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The Binding of ATP to the Catalytic and the Regulatory Site of Ca^{2+},Mg^{2+}-Dependent ATPase of the Sarcoplasmic Reticulum

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Abstract

Two kinds of ATP binding sites were found on the ATPase molecule in deoxycholic acid-treated sarcoplasmic reticulum. One was the catalytic site (1 mol/mol active site) and its affinity was high. Upon addition of Ca^{2+}, all the ATP bound to the catalytic site disappeared at 75 mM KCl, while a significant amount of ATP remained bound to the site at 0–2 mM KCl. The latter binding was found to be due to the formation of a slowly exchanging enzyme–ATP complex, which is in equilibrium with phosphoenzyme + ADP. The other binding site was the regulatory one (1 mol/mol active site) and its affinity was low, changing only insignificantly upon addition of Ca^{2+}. The ATP binding to the regulatory site shifted the equilibrium between the slowly exchanging complex and EP toward EP.

Key Words: Sarcoplasmic reticulum; Ca^{2+},Mg^{2+}-dependent ATPase.

Introduction

The reaction mechanism of Ca^{2+},Mg^{2+}-dependent ATPase of SR[2] has been studied in detail mainly by kinetic methods (Tonomura, 1972; Tada *et al.*, 1978; Yamamoto *et al.*, 1979; de Meis and Vianna, 1979; Inesi, 1979), and only a few studies have been performed on the ATP binding to the ATPase. Meissner (1973) and Yates and Duance (1976) measured the ATP binding in the absence of Ca^{2+} by Millipore filtration and rapid flow-dialysis, respectively, and found that the dissociation constant for ATP binding decreases with increase in the pH value. Dupont (1977) observed two classes of ATP binding, namely at high- and low-affinity sites, in the absence of Ca^{2+}. However, there has been no reliable report on the ATP binding during ATP

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[2]SR, sarcoplasmic reticulum.

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hydrolysis. Therefore, we measured the ATP binding to the enzyme both in the presence and absence of Ca^{2+} using the double-membrane filtration method devised by Yamaguchi and Tonomura (1979).

We found that in the absence of Ca^{2+}, two classes of ATP binding sites exist on the ATPase, one being the catalytic site with high affinity and the other being the regulatory site with low affinity, as already reported by Dupont (1977). Upon addition of Ca^{2+}, EP[3] was formed and the amount of ATP bound to the catalytic site decreased markedly, while the amount of ATP bound to the regulatory site changed insignificantly. At low KCl concentrations, we observed a considerable amount of ATP bound to the catalytic site during the ATPase reaction. The ATP binding was due to formation of a slowly exchanging enzyme–ATP complex, which was in equilibrium with EP + ADP. The equilibrium was shifted to the EP side by the ATP binding to the regulatory site.

Experimental Procedure

Ca^{2+},Mg^{2+}-dependent ATPase was purified from deoxycholic acid-treated sarcoplasmic reticulum by a method described previously (Nakamura and Tonomura, 1982). The amount of active site of the enzyme was estimated to be 6 μmol/g by measuring the maximal amount of EP in 100 μM ATP, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 100 mM KCl, and 100 mM Tris-HCl at pH 8.8 and 0°C. The desalted enzyme was prepared by column chromatography on Sephadex G-50 as described by Shigekawa *et al.* (1978). The desalted enzyme was unstable, and its maximal amount of EP was 3–5 μmol/g. [α-^{32}P]ATP and [^{3}H]glucose were purchased from the Radiochemical Centre, Amersham, England, and New England Nuclear, Boston, Massachusetts, respectively. [γ-^{32}P]ATP was prepared enzymatically by the method of Glynn and Chappell (1964). CK[4] was prepared by the method of Noda *et al.* (1955).

