Effects of Divalent Cations Bound to the Catalytic Site on ATP-Induced Conformational Changes in the Sarcoplasmic Reticulum Ca^{2+}-ATPase: Stopped-Flow Analysis of the Fluorescence of *N*-Acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine Attached to Cysteine-674†

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ABSTRACT: Cys-674 of the sarcoplasmic reticulum Ca^{2+}-ATPase was labeled with *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine without a loss of the catalytic activity. The steady-state fluorescence of the label decreased with increasing concentration of Mg·ATP, Mn·ATP, or Ca·ATP in the presence of Ca^{2+} and 0.1 M KCl at 0 °C, pH 7.0. The maximum extent of the decrease was 23%. The Mg·ATP-, Mn·ATP-, or Ca·ATP-induced fluorescence drop was also determined by the stopped-flow spectrofluorometry. The Ca·ATP-induced fluorescence drop manifested a biphasic time course. The first phase reflects the conformational change which was previously shown [Suzuki, H., Obara, M., Kuwayama, H., & Kanazawa, T. (1987) *J. Biol. Chem. 262,* 15448–15456] to occur upon formation of the calcium·enzyme·substrate complex. The amplitude of the second phase was 2.5% of the fluorescence before addition of Ca·ATP. This phase of the fluorescence drop coincided with the phosphoenzyme formation, which was determined by the continuous flow–rapid quenching method. The phosphoenzyme formed was largely sensitive to ADP. When adenosine 5'-(β,γ-methylenetriphosphate) (a nonhydrolyzable ATP analog incapable of phosphorylating the enzyme) was added in the presence of 5 mM $CaCl_2$ without added $MgCl_2$, the steady-state fluorescence decreased rapidly by 20%. However, this drop lacked the second phase. When phosphoenzyme isomerization from the ADP-sensitive form to the ADP-insensitive form was prevented by the *N*-ethylmaleimide treatment, the second phase of the Ca·ATP-induced fluorescence drop again coincided with the phosphoenzyme formation. These results demonstrate that a conformational change responsible for the second phase of the fluorescence drop occurs upon formation of the ADP-sensitive phosphoenzyme. The data further show that this conformational change is rapid when it is induced by Mg·ATP or Mn·ATP but much slower when induced by Ca·ATP and that, in contrast, the conformational change upon formation of the calcium·enzyme·substrate complex is very rapid irrespective of the kind of metal·ATP complexes tested.

The SR[1] Ca^{2+}-ATPase is a 110-kDa protein which catalyzes ATP hydrolysis coupled to Ca^{2+} transport (Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962), and its primary structure has been revealed (Brandl et al., 1986). According to the predicted functional domain structure (Clarke et al., 1989), this enzyme has one high-affinity ATP-binding site (catalytic site) in the cytoplasmic domain and two high-affinity Ca^{2+}-binding sites (transport sites) in the transmembrane domain. In the initial step of the catalysis, the enzyme is activated by Ca^{2+} binding to the Ca^{2+}-binding sites on the cytoplasmic side. The γ-phosphoryl group of Mg·ATP (the physiological substrate of this enzyme) bound to the catalytic site is transferred to Asp-351 of the activated enzyme (Degani & Boyer, 1973; Bastide et al., 1973) to form ADP-sensitive EP (Makinose, 1967; Yamamoto & Tonomura, 1967; Kanazawa et al., 1970). This EP formation is accompanied by an occlusion of the bound Ca^{2+} (Takakuwa & Kanazawa, 1979; Dupont, 1980; Takisawa & Makinose, 1981). In the next step, this EP is converted to ADP-insensitive EP. Concurrently, the affinity of the Ca^{2+}-binding sites is reduced, and the Ca^{2+} is released into the lumen. Mn·ATP or Ca·ATP can also serve as a substrate of this enzyme (Yamada & Ikemoto, 1980).

Previously, we labeled Cys-674 of this enzyme selectively by EDANS without a loss of the catalytic activity and found that a substantial drop in the fluorescence of the label occurs upon formation of the calcium·enzyme·substrate complex (Suzuki et al., 1987; Kubo et al., 1990). This fluorescence drop reflects a conformational change in the vicinity of the ATP-binding site in the cytoplasmic domain, because Cys-674 is surrounded by amino acid residues which contribute the conformation of the ATP-binding site (Mitchinson et al., 1982; Yamamoto et al., 1989; Clarke et al., 1990; McIntosh et al., 1992; Lacapère et al., 1993; Yamagata et al., 1993; Yamasaki et al., 1994).

In this study, in order to see effects of divalent cations (constituting metal·ATP complexes) on conformational changes in the reaction steps for EP formation from the metal·ATP complex, we have determined the Mg·ATP-, Mn·ATP-, and Ca·ATP-induced fluorescence changes with EDANS-labeled SR vesicles by the stopped-flow spectrofluorometry. We have also determined EP formation by the continuous flow–rapid

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[1] Abbreviations: SR, sarcoplasmic reticulum; EP, phosphoenzyme; EDANS, *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; I-EDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NEM, *N*-ethylmaleimide; AMP-PCP, adenosine 5'-(β,γ-methylenetriphosphate); $C_{12}E_8$, octaethylene glycol dodecyl monoether.

