein) was preincubated in a test tube with medium containing 20 mM Bistris propane (pH 7.40), 0.12 mM KC\(_1\), 5 mM MgCl\(_2\), and 0.015–100 \(\mu\)M Ca\textsuperscript{2+} for 10–60 min at 0 °C. The reaction was manually initiated by the addition of 10 \(\mu\)M to 0.25 mM [\(^{32}\)P]ATP. The volume of the reaction medium was 0.5 ml. The amount of the[^1] labeled phosphoenzyme reached ~80% of its steady-state level within the dead time (~0.2 s) of the experiment after initiation of the reaction and reached the steady-state level 3–5 s later. Thus, the reaction was terminated 5 s after initiation by the addition of 0.5 ml of 10% ice-cold perchloric acid. Aliquots (0.8 ml) of the terminated reaction medium were applied to Millipore HA filters (0.45-\(\mu\)m pore size), which were immersed in 5% perchloric acid containing 0.1 mM ADP and 1 mM Pi. The[^2]labeled enzyme protein on the filter was washed five times with 10 ml of the 5% perchloric acid solution. In the pre-steady-state study of calcium-induced phosphorylation of the enzyme, a rapid filtration method was employed as described previously (28). Briefly, after washing the enzyme (0.2 mg of protein) on the Millipore filter with 0.2 ml EGTA and 10 \(\mu\)M to 0.25 mM [\(^{32}\)P]ATP for ~10 s following 0.2 mM EGTA washing for ~10 s, the phosphorylation reaction was initiated by washing the enzyme on the filter with a phosphorylation solution of 0.015–50 \(\mu\)M Ca\textsuperscript{2+} containing the same concentration of [\(^{32}\)P]ATP as that used before initiation of the reaction. This was achieved by filtering 1–30 ml of the phosphorylation solution at 0 °C. The dead time of the experiment was ~2 s. Compared with the reaction of the ATP-induced phosphorylation described above, the reaction of the calcium-induced phosphorylation was slow and reached its steady-state level 10–30 s after initiation of the reaction (see Fig. 2 and “Discussion” for details).

**RESULTS**

To examine the effect of ATP on calcium binding in the Ca\textsuperscript{2+}-ATPase molecules of the SR, the calcium dependence of the Ca\textsuperscript{2+}-ATPase activity of the ATPase molecules and of the phosphorylation of the ATPase molecules was studied based on a model of two conformational variants (A and B forms) of chemically equivalent Ca\textsuperscript{2+}-ATPase molecules (28). The two forms have been suggested to exist in the SR membrane at a ratio of 1:1 and to independently bind calcium ions in the absence of ATP (Fig. 1) (29).

In Fig. 3A, the calcium dependence of the total Ca\textsuperscript{2+}-ATPase activity, which is composed of the activities of the two forms, was examined at 10 \(\mu\)M ATP and pH 7.40. The Ca\textsuperscript{2+}-ATPase molecules of the SR have been shown to have one catalytic site with a high affinity for ATP (9) and a putative regulatory site(s) at which ATP binding decreases the turnover rate of the ATPase activity with lower affinity (K\textsubscript{m} = 0.1–5.0 mM) (1, 3, 27) for ATP than that at the catalytic site. ATP, which was used here at a concentration of 10 \(\mu\)M, seems to bind to the catalytic site. The Hill plots of the calcium dependence of the total ATPase activity exhibited a biphasic profile with slopes of ~1.8 and ~1.0 and an apparent calcium affinity (calcium concentration for the half-maximum activity (K\textsubscript{0.5})) of ~0.3 \(\mu\)M (Fig. 3B). The two lines of the plots intersect near the zero point of the ordinate, indicating the existence of two different types of ATPase reaction with almost the same level of maximum activity. Such biphasic calcium dependence of the total phosphorylation (slopes of ~1.9 and ~0.9, K\textsubscript{0.5} = ~0.2 \(\mu\)M), which is composed of the phosphorylation of the two forms and which was induced by the addition of ATP to the enzyme molecules preincubated with calcium, was also observed at 10 \(\mu\)M ATP and pH 7.40 (Fig. 4, A and B). On the other hand, it has been found that ATP, which is bound to the catalytic site at 0.89 \(\mu\)M to 0.1 mM ATP, accelerates specific steps in the catalytic cycle (40–43). Therefore, the calcium dependence of the total Ca\textsuperscript{2+}-ATPase activity and of the total phosphorylation was also examined at 0.1 mM ATP. These calcium-dependent profiles (slopes of ~1.9 and ~0.9 and K\textsubscript{0.5} ~0.12 \(\mu\)M for the ATPase activity and slopes of ~1.7 and ~0.7 and K\textsubscript{0.5} = ~0.24 \(\mu\)M for the phosphorylation) (data not shown) were almost the same as those at 10 \(\mu\)M ATP described above (Figs. 3 and 4). In the absence of ATP at pH 7.40, the A form noncooperatively (n\textsubscript{H} = 1) bound calcium ions with an apparent K\textsubscript{0.5} of 2–6 \(\mu\)M, whereas the B form cooperatively (n\textsubscript{H} = 2) bound the ions with a K\textsubscript{0.5} of ~0.2 \(\mu\)M (Fig. 5A; replots of the data in Fig. 3A of Ref. 29). The Hill plots of the calcium dependence of the total calcium binding, which is composed of the binding of the two forms at a ratio of 1:1, were biphasic with slopes of ~1.8 and ~1.0 (Fig. 5B; replots of the data in Fig. 3C of Ref. 29). The two lines of the Hill plots also intersect near the zero point of the ordinate. The K\textsubscript{0.5} of the total binding was ~0.4 \(\mu\)M. This biphasic profile of the total binding in the absence of ATP was close to those of the total ATPase activity and the total phosphor- ylation.