The amount of ATP bound to the enzyme was measured by the double membrane filtration method (Nakamura and Tonomura, 1982), using [α-^{32}P]ATP as a substrate. The reaction was started by mixing 20 μl of the enzyme solution with 80 μl of the ATP solution containing 20 mM CP,[5] 2 mg/ml CK, 1 mM $CaCl_2$ (or 1 mM EGTA[6]), 20 mM $MgCl_2$, 100 mM [^{3}H]glucose, 75 mM KCl, 10% glycerol, and 75 mM Tris-HCl (final concentrations) at pH 8.8 and 0°C. Within 2 min after the start of the reaction, 30-μl portions of the reaction mixture were withdrawn and applied to

[3]EP, phosphorylated intermediate.
[4]CK, creatine kinase.
[5]CP, creatine phosphate.
[6]EGTA, glycoletherdiamine-*N*, *N*, *N'*, *N'*-tetraacetic acid.

a set of two membrane filters (upper, Amicon Diaflo XM-100 A or Millipore filter, pore size 0.3 μm; lower, Millipore filter, pore size 0.8 μm: ϕ = 10 mm each), and suction was applied for 5–15 sec and 0.5–1 sec when XM-100 A and 0.3-μm Millipore were used as the upper filter, respectively. The experiments were carried out in a cold room at 2°C. The lower filter was solubilized with 1 ml of dimethylformamide in a counting vial. After 1 hr, 12 ml of xylene scintillation cocktail was added and the radioactivities were measured. The concentration of ATP in the filtrate was calculated from the ratio of radioactivities ($^{32}P/^{3}H$), and the amount of bound ATP was determined from the difference between the ATP concentrations in the filtrates with and without the enzyme in the reaction mixture.

The amounts of $E^{32}P$[7] and [γ-^{32}P]ATP were measured as follows. The reaction was started by adding 20 μl of [γ-^{32}P]ATP to 180 μl of a solution containing the enzyme and the reagents indicated. For chasing the reaction, 20 μl of a chasing solution, such as ADP, CP, or unlabeled ATP, was added. The reaction was stopped by the addition of 1.8 ml of 5% trichloroacetic acid, and the solution was centrifuged at 2,000 $\times$ g for 20 min. The supernatant was used for the assay of [γ-^{32}P]ATP, and the precipitate was used for the assay of $E^{32}P$ by methods described previously (Nakamura and Tonomura, 1982).

Results

Binding of ATP at 75 mM KCl

The dependence of the amount of ATP bound to the enzyme on the ATP concentration was measured in the presence of 20 mM $MgCl_2$ and 75 mM KCl at pH 8.8 and 0°C (Fig. 1). In the presence of 1 mM EGTA the amount of bound ATP was 1.6 mol/mol of active site at 0.4 mM ATP, and the dependence on the ATP concentration could not be explained by simple binding of ATP to the enzyme. The dependence could be explained by assuming that 1 mol of ATP bound to a high-affinity site per mol of active site with a dissociation constant of 7 μM and another 1 mol of ATP bound to a low-affinity site with a dissociation constant of 80–300 μM. The curves shown in the figure were obtained by assuming that the dissociation constants of the low-affinity binding were 80 and 300 μM, respectively. In the presence of 1 mM $CaCl_2$ the high-affinity binding disappeared and only the low-affinity binding was observed. The curves shown in the figure were calculated by assuming that the dissociation constants were 150 and 400 μM, respectively. Thus, the affinity of ATP binding to the low-affinity site changed insignificantly when the enzyme was phosphorylated. In the presence of $CaCl_2$, no

[7] $E^{32}P$, ^{32}P-labeled EP.