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quenching method and compared its time course with that of the fluorescence change. The results give evidence for the occurrence of a conformational change upon formation of ADP-sensitive EP. The data further show that this conformational change is rapid when it is induced by Mg·ATP or Mn·ATP but much slower when induced by Ca·ATP and that, in contrast, the conformational change occurring upon formation of the calcium·enzyme·substrate complex is very rapid irrespective of the kind of metal·ATP complexes tested.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles. SR vesicles were prepared from rabbit skeletal muscle as described previously (Daiho et al., 1993) with the following two modifications. First, amylase was added to the initial homogenate (1 μg of amylase/mL) to reduce the level of possibly contaminating phosphorylase according to McIntosh et al. (1992). Second, the suspension of SR vesicles in 0.6 M KCl and 5 mM Tris–maleate (pH 6.5) was centrifuged at 15000*g* for 20 min. The supernatant was centrifuged at 125000*g* for 45 min, and the precipitate was homogenized in a buffer containing 1 mM $CaCl_2$, 0.1 M sucrose, and 0.1 M Tris-HCl (pH 7.2). The homogenate was applied to a coarse Sephadex G-50 column (3.2 × 50 cm) equilibrated with the same buffer. The SR vesicles eluted were stored at −80 °C. The Ca^{2+}-dependent ATPase activity determined at 25 °C in a mixture containing 0.01 mg of SR vesicles/mL, 1.33 μM A23187, 0.2 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.4 mM EGTA, 0.1 M KCl, and 20 mM MOPS–Tris (pH 7.0) was 2.22 ± 0.05 μmol of P_i/(mg·min) (n = 3). The content of phosphorylation site determined with [γ-^{32}P]ATP according to Barrabin et al. (1984) was 3.90 ± 0.07 nmol/mg (n = 4).

Labeling of SR Vesicles with I-EDANS. SR vesicles were labeled with I-EDANS as described previously (Suzuki et al., 1987). The content of bound EDANS was about 1 mol/mol of phosphorylation site. The Ca^{2+}-dependent ATPase activity was not impaired by this labeling, being 2.24 ± 0.04 μmol of P_i/(mg·min) (n = 3). The content of phosphorylation site determined as described above was 3.72 ± 0.06 nmol/mg (n = 5).

Treatment of EDANS-Labeled SR Vesicles with NEM. In order to prevent isomerization of EP from the ADP-sensitive form to the ADP-insensitive form, EDANS-labeled SR vesicles were treated with NEM in the presence of adenyl-5′-yl imidodiphosphate according to Kawakita et al. (1980). The Ca^{2+}-dependent ATPase activity was almost completely suppressed by this treatment, while the content of phosphorylation site determined as described above was unaffected.

Steady-State Measurements of Fluorescence. The steady-state fluorescence intensity of bound EDANS was measured on a computer-interfaced RF-5000 spectrofluorometer (Shimadzu, Japan) as described previously (Kubo et al., 1990). The excitation and emission wavelengths were set to 380 and 475 nm, respectively.

Stopped-Flow Measurements of Fluorescence. Rapid kinetic measurements of the fluorescence of bound EDANS were made by using a stopped-flow FSS-300 spectrofluorometer (Unisoku, Japan) interfaced with a personal computer which was programmed to accumulate the digitized data, as described previously (Kubo et al., 1990). The excitation wavelength was 370 nm, and the emitted light was passed through a filter which cut off the light below 450 nm. The reaction was started by mixing equal volumes of solutions from two syringes, one containing EDANS-labeled SR vesicles suspended in a medium described in the figure legends and the other containing ATP or AMP-PCP in the same medium. The measurement was repeated 8–410 times, and the accumulated data were analyzed with the program RS/1 (BBN Software Corp., Cambridge, MA) for nonlinear least-squares fitting on a PS/V personal computer (IBM Corp., Armonk, NY).

Determination of EP. Rapid kinetic measurements of EP formation were made by using a continuous flow–rapid quenching RQS-300 system (Unisoku, Japan), as described previously (Kubo et al., 1990). The reaction was started by mixing equal volumes of solutions from two syringes, one containing EDANS-labeled SR vesicles and the other containing [γ-^{32}P]ATP, and quenched with trichloroacetic acid-containing carrier ATP and P_i. When the reaction was long enough, the above procedures were carried out by manual pipetting. After the reaction was quenched, bovine serum albumin was added. The sample was washed four times by centrifugation with perchloric acid-containing carrier P_i and PP_i and dissolved in 0.5 N NaOH containing 1% sodium dodecyl sulfate, and the radioactivity was measured.