The results indicate that the calcium dependence of the ATPase activities of the A and B forms is near that of their calcium binding in the absence of 10 \(\mu\)M ATP, suggesting that there is no effect of ATP binding at the catalytic site on calcium binding of the two forms. To examine this conclusion, a study was conducted to determine the calcium dependence of the phosphorylation of the A and B forms. The phosphorylation of the two forms was obtained by pre-steady-state analysis of the total calcium-induced total phosphorylation reaction as described previously (28). In the pre-steady-state study, phosphorylation was initiated by the addition of calcium to the enzyme molecules, which were fixed on the filter and preincubated with ATP in the absence of calcium. At pH 7.40, as described previously (28), it is thought that the A and B forms are in the enzyme states of E\textsubscript{1} and E\textsubscript{2}, respectively, before calcium binding.

At a low temperature of 0 °C, the A form, which pre-exists in E\textsubscript{1}, rapidly (<2 s) binds calcium when it is added, whereas

![Image](http://www.jbc.org/).
the B form, which pre-exists in $E_2$, apparently slowly ($\geq 2$ s) binds calcium because of the slow transition of the enzyme state from $E_2$ to $E_1$ (Fig. 2). Therefore, by rapid and slow calcium binding, the phosphorylation of the A and B forms (preincubated with ATP in the absence of calcium) is rapidly and slowly induced, respectively. The calcium-dependent profile of the observed slow phosphorylation ($k_{H} \sim 2.0, K_{0.5} \sim 0.08-0.1 \mu M$) (Fig. 4, A and B) was found to have a maximum level of $\sim 2.5$ nmol of phosphorylated Ca$^{2+}$-ATPase (EP/mg of protein), which is nearly half of the separately obtained maximum total phosphorylation ($\sim 4.9$ nmol of EP/mg of protein), and to be close to that of calcium binding of the B form in the absence of ATP ($k_{H} \sim 2, K_{0.5} \sim 0.20 \mu M$) (Fig. 5, A and B); the slow phosphorylation seems to be fully resolved. The maximum levels of the total phosphorylation ($\sim 4.9$ nmol of EP/mg of protein) and of the slow phosphorylation ($\sim 2.5$ nmol of EP/mg of protein) are thought to be roughly one-half and one-fourth of the density ($\sim 8.5$ nmol/mg of protein) (see “Experimental Procedures” for details) of the total catalytic sites in the ATPase preparation, respectively. In contrast to the observed calcium-induced slow phosphorylation of the B form, the calcium-induced rapid phosphorylation of the A form was not resolved by pre-steady-state analysis using the method of rapid filtration. The steady-state level of the calcium-induced rapid phosphorylation was lower than the level of the rapid phosphorylation, which was calculated by subtracting the calcium-induced slow phosphorylation from the ATP-induced total phosphorylation that was separately measured. For example, at 1.16 $\mu M$ (Fig. 6A) and 10.25 $\mu M$ (Fig. 6B) Ca$^{2+}$, the observed and simulated rapid phosphorylation levels were 0.6–0.8 and $\sim 1.8$ nmol of EP/mg of protein and 1.2–2.0 (also see Fig. 1B in Ref. 28) and $\sim 2.3$ nmol of EP/mg of protein, respectively. The difference between the phosphorylation levels at 1.16 $\mu M$ Ca$^{2+}$ was more significant than that at 10.25 $\mu M$ Ca$^{2+}$. The $K_{0.5}$ of the observed rapid phosphorylation seems to be higher than that of the simulated phosphorylation. In the SR of bullfrog skeletal muscle at 15 °C (43), it has been observed that when the phosphorylation of the SR is induced by the addition of calcium following preincubation of the SR with ATP in the absence of calcium, the steady-state level of the calcium-induced phosphorylation is lower than the level of the ATP-induced phosphorylation following prolonged preincubation with calcium. In the rabbit SR at 25 °C, the phosphorylation level has been found to be independent of the time of preincubation with calcium (26). These observations suggest that the steady-state level of the calcium-induced phosphorylation is lowered by decreasing the

FIG. 4. EP formation of the membranous ATPase molecules at steady state as a function of calcium concentration at 10 $\mu M$ ATP, pH 7.40, and 0 °C. A, ATP-induced total phosphorylation of the A and B forms (○), calculated rapid phosphorylation of the A form (▲), and calcium-induced slow phosphorylation of the B form (△). The amount of the slowly phosphorylated B form was determined by pre-steady-state analysis of the calcium-induced phosphorylation. The amount of the phosphorylated A form was then calculated by subtracting that of the B form, which was separately measured, from that of the total phosphorylation (see “Results” for details). B, Hill plot of the total (○), rapid (▲), and slow (△) phosphorylation. Y is the ratio of each EP at each calcium concentration to the respective maximum levels of EP (4.9, 2.4, and 2.5 nmol/mg of protein for total, rapid, and slow phosphorylation, respectively).