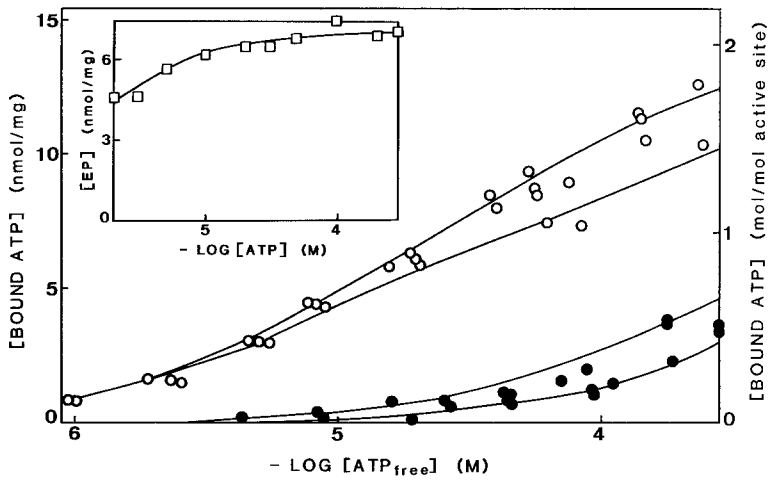

Fig. 1. Dependence of the amount of bound ATP on the concentration of ATP in the presence and absence of Ca^{2+} at 75 mM KCl. The amount of [α-^{32}P]ATP bound to the enzyme (5 mg/ml) was measured in the presence of 1 mM EGTA (○) or 1 mM $CaCl_2$ (●), 2 mg/ml CK, 20 mM CP, 20 mM $MgCl_2$, 75 mM KCl, 100 mM [^{3}H]glucose, 10% glycerol, and 75 mM Tris-HCl at pH 8.8 and 0°C. The curves were obtained by calculation using the equation

$$\frac{[\text{bound ATP}]}{\epsilon} = \frac{1}{1 + K_1/[\text{ATP}]} + \frac{1}{1 + K_2/[\text{ATP}]}$$

and the constants $K_1 = 7\ \mu M$, $K_2 = 80$ or $300\ \mu M$ (○) and $K_1 = \infty$, $K_2 = 150$ or $400\ \mu M$ (●). The inset shows the dependence of the amount of EP on the concentration of ATP. The enzyme (0.1 or 1.0 mg/ml) was phosphorylated with [γ-^{32}P]ATP in the solution containing 1 mM $CaCl_2$ and the same other reagents as the ATP-binding measurements.

ATP binding to the high-affinity site was observed also at pH 8.8 and 20°C or at pH 6.0 or 7.0 and 0°C (data not shown). In the inset of the figure, the amount of EP is plotted against the ATP concentration. The amount of EP was saturated at about 10 μM ATP, and the ATP concentration for half saturation of the EP level was less than 1 μM. The latter value was much smaller than the dissociation constant of ATP to the high-affinity site in the absence of Ca^{2+}, i.e., 7 μM.

The amounts of bound ATP were measured in the presence of 30 μM ATP and 20 mM $MgCl_2$ at various concentrations of free Ca^{2+}. In the presence of an excess amount of EGTA, the amount of bound ATP was about 4 μmol/g. On the other hand, at free Ca^{2+} concentrations of 1 μM to 5 mM, i.e., 50 μM EGTA and 58 μM to 5.05 mM $CaCl_2$, no ATP binding was observed (data not shown). Shigekawa and Dougherty (1978b) and Takisawa and Tonomura (1979) reported that two kinds of EP are formed sequentially, namely ADP-sensitive EP and ADP-insensitive EP, and that the equilibrium between these two kinds of EP shifts to the former with Ca^{2+} and to the latter

with Mg^{2+}. We (Nakamura and Tonomura, 1982) previously found that most of the EP formed was ADP-sensitive in the presence of 5 mM free Ca^{2+} and 20 mM $MgCl_2$, but ADP-insensitive in the presence of 1 μM free Ca^{2+} and 20 mM $MgCl_2$ at pH 8.8. Thus, the enzyme binds no ATP in the presence of more than 1 μM free Ca^{2+} whether the EP formed is ADP-sensitive or ADP-insensitive at 75 mM KCl.

Binding of ATP at 0–2 mM KCl

Figure 2 shows the dependence of the amount of ATP bound to the enzyme (0.5 mg/ml) on the ATP concentration in the presence of 1 mM $MgCl_2$, 20 mM CP, and 2 mM KCl at pH 7.0. In the presence of 2 mM EGTA the amount of bound ATP was essentially equal to that observed in the presence of 75 mM KCl at pH 8.8 (see Fig. 1). Therefore, we assumed that in 2 mM KCl, there are two classes of ATP binding sites as in 75 mM KCl. The profile of ATP binding agreed with the curve obtained by assuming that there is a high-affinity site with a dissociation constant of 8 μM and a low-affinity site with a dissociation constant of 160 μM. In the presence of 5 mM $CaCl_2$ the amount of bound ATP decreased, as was observed in the presence of 75 mM