Miscellaneous. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Free Ca^{2+} concentrations were calculated as described previously (Kubota et al., 1993). Disodium ATP and amylase were purchased from Boehringer Mannheim. Monosodium ADP was obtained from Yamasa Biochemicals (Chiba, Japan). AMP-PCP and adenyl-5′-yl imidodiphosphate were from Sigma. I-EDANS was from Aldrich. $C_{12}E_8$ was purchased from Nikko Chemicals (Tokyo, Japan). [γ-^{32}P]ATP was obtained from Amersham. The pH of solutions of ATP, ADP, and adenyl-5′-yl imidodiphosphate was adjusted to 7.0 with Tris.

RESULTS

Dependence of the Extent of Fluorescence Drop on ATP and AMP-PCP Concentrations. When ATP was added to EDANS-labeled SR vesicles in the presence of 5 mM $MgCl_2$ and 20 μM Ca^{2+}, the fluorescence of bound EDANS decreased with increasing concentration of ATP (Figure 1A). The maximum fluorescence drop was 23%, and the ATP concentration giving a half-maximum fluorescence drop was 0.16 μM. These results are in agreement with our previous findings (Suzuki et al., 1987). Essentially the same results were obtained in the presence of 0.25 mM $MnCl_2$ (in place of $MgCl_2$) and 20 μM $CaCl_2$ without EGTA under the otherwise same conditions as in Figure 1A (data not shown). When ATP was added to the vesicles in the presence of 5 mM $CaCl_2$ without added $MgCl_2$, the maximum fluorescence drop was again 23% and the ATP concentration giving a half-maximum fluorescence drop was 0.11 μM (Figure 1B). These high affinities for ATP in the ATP-induced fluorescence drop are consistent with the previously reported affinities of the catalytic site of the SR Ca^{2+}-ATPase for Mg·ATP, Mn·ATP, and Ca·ATP (Meissner, 1973; Vianna, 1975; Yamada & Ikemoto, 1980; Shigekawa et al., 1983; Lacapère & Guillain, 1990). These results indicate that the fluorescence drop is caused by binding of Mg·ATP, Mn·ATP, or Ca·ATP to the catalytic site of this enzyme.

AMP-PCP (a nonhydrolyzable ATP analog which is incapable of phosphorylating the enzyme) also induced a similar fluorescence drop in the presence of 5 mM $CaCl_2$ without added $MgCl_2$ (Figure 1C). The AMP-PCP concentration giving a half-maximum fluorescence drop was 2.3 μM, being again in fair agreement with the previously reported affinity of the catalytic site for AMP-PCP (Ogawa et al.,

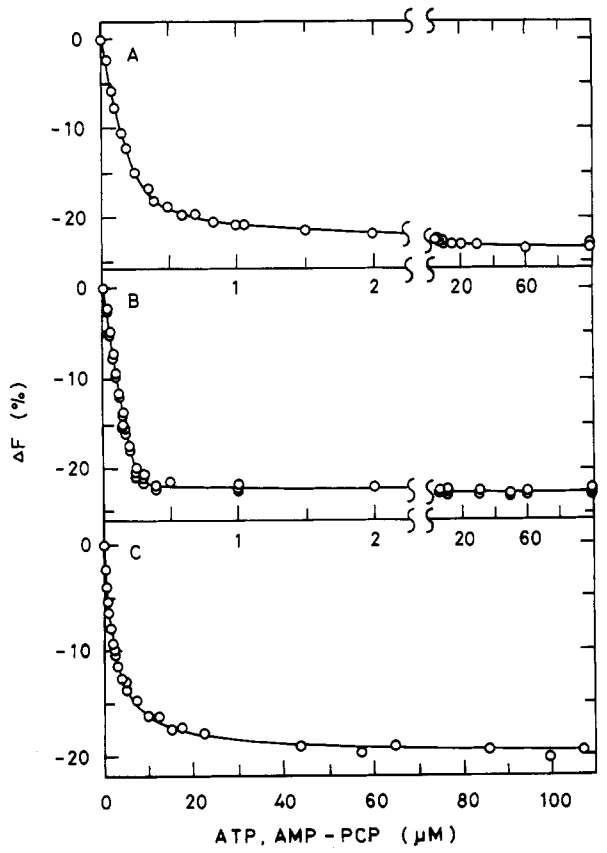

FIGURE 1: Dependence of the extent of fluorescence drop on ATP and AMP-PCP concentrations. (A) The extent of the Mg·ATP-induced fluorescence drop attained in the steady state was determined at 0 °C by adding small volumes of ATP to 2.4 mL of a suspension of EDANS-labeled SR vesicles (0.05 mg/mL) in a medium containing 5 mM $MgCl_2$, 0.511 mM $CaCl_2$, 0.5 mM EGTA (20 μM Ca^{2+}), 0.1 M KCl, and 20 mM MOPS–Tris (pH 7.0). (B) The extent of the Ca·ATP-induced fluorescence drop was determined by adding ATP to a suspension of EDANS-labeled SR vesicles in a medium containing 5 mM $CaCl_2$, 0.1 M KCl, and 20 mM MOPS-Tris (pH 7.0), otherwise as in (A). (C) The extent of the Ca·AMP-PCP-induced fluorescence drop was determined by adding AMP-PCP, otherwise as in (B). In all the measurements, the total volumes of added ATP or AMP-PCP were less than 17 μL. The fluorescence drop was corrected for dilution upon each addition.