FIG. 5. Replots of previous data (29) of calcium binding of the membranous ATPase molecules as a function of calcium concentration in the absence of ATP at pH 7.40 and 0 °C. A, calcium binding to the two forms of ATPase molecules. ○, total binding to the A and B forms; ▲, rapid binding to the A form, which is in an $E/I_{A}$ state dependent on pH and which 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 Fig. 2). B, Hill plot of the total (○), rapid (▲), and slow (△) binding. Y is the ratio of calcium binding at each calcium concentration to the maximum levels of binding (10.3, 4.8, and 5.5 nmol/mg of protein for total, rapid, and slow binding, respectively).
reaction temperature. In this study, the reaction of the calcium-induced phosphorylation was initiated by the addition of calcium to the enzyme preparation on the filter, which was prewashed with ATP in the absence of calcium for ~10 s following washing with EGTA for ~10 s at 0 °C, similar to the case of the reaction in the bullfrog SR (43) mentioned above. It is therefore likely that at the low temperature, such a progressive ATP loading on the A form in the absence of calcium lowers the calcium affinity of the form, whereas the B form is not affected by the progressive loading. Thus, the rapid phosphorylation was determined by subtracting the calcium-induced slow phosphorylation from the separately obtained ATP-induced total phosphorylation. The calcium dependence of the calculated rapid phosphorylation (Fig. 4, A and B) exhibited a noncooperative profile with $n_{H} = 1.1$ and $K_{0.5} = 5 \mu M$ and was near that of the rapid calcium binding of the A form in the absence of ATP ($n_{H} = 1$, $K_{0.5} = 2-6 \mu M$) (Fig. 5, A and B). The consistency in the calcium-dependent profiles of phosphorylation and binding in the absence of ATP confirms the above conclusion of the lack of effect of ATP binding to the catalytic site on calcium binding. On the other hand, the phosphorylation reaction of the two forms (induced by ATP after preincubation with calcium) was too fast to be resolved in our experiments, which was manually operated; the amount of the phosphorylated enzyme reached ~80% of its steady-state level within the dead time (~0.2 s) of the experiment (see “Experimental Procedures”) at 0 °C. To understand the phosphorylation reaction of the two forms, the ATP-induced phosphorylation reaction should be analyzed by a method that has a shorter dead time and a higher power of time resolution than those of the method employed here (~0.2 and 1 s, respectively).

In Fig. 7A, the calcium dependence of the total Ca$^{2+}$-ATPase activity of the two forms was examined at 0.25 mM ATP. The calcium-dependent profile of the total ATPase activity was monophasic with $n_{H} = 2.1$ and $K_{0.5} = 0.3 \mu M$ (Fig. 7B). It is thought that in the presence of 0.25 mM ATP, both the A and B forms cooperatively bind two calcium ions with the same calcium affinity and that the calcium-dependent profile of the total ATPase activity is the same as those of the two forms. However, the calcium dependence of the ATP-induced total phosphorylation of the two forms at steady state exhibited a monophasic profile with a lower $n_{H}$ value (~1.6) than the value of the total Ca$^{2+}$-ATPase activity (~2.1), although the $K_{0.5}$ (~0.2 \mu M) was almost the same as that of the ATPase activity (~0.3 \mu M) (data not shown). Phosphorylation reached its maximum level at ~10 \mu M Ca$^{2+}$, whereas the ATPase activity reached its maximum at ~1.9 \mu M Ca$^{2+}$ and decreased at >10 \mu M Ca$^{2+}$. As mentioned above, at 10 \mu M (Figs. 3B and 4B) and 0.1 mM (data not shown) ATP, no significant difference in the Hill plots of the calcium dependence of the ATPase activity and of phosphorylation was observed; for example, at 0.1 mM ATP, the Hill plots for the ATPase activity and phosphorylation were biphasic with slopes of ~1.9 and ~0.9 and of ~1.7 and ~0.7, respectively. In this study, the reactions of the ATPase activity and the ATP-induced phosphorylation were assayed at 25 and 0 °C, respectively. The previous (26, 43) and present (Fig. 6) observations suggest that the steady-state level of the calcium-induced phosphorylation of the A form is lowered by decreasing the reaction temperature, although the phosphorylation of the B form is not sensitive to temperature as described above. Taking these observations into account, it is likely that the level of the ATP-induced phosphorylation of the A form is also lowered by decreasing the temperature at the higher ATP concentration (0.25 mM), but not at the lower ATP concentration (~0.1 mM); the reduction of the phosphorylation level might make the slope ($n_{H}$ value) of the Hill plots of the calcium dependence of the total phosphorylation (~1.6) lower than that of the plots of the calcium dependence of the total ATPase activity (~2.1). Similar to the case at 10 \mu M ATP (28), the two forms (which pre-exist in $E_{1}$ and $E_{2}$, respectively, at pH 7.40 and 0 °C) are predicted to be rapidly (t < 2 s) and slowly (t < 3 s) phosphorylated with 0.25 mM ATP by the addition of calcium (Fig. 2), if the ATP does not significantly affect the equilibrium of the forms between $E_{1}$ and $E_{2}$ before calcium binding. In Fig. 8A, pre-steady-state analysis of the calcium-induced total phosphorylation of the two forms was carried out at 50 \mu M Ca$^{2+}$. In addition to the rapid phosphorylation (~< 2 s), slow phosphorylation at $t_{s} = 2$ s was observed. The observed maximum level of the slow phosphorylation (~2.5 nmol of EP/mg of protein) (Fig. 8A) was close to half of the maximum total phosphorylation (~4.9 nmol of EP/mg of protein) (data not shown), which was separately obtained by analyzing the ATP-induced phosphorylation reaction at 50 \mu M Ca$^{2+}$. The calcium-dependent profile of the slow phosphorylation ($n_{H} = 1.8$, $K_{0.5} = 0.1 \mu M$) (Fig. 9, A and B) was also close to that of the slow calcium binding of the B form in the absence of ATP ($n_{H} = 2$, $K_{0.5} = 0.2 \mu M$) (Fig. 5, A and B). The results suggest that the observed slow phosphorylation is that of the B form. The steady-state level of the calcium-induced rapid phosphorylation was, however, observed to be lower than that expected from the value of the separately obtained total phosphorylation, similar to the case of the rapid phosphorylation at 10 \mu M ATP.
ATP (Fig. 6, A and B). For example, at 1.9 μM Ca²⁺, where the ATPase activity reached its maximum (Fig. 7A), the observed level of the rapid phosphorylation (~0.3 nmol of EP/mg of protein) was much lower than the expected half-maximum level of the total phosphorylation (~2.5 nmol of EP/mg of protein); and even at 50 μM Ca²⁺ (cf. Fig. 8A), the observed rapid phosphorylation level increased only to ~1.6 nmol of EP/mg of protein (data not shown). As with the case at 10 μM ATP described above, it is probable that at a higher ATP concentration of 0.25 mM and 0 °C, the progressive ATP loading on the A form lowers the calcium affinity of the form, whereas the B form is not affected by the ATP loading. Nevertheless, the obtained results suggest that at 0.25 mM ATP, halves of the molecules that can be phosphorylated are slowly and rapidly phosphorylated, respectively. It is thought that 0.25 mM ATP does not significantly change the enzyme state of the A and B forms (which pre-exist in E₂ and E₂⁻, respectively) before calcium binding.