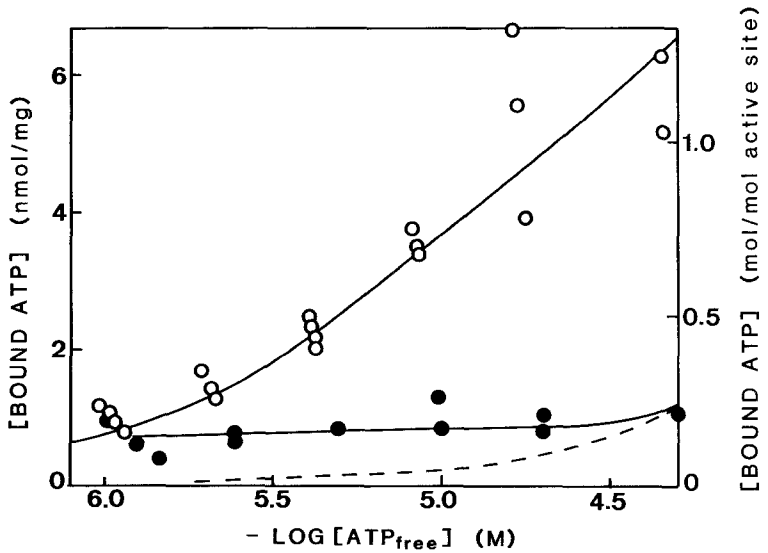

Fig. 2. Dependence of the amount of bound ATP on the concentration of ATP in the presence and absence of Ca^{2+} at 2 mM KCl. The amount of ATP bound to the enzyme (0.5 mg/ml) was measured in the presence of 2 mM EGTA (○) or 5 mM $CaCl_2$ (●), 20 mM CP, 1 mM $MgCl_2$, 2 mM KCl, 100 mM [^{3}H]glucose, and 60 mM imidazole-HCl, at pH 7.0 and 0°C. The curves were obtained by calculation using the equation given in Fig. 1 and the constants $K_1 = 8$ μM, $K_2 = 160$ μM (○) and $K_1 = \infty$, $K_2 = 160$ μM (●).

KCl. However, the amount of bound ATP was apparently larger than the value obtained by assuming that only the low-affinity binding remained with the dissociation constant of 160 μM (broken line in the figure). This finding indicates that a significant amount of ATP remains bound to the high-affinity site during the ATPase reaction at 0–2 mM KCl.

Effect of ADP on EP Level in the Absence of KCl

Figure 3A shows the decrease in the amount of EP after addition of ADP. The desalted enzyme (0.5 mg/ml) was phosphorylated with 5 μM [γ-^{32}P]ATP in the presence of 1 mg/ml CK, 6 mM $CaCl_2$, 1 mM EGTA, and 1 mM $MgCl_2$. Under the conditions used, the rate of P_i liberation was very low (less than 0.2 μmol/g · min). When ADP at 9.1, 46, or 91 μM was added to $E^{32}P$ 20 sec after the start of the reaction, the amount of $E^{32}P$ decreased from 3.4 to 1.8, 0.65, or 0.45 μmol/g, respectively. When 20 mM CP instead of ADP was added to remove ADP from the reaction mixture, the amount of $E^{32}P$ increased to 5.0 μmol/g. The change in the total amount of [γ-^{32}P]ATP was