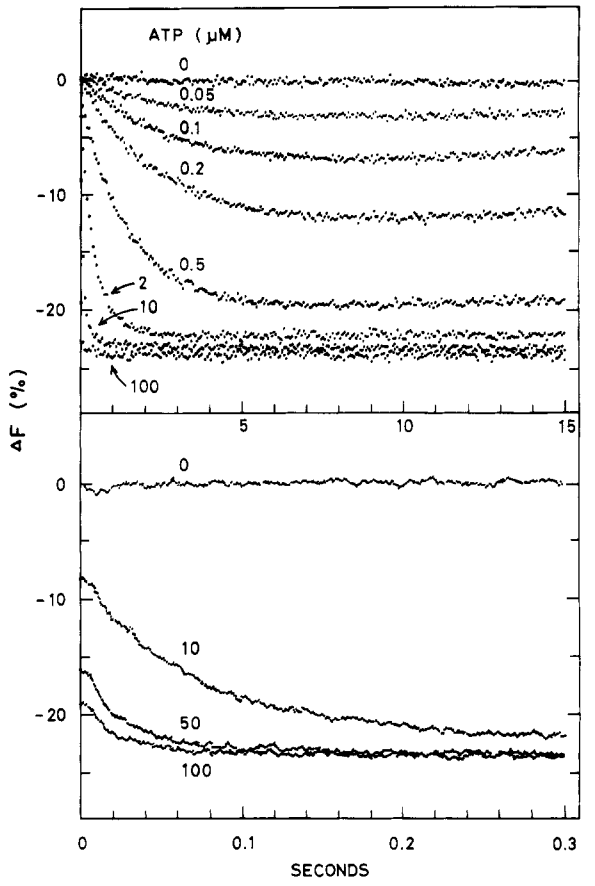

FIGURE 2: Time course of Mg·ATP-induced fluorescence drop. EDANS-labeled SR vesicles (0.1 mg/mL) in the same medium as in Figure 1A were mixed with ATP at 0 °C by the stopped-flow method. The reaction was followed for 15 (upper panel) or 0.3 (lower panel) s. The extents of the fluorescence change are normalized to those obtained in the steady-state measurement. ATP concentrations after the mixing are indicated in the figure.

1986). This finding indicates that the ATP-induced fluorescence drop occurs upon formation of the calcium·enzyme·substrate complex. However, the maximum fluorescence drop (20%) obtained by Ca·AMP-PCP was significantly smaller than that obtained with Mg·ATP, Mn·ATP, or Ca·ATP. This suggests that an additional fluorescence drop may occur after the formation of the calcium·enzyme·substrate complex. This probability has been supported by the following experiments.

Time Course of Mg·ATP-, Mn·ATP-, or Ca·ATP-Induced Fluorescence Drop. The Mg·ATP-induced fluorescence drop was followed in the presence of 5 mM $MgCl_2$ and 20 μM Ca^{2+} by the stopped-flow method (Figure 2). The initial rate of the fluorescence drop increased with increasing concentration of ATP, and it was unsaturated even with 50 μM ATP added. The fluorescence drop with 100 μM ATP added was almost completed in 0.3 s. These results are in agreement with our previous observations (Suzuki et al., 1987). Essentially the same results were obtained with Mn·ATP in the presence of 0.25 mM $MnCl_2$ (in place of $MgCl_2$) and 20 μM $CaCl_2$ without EGTA under the otherwise same conditions as in Figure 2 (data not shown).

On the other hand, the Ca·ATP-induced fluorescence drop in the presence of 5 mM $CaCl_2$ without added $MgCl_2$ manifested a biphasic time course (Figure 3). When ATP was added to give 100 μM, the first phase of the fluorescence drop was completed within the instrumental dead time (less than 15 ms) (lower panel of Figure 3). This phase accounted for most of the total fluorescence drop. The second phase of the drop was completed in some 8 s, and its maximum amplitude was 2.5% of the initial fluorescence intensity before the addition of ATP (upper panel of Figure 3).

Comparison between the Time Course of Mg·ATP-, Mn·ATP-, or Ca·ATP-Induced Fluorescence Drop and the Time Course of EP Formation. The time course of the Mg·ATP-, Mn·ATP-, or Ca·ATP-induced fluorescence drop was compared with that of EP formation. When ATP was added to give 0.2 μM (Figure 4A) or 0.5 μM (Figure 4B) in the presence of 5 mM $MgCl_2$ and 20 μM Ca^{2+}, the Mg·ATP-induced fluorescence drop was slow and coincided with EP formation. When ATP was added to give 100 μM (Figure 4C), the fluorescence drop became very rapid and mostly preceded EP formation. Essentially the same results were obtained with Mn·ATP in the presence of 0.25 mM $MnCl_2$ (in place of $MgCl_2$) and 20 μM $CaCl_2$ without EGTA under the otherwise same conditions as in Figure 4B,C (data not shown).