Fig. 10A shows the calcium dependence of the total Ca²⁺-ATPase activity at 5 mM ATP and pH 7.40. The Hill plots of the calcium dependence exhibited a profile with nₜ₁ = 2.0–2.2 and K₀.₅ = 0.04–0.08 μM (Fig. 10B). This profile was almost the same as that previously observed by Möllering et al. (27) at pH 7.5 (nₜ₁ ~ 1.8, K₀.₅ ~ 0.01 μM).

At pH 6.23, the calcium dependence of the ATP-induced total phosphorylation, which is composed of the phosphorylation of the two forms, was examined in the presence of 0.25 mM ATP (Fig. 11A). The calcium dependence of the phosphorylation exhibited a monophasic profile with nₜ₁ ~ 1.3 and K₀.₅ ~ 4 μM (Fig. 11B). Such a monophasic profile of the total Ca²⁺-ATPase activity at 0.25 mM ATP (nₜ₁ ~ 1.5, K₀.₅ ~ 2 μM) was also observed (data not shown). As shown previously (28), at this acidic pH of 6.23, it is thought that the A and B forms are predominantly in E₂ before calcium binding and that at a low temperature of 0 °C, the two forms apparently slowly (t₁/₂ ~ 8 s) bind calcium because of a slow transition of the two forms from E₂ to E₁ (Fig. 2). To examine the effect of 0.25 mM ATP on the enzyme state (E₂) of the two forms before calcium binding, the calcium-induced phosphorylation of the forms was examined at 0 °C (Fig. 5B). The enzyme molecules were slowly and monophasically phosphorylated at t₁/₂ ~ 2 s without the rapid phosphorylation (<2 s) (Fig. 5A), which was observed at pH 7.40. The equilibrium of the two forms between E₂ and E₂⁻ does not seem to significantly change at 0.25 mM ATP. At 5 mM ATP and the acidic pH, the calcium-dependent profile of the total Ca²⁺-ATPase activity at 25 °C was monophasic with nₜ₁ ~ 1.9 and K₀.₅ ~ 0.4–0.8 μM (Fig. 12, A and B).

In a previous study (30), the calcium dependence of calcium binding of the enzyme molecules (solubilized with C₁₂E₈) was examined at 10 μM and 5 mM ATP and pH 7.40. In Fig. 13A, the calcium dependence of the Ca²⁺-ATPase activity of the solubilized molecules was examined at 0.25 mM ATP. Almost all of the Hill plots of the calcium dependence were monophasic with nₜ₁ = 0.8–0.9 and K₀.₅ = 1–2 μM, although a small deviation from the linear line was observed at ~0.35 μM Ca²⁺ (Fig. 13B). Such a monophasic profile of the ATPase activity with nₜ₁ ~ 1 was not observed at 0.1 mM ATP; the profile was almost the
same with $n_H < 1$ (0.5–0.6) and $K_{0.5} \sim 5 \mu$M (data not shown) as that at 10 $\mu$M ATP. The effect of ATP on calcium binding of the solubilized enzyme was not examined at pH 6.23 because of inactivation of the enzyme at this acidic pH.

**DISCUSSION**

Ogawa (24) and Ogawa and Ebashi (25) earlier reported that ATP increases the calcium affinity of the Ca$^{2+}$-ATPase molecule depending on the ATP concentration (0.3 $\mu$M to 2.7 mM). Here, to clarify whether ATP changes calcium binding of the ATPase molecules, we studied the effect of ATP on calcium binding of the enzyme molecules based on a model of two conformational variants (A and B forms) of the molecules (Fig. 1) (28).