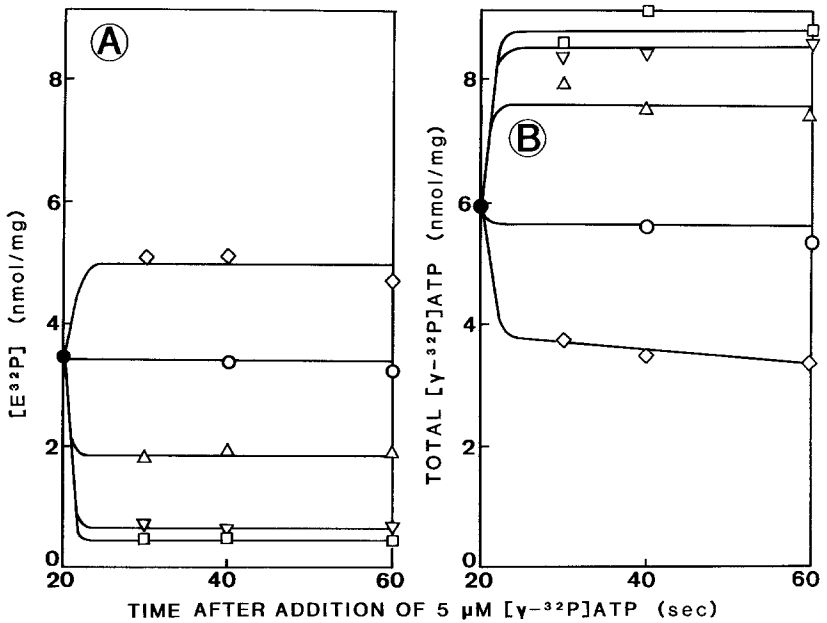

Fig. 3. Changes in the amount of $E^{32}P$ and [γ-^{32}P]ATP after addition of ADP. The desalted enzyme (0.5 mg/ml) was phorphorylated with 5 μM [γ-^{32}P]ATP in the presence of 1 mg/ml CK, 6 mM $CaCl_2$, 1 mM EGTA, 1 mM $MgCl_2$, 20 mM glucose, and 20 mM imidazole-HCl at pH 7.0 and 0°C. At 20 sec, 0 (○), 9.1 (△), 46 (▽), or 91 μM ADP (□) or 20 mM CP (◇) were added, and the changes in the amount of $E^{32}P$ (A) and the total amount of [γ-^{32}P]ATP (B) were measured.

measured simultaneously with the change in the amount of $E^{32}P$ (Fig. 3B). The total amount of [γ-^{32}P]ATP increased from 5.9 to 7.5, 8.4, or 8.7 μmol/g when ADP at 9.1, 46, or 91 μM was added, respectively. Thus, the increase in the [γ-^{32}P]ATP was almost equal to the decrease in the $E^{32}P$. The total amount of [γ-^{32}P]ATP decreased from 5.9 to about 3.5 μmol/g when 20 mM CP instead of ADP was added. The decrease in [γ-^{32}P]ATP was slightly larger than the increase in $E^{32}P$. This might be due to the CK-catalyzed phosphate-exchange reaction between ATP and CP. The results shown in Fig. 3 indicate that most of the EP formed was ADP-sensitive and that the concentration of ADP necessary to convert EP into an enzyme–ATP complex was several tens of micromolar under the conditions used.

In the experiments shown in Fig. 4, the reaction was started by the addition of 5 μM [γ-^{32}P]ATP to the enzyme solution containing 0.5 mg/ml desalted enzyme, 5 mg/ml CK, 6 mM $CaCl_2$, 1 mM EGTA, and 1 mM $MgCl_2$. At 20 sec after the start of the reaction, 46 μM ADP was added. The amount of $E^{32}P$ decreased from 2.0 to 0.7 μmol/g and then remained constant, as already seen in Fig. 3. When 20 mM CP was added to remove ADP from the reaction mixture, the amount of $E^{32}P$ increased rapidly and then decreased very slowly. The amount of $E^{32}P$ increased from 0.7 to 3.3 μmol/g with the addition of CP at 25 sec, then decreased gradually with an increase in the time interval between the ADP addition and the CP addition. It was 1.9 μmol/g with the addition of CP at 40 sec.

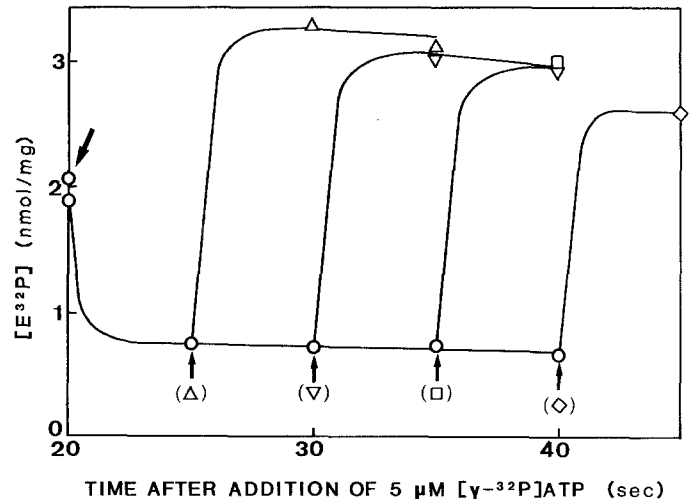

Fig. 4. Effects of ADP and CP on the amount of $E^{32}P$. The desalted enzyme (0.5 mg/ml) was phosphorylated with 5 μM [γ-^{32}P]ATP in the presence of 5 mg/ml CK under the conditions given in Fig. 3. At 20 sec, 46 μM ADP was added (○), and then 20 mM CP was added at 25 (△), 30 (▽), 35 (□), and 40 sec (◇).