When ATP was added to give 0.2 μM in the presence of 5 mM $CaCl_2$ without added $MgCl_2$ (Figure 4D), the Ca·ATP-induced fluorescence drop occurred at nearly the same rate

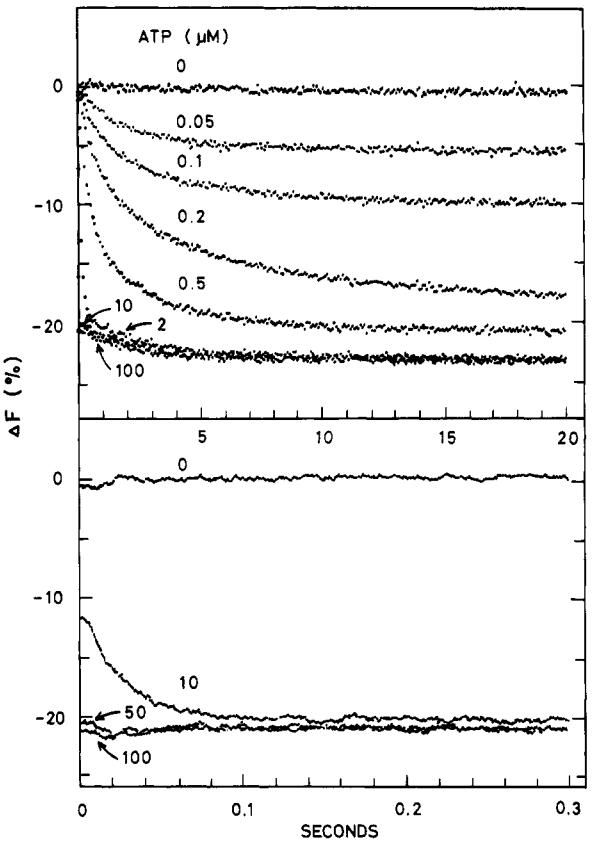

FIGURE 3: Time course of Ca·ATP-induced fluorescence drop. EDANS-labeled SR vesicles in the same medium as in Figure 1B were mixed with ATP, otherwise as in Figure 2. The reaction was followed for 20 (upper panel) or 0.3 (lower panel) s.

as in the presence of 5 mM $MgCl_2$ (cf. Figure 4A). However, EP formation was much slower than that in the presence of 5 mM $MgCl_2$ (cf. Figure 4A), and in consequence the fluorescence drop appreciably preceded EP formation.

These results show that a conformational change involved in most of the Mg·ATP-, Mn·ATP-, or Ca·ATP-induced fluorescence drop occurs upon formation of the calcium·enzyme·substrate complex and that this conformational change becomes a rate-limiting step for EP formation when it is slow relative to EP formation.

Coincidence between the Second Phase of Ca·ATP-Induced Fluorescence Drop and EP Formation. The time course of the second phase of the Ca·ATP-induced fluorescence drop was compared with that of EP formation in the presence of 0.25 mM (Figure 5A) or 5 mM (Figure 5B) $CaCl_2$ without added $MgCl_2$ at 0 °C. In this experiment, ATP was added to give 100 μM. Under these conditions, the first phase of the Ca·ATP-induced fluorescence drop was completed within the instrumental dead time (cf. lower panel of Figure 3). The second phase of the fluorescence drop coincided with EP formation and was well fitted with a single exponential. The ratio of the amplitude of this second phase to the amount of EP in the presence of 5 mM $CaCl_2$ was approximately the same as that in the presence of 0.25 mM $CaCl_2$. Thus, the increase in the Ca^{2+} concentration from 0.25 to 5 mM had no significant effects on the kinetic and quantitative relationships between the fluorescence drop in the second phase and EP formation. Almost all of EP formed under these conditions was sensitive to ADP.

When the reaction was performed at 8 °C otherwise as in Figure 5B, both the second phase of the fluorescence drop and EP formation were about twice as fast as those at 0 °C (Figure 5C). Irrespective of this elevated reaction temperature, the second phase of the fluorescence drop coincided with EP formation. EP formed was again almost entirely sensitive to ADP. When AMP-PCP was added to give 100 μM in the presence of 5 mM $CaCl_2$ without added $MgCl_2$ at 0 °C, the