The ATPase molecules have been shown to have one catalytic site with a high affinity for ATP ($K_\text{m} = 2–10 \mu$M) (8, 9) and a regulatory site(s) at which ATP binding accelerates the catalytic cycle of the ATPase molecules with lower affinity ($K_\text{m} = 0.1–5.0 \mu$M) (1, 3, 27) for ATP than at the catalytic site. First, the effect of ATP binding to the catalytic site on calcium binding of the A and B forms was examined at 10 $\mu$M ATP and pH 7.40 (Fig. 3A). The Hill plots of the calcium dependence of the total Ca$^{2+}$-ATPase activity were biphasic with slopes of $-1.8$ and $-1.0$ (Fig. 3B). The two lines of the plots intersect near the zero point of the ordinate. The $K_{0.5}$ of the total activity was $-0.3 \mu$M. Such a biphasic profile of the ATPase-induced total phosphorylation (slopes of $-1.9$ and $-0.9$, $K_{0.5} \sim 0.2 \mu$M) was also observed at 10 $\mu$M ATP (Fig. 4). In the absence of ATP at pH 7.40, it has been shown that the A form noncooperatively ($n_H = 1$) binds two calcium ions with $K_{0.5} \sim 2–6 \mu$M, whereas the B form cooperatively ($n_H = 2$) binds the ions with $K_{0.5} \sim 0.2 \mu$M (Fig. 5A; replots of the data in Fig. 3A of Ref. 29). The Hill plots of the calcium dependence of the total calcium binding were biphasic with slopes of $-1.8$ and $-0.8$ (Fig. 5B; replots of the data in Fig. 3C of Ref. 29). The two lines of the Hill plots also intersect near the zero point of the ordinate. The $K_{0.5}$ of the total binding was $-0.4 \mu$M. The observed calcium-dependent profile of the total calcium binding was similar to the profiles of the total ATPase activity and the ATP-induced total phosphorylation. This suggests that there is no effect of ATP binding at the catalytic site on calcium binding of the two forms. This conclusion was confirmed by the observations that the calcium-dependent profiles of the rapid and slow phosphorylation ($n_H = 1$, $K_{0.5} \sim 5 \mu$M and $n_H = 2$, $K_{0.5} \sim 0.08–0.1 \mu$M, respectively) of the A and B forms in the presence of 10 $\mu$M ATP (Fig. 4) were close to those of calcium binding ($n_H = 1$ and $K_{0.5} = 2–6 \mu$M and $n_H = 2$ and $K_{0.5} \sim 0.2 \mu$M, respectively) of the two forms in the absence of ATP, respectively (Fig. 5). The observations also suggest that there is no effect of 10 $\mu$M ATP on the enzymes (E_A and E_B) of the two forms in the absence of ATP before calcium binding. Such an absence of effect of 10 $\mu$M ATP on calcium binding has also been shown for detergent-solubilized ATPase molecules, although the solubilized molecules negatively cooperatively ($n_H = 0.5–0.6$) bind two calcium ions (30). Therefore, ATP binding at the catalytic site does not seem to affect calcium binding of the enzyme molecules, irrespective of the aggregation state of the enzyme molecules.

The affinity ($K_\text{m}$) of the regulatory site(s) of the membranous Ca$^{2+}$-ATPase for ATP has been shown to be 0.1–5.0 $\mu$M for ATP (1, 3, 27). Here, the effect of ATP on calcium binding of the two forms was studied at 0.25 and 5 mM ATP. The calcium dependence of the total ATPase activity at 0.25 mM ATP and pH 7.40 exhibited a monophasic profile with $n_H \sim 2.1$ and $K_{0.5} \sim 0.3 \mu$M (Fig. 7). As mentioned under “Results,” it was found that at 0.25 mM ATP and 0 °C, the rapid phosphorylation of the A form and the total phosphorylation of the two forms were not resolved by the pre-steady-state and steady-state analyses of phosphorylation, respectively, whereas the slow phosphorylation of the B form was fully resolved by the pre-steady-state analysis. The calcium dependence of the slow phosphorylation of the B form ($n_H \sim 2.0$, $K_{0.5} \sim 0.1 \mu$M) (Fig. 9) exhibited almost the same profile as that of the total ATPase activity ($n_H \sim 2.1$, $K_{0.5} \sim 0.3 \mu$M) (Fig. 7). Therefore, the results suggest that the calcium-dependent profile of the ATPase activity of the A form is also cooperative and is close to that of the activity of the B form. This indicates that the cooperative profile of the A form at 0.25 mM ATP is different from the noncooperative profile of calcium binding of the A form in the absence of ATP ($n_H \sim 1$, $K_{0.5} \sim 2–6 \mu$M) (Fig. 5) and that the cooperative calcium-dependent profile ($n_H \sim 1.8$, $K_{0.5} \sim 0.1 \mu$M) of the phosphorylation of the B form at 0.25 mM ATP is near that ($n_H \sim 2.0$, $K_{0.5} \sim 0.2 \mu$M) of calcium binding of the B form in the absence of ATP (Fig. 5). It has been shown that ATP binding to the catalytic site at 0.1 mM ATP accelerates the catalytic reaction of the enzyme molecules (40, 43). Therefore, to examine whether the observed regulatory effect of 0.25 mM ATP results from ATP binding to the regulatory site or to the catalytic site, the calcium dependence of the total ATPase activity and the total phosphorylation was examined at 0.1 mM ATP. The calcium dependence exhibited almost the same biphasic profile (slopes of $-1.9$ and $-0.9$ and $K_{0.5} \sim 0.12 \mu$M for the ATPase activity and slopes of $-1.7$ and $-0.7$ and $K_{0.5} \sim 0.24 \mu$M for the phosphorylation) (data not shown) as that at 10 $\mu$M ATP (Figs. 3 and 4); it was distinct from the monophasic profile (Fig. 7) of the ATPase activity at 0.25 mM ATP. Similar to the case at 10 $\mu$M ATP, 0.1 mM ATP did not seem to affect calcium binding of the enzyme molecules. It is therefore thought that at 0.25 mM ATP, ATP binding to the regulatory site (but not to the catalytic site) changes the calcium binding manner of the A form from noncooperative to cooperative, concurrent with a decrease in the $K_{0.5}$ of 2–6 to 0.1–0.3 $\mu$M, whereas ATP binding does not significantly affect the cooperative binding of the B form. These observations show the existence of two pools of enzyme molecules, the calcium binding of which differs in sensitivity to 0.25 mM ATP, further supporting the model of two different conformations of the molecules (28).