Effects of a High Concentration of ATP on Elementary Steps of ATPase Reaction

We examined the effect of ATP on the EP level at a fixed concentration of ADP. The desalted enzyme (1 mg/ml) was phosphorylated with 8 μM [γ-^{32}P]ATP for 20 sec in the presence of 1 μM ADP in a buffer solution (6 mM $CaCl_2$, 1 mM EGTA, and 1 mM $MgCl_2$ at pH 7.0) (Fig. 5). Next, 20 volumes of the buffer solution containing 1 μM ADP and various concentra-

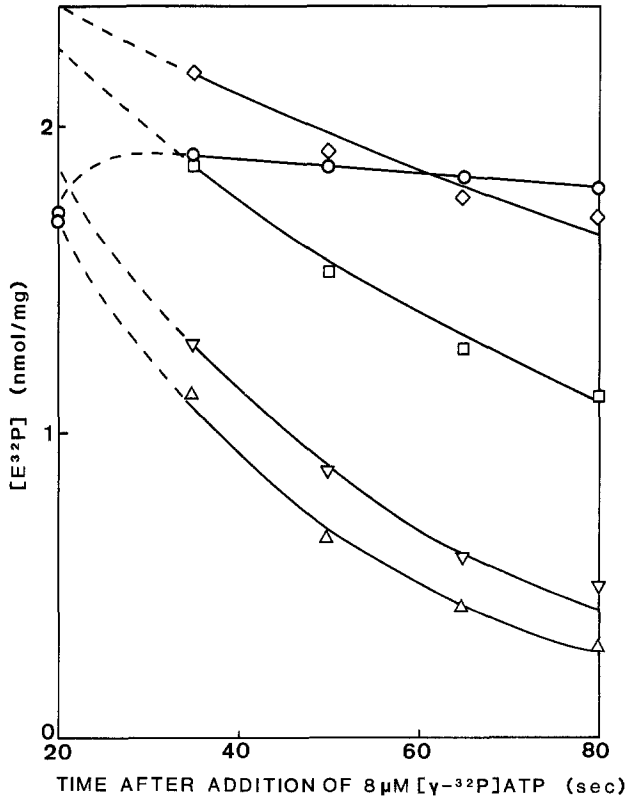

Fig. 5. Effect of a high concentration of ATP on the EP level at a fixed concentration of ADP. The desalted enzyme (1 mg/ml) was phosphorylated with 8 μM [γ-^{32}P]ATP in the presence of 1 μM ADP in a buffer solution (6 mM $CaCl_2$, 1 mM EGTA, 1 mM $MgCl_2$, 20 mM glucose, and 40 mM imidazole-HCl) at pH 7.0 and 0°C. After 20 sec, 20 volumes of the buffer solution containing 1 μM ADP with 0 (○), 8 (△), 40 (▽), 200 (□), or 1000 μM unlabeled ATP (◇) were added, and the amounts of $E^{32}P$ were measured. The unlabeled ATP (5 mM) was pretreated with 0.002 mg/ml CK and 5 mM CP for 12 hr in the presence of 1 mM $MgCl_2$ and 40 mM imidazole-HCl at pH 7.0 and 0°C and added to the diluting solution just before use.

tions of unlabeled ATP was added, and the change in the amount of $E^{32}P$ was followed. The unlabeled ATP (5 mM) was pretreated with 0.002 mg/ml CK and 5 mM CP for 12 hr at 0°C and added to the diluting solution just before use. When the reaction was diluted with 0 μM ATP + 1 μM ADP, the amount of $E^{32}P$ increased slightly, then decreased slowly with an apparent rate constant of 0.065 min^{-1}. When the reaction mixture was diluted with 1 μM ADP + 8 μM unlabeled ATP, the amount of $E^{32}P$ decreased from the original level with a first-order rate constant of 1.84 min^{-1}. When the diluting solution contained 40, 200, or 1000 μM unlabeled ATP with 1 μM ADP, the rate constants of the decrease in the amount of $E^{32}P$ were 1.51, 0.72, and 0.38 min^{-1}, respectively. The amounts of $E^{32}P$ estimated by extrapolating to the time of the dilution increased with increase in the concentration of unlabeled ATP. They were 1.8, 2.2, and 2.4 μmol/g when 40, 200, and 1000 μM unlabeled ATP were used, respectively. We measured the time course of decomposition of $E^{32}P$ into E + $^{32}P_i$ after diluting the reaction mixture with a solution containing unlabeled ATP and enough amounts of CK and CP. We found that the rate constant of decomposition of $E^{32}P$ into E + $^{32}P_i$ under the conditions used in the experiments of Fig. 5 was almost independent of the concentration of unlabeled ATP (20 μM to 1 mM) and was very small, i.e., 0.06 min^{-1}.