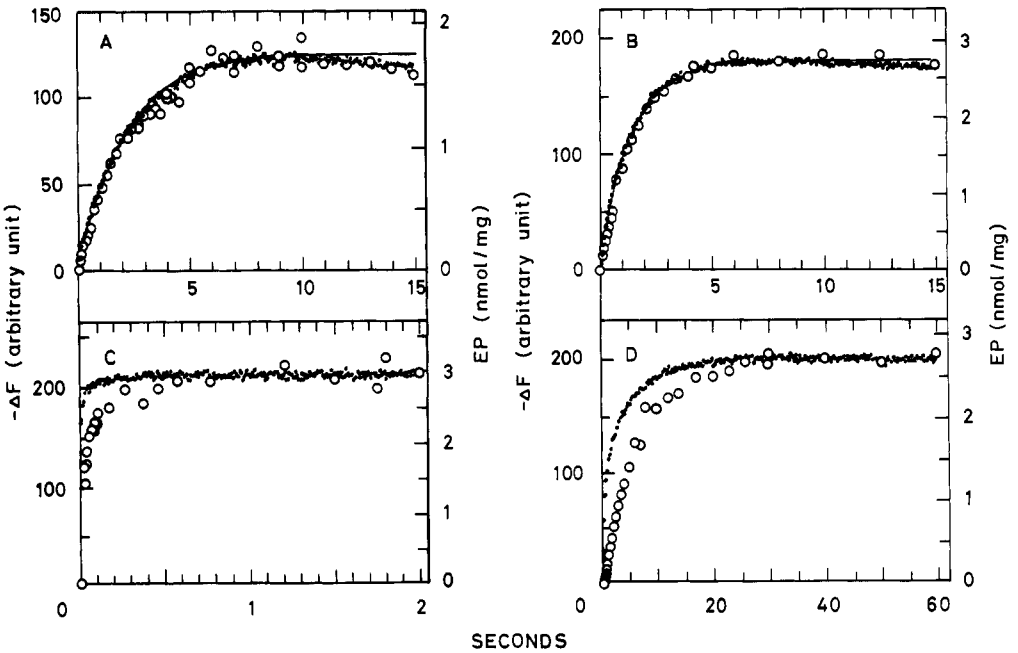

FIGURE 4: Comparison between the time course of Mg·ATP- or Ca·ATP-induced fluorescence drop and the time course of EP formation. Stopped-flow measurements were performed by mixing EDANS-labeled SR vesicles with ATP under the following conditions, otherwise as in Figure 2. Compositions of the media: (A–C) 5 mM $MgCl_2$, 0.511 mM $CaCl_2$, 0.5 mM EGTA (20 μM Ca^{2+}), 0.1 M KCl, and 20 mM MOPS–Tris (pH 7.0); (D) 5 mM $CaCl_2$, 0.1 M KCl, and 20 mM MOPS-Tris (pH 7.0). ATP concentrations after the mixing (μM): (A) 0.2; (B) 0.5; (C) 100; (D) 0.2. The Mg·ATP- or Ca·ATP-induced fluorescence change (···) was obtained by subtracting the base-line level, which was determined by mixing EDANS-labeled SR vesicles with the ATP-free medium. Solid lines in (A) and (B) show least-squares fit of a single exponential to the Mg·ATP-induced fluorescence changes, in which the first-order rate constants were 0.47 s^{-1} and 0.73 s^{-1}, respectively. EP formation (O) was performed with [γ-^{32}P]ATP by the continuous flow–rapid quenching method or by manual pipetting under the otherwise same conditions as in the stopped-flow measurement.