At pH 6.23, the calcium dependence of the ATP-induced total phosphorylation at 0.25 mM ATP exhibited a monophasic profile with $n_H \sim 1.3$ and $K_{0.5} \sim 4 \mu$M (Fig. 11). The calcium-dependent profile of the total Ca$^{2+}$-ATPase activity at 0.25 mM ATP was also monophasic ($n_H \sim 1.5$, $K_{0.5} \sim 2 \mu$M) (data not shown) and was the same as that of the total phosphorylation. As reported previously (29), at the acidic pH of 6.23, it is
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**Fig. 13.** Ca\(^{2+}\)-ATPase activity of the detergent-solubilized ATPase molecules as a function of calcium concentration at 0.25 mM ATP and pH 7.40. The ATPase activity was assayed in 20 mM PIPES buffer solution (pH 7.40) containing 0.01 mg/ml enzyme protein, 2.0 mg/ml C\(_{12}\)E\(_{6}\), 0.12 mM KCl, 5 mM MgCl\(_2\), 0.25 mM ATP, and 0.052–442 mM Ca\(^{2+}\). The enzyme was preincubated in the medium for 2 min before the addition of ATP. The reaction time was 1 min. A, calcium dependence of the total ATPase activity, which consists of the activities of the two forms; B, Hill plot of the total activity. Y is the ratio of the activity at each calcium concentration to the maximum level of the activity (4.69 \(\mu\)mol of P/mg of protein/min).

![Graph A](image1.png)

**Fig. 14.** Replots of previous data (29) of calcium binding of the membranous ATPase molecules as a function of calcium concentration in the absence of ATP at pH 6.23 and 0 °C. A, total calcium binding to the two forms of ATPase molecules. Calcium binding to the A form (\(\times\)), which is in an E\(_1\)/E\(_0\) state dependent on pH and which pre-exists in E\(_0\) at this acidic pH, was obtained by subtracting the binding to the B form (\(––\)), which pre-exists in E\(_0\) independent of pH, from the total binding (\(\bigcirc\)). Calcium binding to the B form was simulated using parameters of \(n_{H1} = 2.0\) and \(K_{0.5} = 5.3 \mu\)M, obtained on the basis of observations of the calcium-dependent change in fluorescence intensity of the molecules and a maximum binding capacity of half (4.9 nmol/mg of protein) of the capacity of the observed total binding (9.8 nmol/mg of protein) (cf. “Discussion” in Ref. 29). B, Hill plot of the total binding. Y is the ratio of calcium binding at each calcium concentration to the maximum level of binding (9.8 nmol/mg of protein).

thought that the A form noncooperatively (\(n_H = 1\)) binds calcium ions with \(K_{0.5} = 2–6 \mu\)M, whereas the B form cooperatively (\(n_H = 2\)) binds calcium with almost the same \(K_{0.5}\) value (\(7 \mu\)M) as that of the A form (Fig. 14A; replots of the data in Fig. 1A of Ref. 29). The calcium dependence of the total binding profile is monophasic with \(n_H = 1.3\) and \(K_{0.5} = 6 \mu\)M (Fig. 14B; replots of the data in Fig. 1C of Ref. 29). The calcium-dependent profile of the total binding at the acidic pH was almost the same as the profiles of the total phosphorylation and the total ATPase activity. Therefore, in contrast to the above-mentioned case at alkaline pH, submillimolar ATP does not seem to affect calcium binding of the two forms at acidic pH.

The solubilized form of the enzyme molecules has also been shown to have a regulatory site (\(K_m = 0.1 \text{ mM for ATP}\)) in addition to the catalytic site with \(K_m = 7 \mu\)M (27). Most of the Hill plots of the calcium dependence of the Ca\(^{2+}\)-ATPase activity of the solubilized molecules at 0.25 mM ATP exhibited a noncooperative profile with \(n_H = 0.8–0.9\) and \(K_{0.5} = 1–2 \mu\)M (Fig. 13). It was found earlier (30) that in the presence of 10 \(\mu\)M ATP, the solubilized molecules are predominantly in E\(_0\) before binding calcium and that the molecules negatively cooperatively (\(n_H = 0.5\)) bind calcium ions with \(K_{0.5} = 3–5 \mu\)M, similar to the case in the absence of ATP. In the present study, the calcium-dependent profile of the ATPase activity at 0.1 mM ATP (\(n_H = 0.5–0.6, K_{0.5} = 5 \mu\)M) (data not shown) was found to be close to that at 10 \(\mu\)M ATP. These results show that at 0.25 mM ATP, ATP binding to the regulatory site of the solubilized molecules changes the calcium binding manner of the molecules from negatively cooperative to noncooperative, although it does not significantly change the calcium affinity of the molecules (\(K_{0.5} = 3–5 \mu\)M). It is therefore thought that the regulatory site with submillimolar affinity for ATP belongs to the solubilized molecules, whereas in the SR membrane, it belongs only to the A form as a result of intermolecular interaction of the molecules.