We measured the amount of $E^{32}P$ formed with various concentrations of [γ-^{32}P]ATP at steady state under optimal conditions, i.e., in the presence of 50 μM Ca^{2+} and 90 mM KCl at pH 7.0. We used the CK–CP system to keep the ATP concentration constant, and this led to a decrease in the specific radioactivity of [γ-^{32}P]ATP with time. Therefore, we evaluated the EP level at steady state on the basis of the specific radioactivity of [γ-^{32}P]ATP at the time when the reaction was stopped. The double reciprocal plot of the EP level at steady state against the concentration of ATP deviated downward from a straight line above 10 μM ATP (data not shown).

Discussion

The usual SR-ATPase preparations contain inactive ATPase fractions, and to determine the amount of the active site of ATPase, we measured the maximum amount of EP formed under conditions where the rate of EP decomposition was extremely small (see Experimental Procedure). We cannot exclude the possibility that ATP binds to the inactive enzyme fraction with low affinity. However, we assumed that the low-affinity binding of ATP occurred at the regulatory site of the active enzyme, since the binding of ATP with low affinity affected markedly the reaction kinetics of the SR-ATPase, as mentioned below.

The reaction covered by our experimentation can be discussed conveniently with the aid of a minimal scheme for the SR-ATPase reaction:

$$E \overset{ATP}{\rightleftharpoons} E\,ATP \rightleftharpoons E^*ATP \overset{ADP}{\rightleftharpoons} EP \xrightarrow{P_i} E$$

In this scheme, the ATP bound to a loose enzyme–substrate complex (E ATP) is rapidly exchanged, while the ATP bound to the second enzyme–substrate complex (E*ATP[8]) is exchanged very slowly with medium ATP, since the formation of $E^*[\gamma\text{-}^{32}P]ATP + E^{32}P$ is known to be immediately stopped by the addition of unlabeled ATP (Sumida *et al.*, 1976; also see p. 31).

In the absence of Ca^{2+}, ATP bound to the enzyme at a high-affinity site with a dissociation constant of 7–8 μM and formed E ATP. Further reaction proceeded upon addition of Ca^{2+}. At 75 mM KCl, all of the ATP bound to the high-affinity site was converted into EP by the addition of Ca^{2+} (Fig. 1). Furthermore, the dissociation constant of ATP in the absence of Ca^{2+} was much larger than the ATP concentration for half saturation of the EP level in the presence of Ca^{2+}. These findings indicate that the high-affinity site is the catalytic site, and that the equilibrium between E*ATP and EP shifts extremely toward EP in the presence of Ca^{2+}.

At low KCl concentrations, the reaction mechanism of the SR-ATPase is modified at several reaction steps, and the rate of EP decomposition into E + P_i is extremely small (Shigekawa and Pearl, 1976; Shigekawa *et al.*, 1978; Shigekawa and Dougherty, 1978a, b; Shigekawa and Akowitz, 1979). We found that at 0–2 mM KCl and 5 mM $CaCl_2$, a significant amount of ATP remained bound at the high-affinity site even after the addition of Ca^{2+} (Fig. 2). Therefore, we suggest that at 0–2 mM KCl, the equilibrium constant between E*ATP and EP is of the order of magnitude of 1, while at 75 mM KCl, it is much larger than 10, as mentioned above.