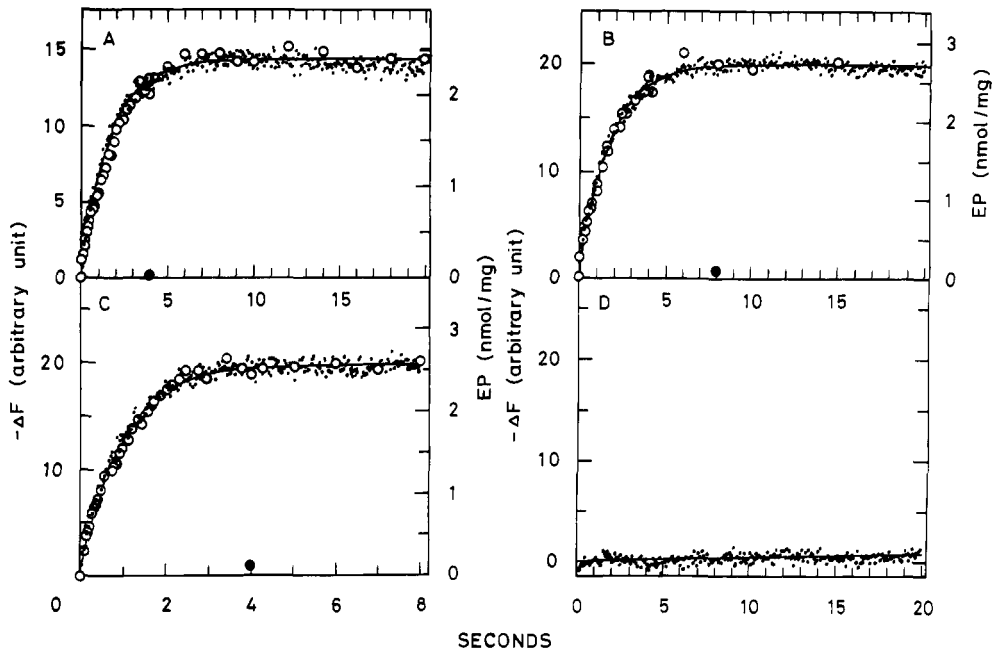

FIGURE 5: Coincidence between the second phase of Ca·ATP-induced fluorescence drop and EP formation. Stopped-flow measurements were performed by mixing EDANS-labeled SR vesicles with ATP or AMP-PCP under the following conditions, otherwise as in Figure 2. Compositions of the media: (A) 0.25 mM $CaCl_2$, 0.1 M KCl, and 20 mM MOPS–Tris (pH 7.0); (B–D) 5 mM $CaCl_2$, 0.1 M KCl, and 20 mM MOPS-Tris (pH 7.0). Reaction temperatures: (A, B, D) 0 °C; (C) 8 °C. Nucleotides used and the concentrations after the mixing: (A–C) 100 μM ATP; (D) 100 μM AMP-PCP. The Ca·ATP- or Ca·AMP-PCP-induced fluorescence change (···) was obtained by subtracting the base-line level as in Figure 4. The first phase of the fluorescence change finished within the instrumental dead time. The data in the figure represent only the second phase of the fluorescence change. Solid lines in (A), (B), and (C) show least-squares fit of a single exponential to the Ca·ATP-induced fluorescence changes, in which the first-order rate constants were 0.61 s^{-1}, 0.58 s^{-1}, and 1.05 s^{-1}, respectively. EP formation was performed with [γ-^{32}P]ATP under the otherwise same conditions as in the stopped-flow measurement, and the total amount of EP (○) was measured. For the assessment of ADP-insensitive EP (●), 9 volumes of a mixture of ADP and nonradioactive ATP in the same medium as above were added to give 5 mM ADP and 200 μM nonradioactive ATP at the time indicated. The reaction was quenched with acid 5 s after the addition of the ADP/ATP mixture, and the amount of EP remaining was measured.

fluorescence drop (cf. Figure 1C) finished within the instrumental dead time and no further fluorescence change was detected (Figure 5D). All the above findings demonstrate that the second phase of the Ca·ATP-induced fluorescence drop reflects a conformational change associated with EP formation.

The Second Phase of Ca·ATP-Induced Fluorescence Drop Is Attributed to Formation of ADP-Sensitive EP. The data given in Figure 5 suggest that the second phase of the Ca·ATP-induced fluorescence drop is due to the formation of ADP-sensitive EP, because EP formed is largely sensitive to ADP. In order to ascertain whether this idea is valid, EDANS-labeled vesicles were pretreated with NEM under the conditions in which isomerization of ADP-sensitive EP to ADP-insensitive EP should be prevented (Kawakita et al., 1980). In agreement with our previous observations (Obara et al., 1988), when ATP was added to the NEM-untreated vesicles in the absence of K^+ and presence of $C_{12}E_8$, ADP-insensitive EP accumulated to a large extent (Figure 6). In contrast, when ATP was added to the NEM-treated vesicles, no ADP-insensitive EP accumulated although the total amount of EP decreased only to a small extent. These findings show that isomerization of ADP-sensitive EP to ADP-insensitive EP was in fact almost completely prevented by the NEM treatment.

When ATP was added to the NEM-treated vesicles under the otherwise same conditions as in Figure 5A, the Ca·ATP-induced fluorescence drop again showed a biphasic time course. The first phase of the fluorescence drop was completed within the instrumental dead time. The rate and amplitude of the second phase were nearly the same as those obtained with the untreated vesicles (Figure 7, and cf. Figure 5A). The time course of this second phase agreed well with that of the formation of ADP-sensitive EP. These findings demonstrate that the second phase of the Ca·ATP-induced fluorescence drop is attributed to the formation of ADP-sensitive EP.

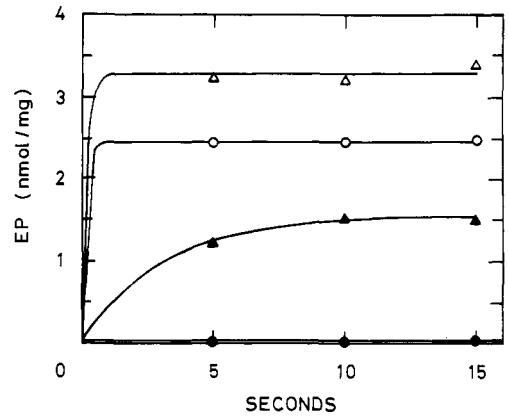

FIGURE 6: Inhibition of EP isomerization from the ADP-sensitive form to the ADP-insensitive form by the NEM treatment. NEM-treated EDANS-labeled SR vesicles (0.1 mg/mL) (○, ●) or untreated EDANS-labeled SR vesicles (0.1 mg/mL) (△, ▲) in a medium containing 33 μg/mL $C_{12}E_8$, 10 mM $MgCl_2$, 50 μM $CaCl_2$, 0.1 M LiCl, and 20 mM MOPS–Tris (pH 7.0) were mixed at 0 °C with an equal volume of 200 μM [γ-^{32}P]ATP in the same medium, and the total amount of EP (○, △) was measured. The amount of ADP-insensitive EP (●, ▲) was measured as in Figure 5 except that a half volume of an ADP/EGTA mixture (in place of the ADP/ATP mixture) was added to give 10 mM ADP and 5 mM EGTA.