At 5 mM ATP and pH 7.40, the calcium dependence of the total ATPase activity of the two forms exhibited a monophasic profile with \(n_H = 2.0–2.2\) and \(K_{0.5} = 0.04–0.08 \mu\)M (Fig. 10). The \(K_{0.5}\) was lower than that of the ATPase activity at 0.25 mM ATP (Fig. 7), although both calcium-dependent profiles of the activities at 0.25 and 5 mM ATP were positively cooperative with the same \(n_H\) value (2). It is thought that 5 mM ATP further decreases the \(K_{0.5}\) of the A form, which is already decreased by 0.25 mM ATP, and that it also decreases the \(K_{0.5}\) of the B form, which is not affected by 0.25 mM ATP, to the same level as the further decreased \(K_{0.5}\) of the A form. At an acidic pH of 6.23, the calcium-dependent profile of the total ATPase at 5 mM ATP was monophasic with \(n_H = 1.9\) and \(K_{0.5} = 0.4–0.8 \mu\)M (Fig. 12); the \(K_{0.5}\) was smaller than the \(K_{0.5}\) values of the ATPase activity at 0.25 mM ATP and calcium binding (29) in the absence of ATP (2–6 \(\mu\)M). These results at pH 6.23 and 7.40 suggest that the A and B forms equally and positively cooperatively bind two calcium ions at 5 mM ATP, irrespective of pH, although the calcium affinities of the forms at the acidic and alkaline pH values are different. As for the solubilized enzyme molecules, the previous (30) and present (Fig. 13) studies show that the calcium-dependent profile of calcium binding in the presence and absence of 10 \(\mu\)M to 0.1 mM ATP is negatively cooperative (\(n_H = 0.5–0.6\) with \(K_{0.5} = 3–5 \mu\)M (30), that the calcium-dependent profile of the Ca\(^{2+}\)-ATPase activity at 0.25 mM ATP is noncooperative (\(n_H = 1\)) with \(K_{0.5} = 1–2 \mu\)M, and that the profile of the ATPase activity at 5 mM ATP is positively cooperative (\(n_H = 2\)) with \(K_{0.5} = 0.1 \mu\)M. Based on these data, it is thought that 5 mM ATP further changes the noncooperative binding manner of the solubilized enzyme molecules (which have already been converted by 0.25 mM ATP from the negatively cooperative manner without any significant change in
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(ii) This site is the locus of the catalytic site (21–23). (iii) The catalytic site belongs to half of the molecules (44). Recently, Toyoshima et al. (15) distinctly showed that the enzyme molecule has one nucleotide-binding site. It is therefore probable that the regulatory site is a manifestation of the catalytic site. In a previous study (30) as well as the present one, it was observed that ATP regulation also exists in the presence of C\textsubscript{12}E\textsubscript{8}, which produces the enzyme monomer (45), and that detergent-solubilized molecules exist in E\textsubscript{2} before calcium binding. It is therefore thought that the regulatory site belongs to the monomeric form of the ATPase molecule in an alternate enzyme state of E\textsubscript{2} in the catalytic cycle and that the fundamentals of ATP regulation reside in the monomeric molecule. However, it is difficult to understand the coexistence of two types of regulatory site (as observed here) on one enzyme molecule. The E\textsubscript{2} state may be in equilibrium between two different E\textsubscript{2} states that have high and low affinity for ATP, respectively. Based on previous (29, 30) and present (Figs. 1 and 2) data on the enzyme molecules regarding their enzyme states (E\textsubscript{1} or E\textsubscript{2}) before calcium binding, calcium affinities, and manners of calcium binding under various conditions of ATP concentrations and pH, the reaction sequence of ATP regulation can be therefore described as follows. Here, for simplicity, the ATPase conformers that negatively cooperatively (n\textsubscript{H} < 1), noncooperatively (n\textsubscript{H} = 1), and positively cooperatively (n\textsubscript{H} > 1) bind calcium ions are termed a, A, and B, respectively.

![Image of Schematic representation of the ATP effect on calcium binding](http://www.jbc.org/)

**Fig. 15.** Schematic representation of the ATP effect on calcium binding of the membranous Ca\textsuperscript{2+}-ATPase molecules at pH 7.40 (A) or 6.23 (B) and of the detergent-solubilized molecules at pH 7.40 (C). The scheme below each panel shows the enzyme state (E\textsubscript{1} or E\textsubscript{2}) of the ATPase molecules before and after the addition of calcium and the association state of the molecules under the different conditions of ATP concentrations and pH (see “Discussion” for details). For simplicity, the ATPase conformers that negatively cooperatively (n\textsubscript{H} < 1), noncooperatively (n\textsubscript{H} = 1), and positively cooperatively (n\textsubscript{H} > 1) bind calcium ions are termed a, A, and B, respectively.

Calcium affinity (from K\textsubscript{0.5} = 3–5 to 1–2 μM) to positively cooperative, concurrent with a 10–20-fold decrease in the K\textsubscript{0.5} from 1–2 to ∼0.1 μM (30). In contrast to the regulation site with submillimolar affinity for ATP described above, the regulatory site with millimolar affinity for ATP is thought to belong to the enzyme molecules, irrespective of pH and of the aggregation state of the molecules.

The results obtained here confirm the earlier reports by Ogawa (24) and Ogawa and Ebashi (25) that ATP increases the calcium affinity of the Ca\textsuperscript{2+}-ATPase molecule depending on the ATP concentration. Data on ATP regulation of calcium binding of the membranous and solubilized ATPase molecules are schematically represented in Fig. 15.

The data shown in Fig. 15 suggest the existence of two types of regulatory site of the Ca\textsuperscript{2+}-ATPase molecules; ATP binding to these sites improves the calcium binding performance of the ATPase molecules at 0.25 and 5 mM ATP. The regulatory sites for calcium binding in a and A correspond to the regulatory sites (1, 3, 6, 7) of the enzyme molecules; ATP binding to these sites also accelerates the ATP hydrolysis cycle of the molecules at concentrations of 0.1–5 mM (1, 3, 27). The following observations have been reported for the molecular basis of the regulatory site for ATP hydrolysis. (i) The regulatory site coexists with the catalytic site in the same enzyme molecule (16–20).
In the solubilized molecules at pH 7.40 (Fig. 15C), the A form is in equilibrium between $E_2$ and $A(E_2)$; the equilibrium shifts to the $E_2$ side before calcium binding (Scheme I, part C); and the A form negatively cooperatively ($n_H < 1$) binds calcium ions. At submillimolar ATP, ATP binds to the A form in $E_2$, $A(E_2)$, before calcium binding and converts it to $A^*E_2$; in the presence of calcium, the A form noncooperatively ($n_H - 1$) binds calcium ions with almost the same $K_{0.5}$ (1–2 $\mu$M) as that of a form in the absence of ATP (3–5 $\mu$M). $A^*E_2$ is in equilibrium with $A^*E_2$ before calcium binding, and the equilibrium shifts to the $A^*E_2$ side. At millimolar ATP, ATP that is bound to $A^*E_2$ converts it to $B^*E_2$ and accelerates the transition of $B^*E_2$ to $E_1$, $E_{(Ca^2+)}$, via $E_1$ with the addition of calcium; millimolar ATP further converts the binding manner of the molecules from noncooperative ($n_H - 1$) to positively cooperative ($n_H = 2$), accompanying a decrease in the $K_{0.5}$ (1–2 $\mu$M) to ~0.1 $\mu$M. As mentioned above, ATP is presumed to work as a kinetic and conformational effector in regulating calcium binding. As shown in Scheme I (parts A and B), the present data suggest the existence of heterologous conformational interactions of the membranous $Ca^{2+}$-ATPase conformers in their ATP regulation reactions. Hobbs et al. (46) found such heterologous conformational interactions in the catalytic cycle in the Na$^+$,K$^+$-ATPase molecules.