The EP formed in the presence of 1 or 5 mg/ml CK, 5 mM $CaCl_2$, and 1 mM $MgCl_2$ at pH 7.0 was ADP-sensitive even in the absence of KCl, owing to the high concentration of Ca^{2+} (Shigekawa and Dougherty, 1978b). Upon addition of ADP, the amount of EP decreased and the amount of ATP formed was equal to the decrease in the amount of EP (Fig. 3). However, when CP was added several tens of seconds after the addition of ADP, the amount of EP increased markedly (Fig. 4). These findings indicate that EP is converted into E*ATP by the addition of ADP and that all the E*ATP (newly converted one + originally present one) is reconverted into EP by removal of ADP. The amount of reconverted EP decreased very slowly with increase in the time interval between the ADP addition and the CP addition. This decrease is attributed to the slow exchange of ATP in E*ATP with medium ADP and/or

[8]E*ATP, slowly exchanging enzyme–ATP complex.

ATP, since the rate of EP decomposition into E + P_i is negligible under the conditions used. Thus, E*ATP is in equilibrium with EP + ADP, and the equilibrium shifts toward EP by removing ADP. Moreover, E*ATP is a slowly exchanging enzyme–ATP complex, and the [γ-^{32}P]ATP in the complex is hardly exchanged with the large amount of unlabeled ATP produced immediately upon addition of CP.

We found that two kinds of ATP-binding sites exist on the ATPase molecule (Figs. 1 and 2). The high-affinity site is the catalytic site as mentioned above, while the low-affinity ATP-binding site is the regulatory site and the binding of ATP to it affects several elementary steps in the Ca^{2+},Mg^{2+}-dependent ATPase reaction. Thus, when an excess amount of unlabeled ATP was added to $E^{32}P$ at a constant concentration of ADP, the amount of $E^{32}P$ increased and then decreased gradually with first-order kinetics (Fig. 5). The rate constant of the $E^{32}P$ decay decreased, while the amount of $E^{32}P$ estimated by extrapolating to the time of the addition of unlabeled ATP increased with increase in the concentration of unlabeled ATP added. These findings can be explained by the following scheme for the backward reaction:

$$EP + ADP \rightleftharpoons E^*ATP \longrightarrow E\,ATP \rightleftharpoons E + ATP$$

The first step is in rapid equilibrium, while the rate of E*ATP $\rightarrow$ E ATP is slow, as mentioned above. The apparent rate constant of $E^{32}P$ decay is proportional to the concentration of E*[γ-^{32}P]ATP, since the rate of $E^{32}P$ decomposition is negligible under the conditions used. When the equilibrium of the first step shifts to the EP side upon the addition of ATP at a constant concentration of ADP, the apparent rate constant of $E^{32}P$ decay decreases, since the amount of E*[γ-^{32}P]ATP decreases by the shift of the equilibrium. Shigekawa and Kanazawa (1981) recently reported that the amount of $E^{32}P$ increases upon the addition of a high concentration of unlabeled ATP. However, the concentration of ADP during their experiments was not kept constant.

At low KCl concentrations, the amount of ATP binding in the presence of 5 mM $CaCl_2$ was almost constant over a wide range of ATP concentrations (Fig. 2). This is because the amount of ATP bound to the catalytic site decreases owing to the shift of the equilibrium between E*ATP and EP to the EP side, while the amount of ATP bound to the regulatory site increases with increase in the ATP concentration.

The double reciprocal plot of the ATPase activity (v_0) against the concentration of ATP deviates downward from a straight line at high ATP concentrations. Similar deviation on the double reciprocal plot of the EP level at steady state against the concentration of ATP was observed, even when the equilibrium between E*ATP and EP shifted extremely toward EP with 90

mM KCl and an ATP-regenerating system. Kanazawa *et al.* (1971) previously reported that the rate constant of EP decomposition (v_0/[EP]) is independent of the concentration of ATP in the presence of a high concentration of KCl. Therefore, our finding on the downward deviation of $[EP]^{-1}$ versus $[ATP]^{-1}$ indicates that the other step(s) besides $E^*ATP \rightleftharpoons EP + ADP$ is also affected by the ATP binding to the regulatory site. However, the dissociation constant of ATP binding to the regulatory site measured in 1 mM $CaCl_2$ at pH 8.8 was 150–400 μM (Fig. 1), while the double reciprocal plot of [EP] versus [ATP] in 50 μM free Ca^{2+} at pH 7.0 deviated downward above 10 μM ATP. These findings indicate that the dissociation constant of ATP binding to the regulatory site depends on pH and/or the Ca^{2+} concentration.

Acknowledgments

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