DISCUSSION

Effects of divalent cations on the conformational changes in the reaction steps for EP formation from the metal·ATP

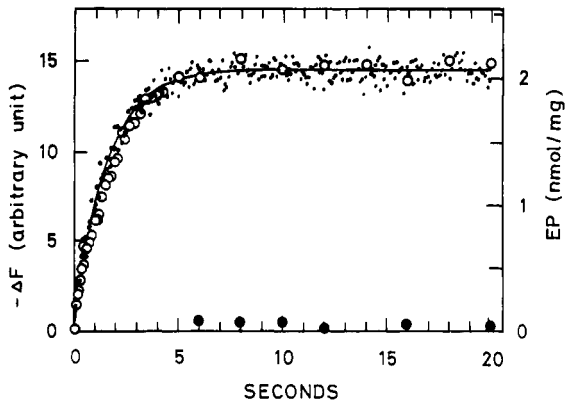

FIGURE 7: Coincidence between the second phase of Ca·ATP-induced fluorescence drop and EP formation with NEM-treated EDANS-labeled SR vesicles. The second phase of Ca·ATP-induced fluorescence drop, the total amount of EP (○), and the amount of ADP-insensitive EP (●) were determined as in Figure 5A except that NEM-treated EDANS-labeled SR vesicles were used. The solid line shows least-squares fit of a single exponential to the Ca·ATP-induced fluorescence change, in which the first-order rate constant was 0.66 s^{-1}.

complex may be conveniently discussed in terms of the following scheme:

$$Ca_2{\cdot}E + S \underset{(1)}{\rightleftharpoons} Ca_2{\cdot}E{\cdot}S \underset{(2)}{\rightleftharpoons} Ca_2{\cdot}E'{\cdot}S \underset{(3)}{\rightleftharpoons} Ca_2{\cdot}E''P$$

where E, E′, and E″ denote different conformational states of the Ca^{2+}-activated enzyme, and S denotes the substrate (metal·ATP complex). E″P represents ADP-sensitive EP. According to this scheme, the first phase of the fluorescence drop occurs in step 2, and the second phase in step 3. The data indicate that the rate of the fluorescence drop in the first phase is unsaturated at substrate concentrations where the extent of the fluorescence drop is saturated (Figures 1–3). This finding suggests that step 1 is favorable to dissociation of Ca_2·E·S and that step 2 is greatly favorable to formation of Ca_2·E′·S, as proposed earlier (Suzuki et al., 1987).

The present results show that the conformational change in step 2 is very rapid irrespective of the kind of metal·ATP complexes tested (Figures 2 and 3) and that, in contrast, the conformational change in step 3 is rapid when it is induced by Mg·ATP or Mn·ATP but much slower when induced by Ca·ATP (compare Figure 4A with Figure 4D, and compare Figure 4C with Figure 5B). These findings are consistent with the previously reported observations that EP formation with Ca·ATP is much slower than that with Mg·ATP or Mn·ATP (Yamada & Ikemoto, 1980; Shigekawa et al., 1983; Lacapère & Guillain, 1990). It is certain that the observed effects of divalent cations are due to binding of divalent cations (constituting metal·ATP complexes) to the catalytic site, but not due to binding of divalent cations to the high-affinity Ca^{2+}-binding sites, because all the experiments in the present study have been carried out under the conditions in which the high-affinity Ca^{2+}-binding sites have always been occupied by Ca^{2+} (Chiesi & Inesi, 1980; Gomes Da Costa & Madeira, 1986).

Previously, on the basis of the kinetic analysis of EP formation from ATP, Petithory and Jencks (1986) suggested the existence of a rate-limiting conformational change followed by rapid phosphoryl transfer from ATP to the enzyme. It is possible that the conformational change predicted by Petithory and Jencks corresponds to the conformational change which is involved in the second phase of the ATP-induced fluorescence drop shown in the present experiments. However, it is also possible that the conformational change responsible for the second phase of the ATP-induced fluorescence drop is directly associated with the process of phosphoryl transfer.

Quite recently, we have found that the ATP-induced drop in the intrinsic tryptophan fluorescence of the Ca^{2+}-ATPase reported earlier (Dupont & Leigh, 1978; Fernandez-Belda et al., 1984) reflects a conformational change occurring, probably in or near the transmembrane domain, upon formation of ADP-sensitive EP (Nakamura et al., 1994). It appears likely that the conformational change (responsible for the second phase of the ATP-induced drop in the fluorescence of bound EDANS) upon formation of ADP-sensitive EP in the catalytic site in the cytoplasmic domain is transmitted to the transmembrane domain and induces Ca^{2+} occlusion in the Ca^{2+}-binding sites.

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