In the monomeric pathway model described above, the ATPase molecules in $E_1$ and $E_2$, which are the alternate enzyme states in the catalytic cycle (1–3, 5), are dealt with in the discussion of ATP regulation, and the putative regulatory site is allotted to the monomeric molecule in $E_2$. Yokoyama et al. (47) have recently suggested the existence, in the Na$^+$,K$^+$-ATPase, of ATP binding to the $a$-subunit, which is not involved in the catalytic phosphorylation-dephosphorylation reaction, in addition to the binding that is involved in the catalytic reaction. It has been observed in the $Ca^{2+}$-ATPase that the ATPase molecules phosphorylated with ATP or $P_i$ are only half of the total enzyme molecules in the SR membrane (44); it is not known whether half of the total molecules are phosphorylated with ATP or $P_i$. However, based on the above-mentioned analogy of the Na$^+$,K$^+$-ATPase molecules, the $Ca^{2+}$-ATPase molecules might also be composed of catalytic and non-catalytic molecules. ATP sites for the phosphorylation and ATP regulation of calcium binding might possibly belong to the two different $Ca^{2+}$-ATPase molecules (catalytic and regulatory molecules) that are involved and not involved in the catalytic reaction, respectively. The ATP regulation pathway can therefore also be described based on the following oligomeric model.

The two types of regulatory site with submillimolar and millimolar affinities for ATP, respectively, reside in the two different states of the regulatory molecule, which are in equilibrium depending on pH, as discussed above. The regulatory molecule is adjacent to the catalytic molecule in an oligomer of the ATPase molecules. ATP binding to the regulatory subunit indirectly improves the calcium binding performance of the catalytic subunit. Similar to the case in the monomeric pathway model, the catalytic membranous molecules associate to form a stable pair of molecules in $E_1$ and/or $E_2$ in the reaction sequence of ATP regulation. It is therefore predicted that a trimmer of two catalytic subunits (which are in $E_1$ and/or $E_2$) and one regulatory subunit (which is in a high or low affinity state for ATP) is formed. In the regulation model, a specific oligomerization of the catalytic and regulatory subunits is required to produce ATP regulation of the catalytic subunit. However, ATP regulation at submillimolar and millimolar ATP was also observed in the detergent-solubilized enzyme molecules (Fig. 15C). Such a specific intermolecular interaction of the molecules does not seem to occur in the solubilized molecules because they have been shown to be in a monomeric state (45). The oligomeric pathway model is therefore unlikely to be suitable to explain the results obtained. However, the data (summarized in Fig. 15) show that at pH 7.40, the regulatory effect of submillimolar ATP was relatively lowered by the solubilization of the membranous enzyme molecules with detergent. Submillimolar ATP changed the calcium binding manner of the membranous A form in the absence of ATP from noncooperative to positively cooperative, whereas it also changed the manner of the solubilized molecules in the absence of ATP from noncooperative to cooperative. As for the affinity of the enzyme molecules for calcium, ATP decreased the $K_{0.5}$ of the membranous A form in the absence of ATP from a micromolar ($2–6 \mu$M) to a submicromolar ($0.1–0.3 \mu$M) level, although it scarcely decreased the $K_{0.5}$ of the solubilized molecules in the absence of ATP (from 3–5 to 1–2 $\mu$M). The cooperative binding of the membranous B form ($n_H = 2$, $K_{0.5} = 0.2 \mu$M) was not affected by ATP. These observations regarding the membranous A form and the solubilized molecules suggest that the expression of the regulatory effect of submillimolar ATP is related to the intermolecular interaction of the enzyme molecules; the solubilized molecules might interact with each other in the presence of the detergent, although the interaction is much weakened by the solubilization. In the Na$^+$,K$^+$-ATPase, which is composed of an $a$-subunit (catalytic) and a $\beta$-subunit (non-catalytic), even after the solubilization of the ATPase sub-
units with detergent, αβ-protemers of the subunits have been observed to associate to form a diprotomer ((αβ)p) and a higher oligomer accompanying transition of the enzyme state of the Na+-K+-ATPase from E1 to E2 (48). Re-examination of the possibility of the interaction of the solubilized Ca2+-ATPase molecules on the basis of the data obtained here is worthwhile.

For the sequence of the catalytic reaction of the Ca2+-ATPase molecules, Froehlich and Taylor (49) and Ikemoto et al. (50) have proposed an alternating or flip-flop model of the ATPase molecules in an enzyme oligomer (dimer or 2×-n-mer); half of the enzyme molecules are one step ahead of the other in carrying out the same sequential steps in the reaction of ATP hydrolysis. We previously proposed a model in which the A and B forms of the enzyme molecules independently carry out their reaction sequences of acetyl phosphate hydrolysis without change in their calcium binding performances (51). In the monomeric pathway model of ATP regulation that is described above, the data are discussed according to the alternating model of the two types of ATPase conformer, one of which converts to the other in the presence of ATP at concentrations higher than those required for saturation of the catalytic site. However, further study is required to understand the whole sequence of the ATP hydrolysis reaction of the ATPase molecules.

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