A Conformational Change of \(N\text{-Iodoacetyl-N'-(5-sulfo-1-naphthyl)}\text{ethylenediamine-labeled Sarcoplasmic Reticulum Ca}^{2+}\text{-ATPase upon ATP Binding to the Catalytic Site*}

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Sarcoplasmic reticulum vesicles were modified with a fluorescent thiol reagent, \(N\text{-Iodoacetyl-N'-(5-sulfo-1-naphthyl)}\text{ethylenediamine. One mol of readily reactive thiols per mol of the Ca}^{2+}\text{-ATPase was labeled without a loss of the catalytic activity. The fluorescence of the label increased by 8% upon binding of Ca}^{2+}\text{ to the high affinity sites of the enzyme. This fluorescence enhancement probably reflects a conformational change responsible for Ca}^{2+}\text{-induced enzyme activation. Upon addition of ATP to the Ca}^{2+}\text{-activated enzyme, the fluorescence decreased by 15%. This fluorescence drop and formation of the phosphoenzyme intermediate were determined under the same conditions with a stopped-flow apparatus and a rapid quenching system. The amplitude of the fluorescence drop thus determined was saturated with 3 \(\mu M\) ATP. This shows that the fluorescence drop was caused by ATP binding to the catalytic site. In contrast, the rate of the fluorescence drop was not saturated even with 50 \(\mu M\) ATP. The fluorescence drop coincided with phosphoenzyme formation at 0.5 or 3 \(\mu M\) ATP, but it became much faster than phosphoenzyme formation when the ATP concentration was raised to 100 \(\mu M\). These results indicate that the ATP-induced fluorescence drop reflects a conformational change in the enzyme-ATP complex. The fluorescence drop was accompanied by a red spectrum shift, which suggests that the label was exposed to a more hydrophilic environment. The electrophoretic analysis of the tryptic digest of the labeled enzyme (10.9 kDa) showed that almost all of the label was located on the 5.2-kDa fragment which includes the carboxyl terminus and the putative ATP-binding domain. The sequencing of the two major labeled peptides, which were isolated from the thermolytic digest of the labeled enzyme, revealed that the labeled site in either of these peptides was Cys\(^{677}\). It seems likely that the label bound to this Cys\(^{677}\) could be involved in the observed fluorescence changes.

The Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum catalyzes ATP hydrolysis which is coupled to the Ca\(^{2+}\) transport (1–3). During the catalytic cycle, the enzyme is phosphorylated by ATP to form EP\(^{+}\) intermediates. Recently, the primary structure of this enzyme was entirely revealed by sequencing cDNA (4). This opened the way anew for much fruitful investigation of the transport mechanism on the submolecular level. From this aspect, it is now important to examine the conformational behavior of a specific site of the enzyme during the catalytic process. Some fluorescent probes could be useful for this purpose.

An enhancement of intrinsic fluorescence upon binding of Ca\(^{2+}\) to the enzyme was originally reported by Dupont (5) and ascribed to a conformational change responsible for the enzyme activation (5–9). Subsequently, a drop in intrinsic fluorescence following addition of ATP was found (6), but it was assigned to different reaction steps by different authors (9–13). This discrepancy is likely due to a dominant contribution of different tryptophan residues to the fluorescence drop under different conditions. In addition, it seems difficult to locate the site responsible for this fluorescence drop since the enzyme contains many tryptophan residues (4). Therefore, the intrinsic fluorescence is not very useful for investigation of a conformational event at a specific site in a single reaction step, as suggested by Champeil et al. (13).

On the other hand, functionally distinct thiols of this enzyme can be specifically labeled with various thiol reagents under controlled conditions (14–18). Thus, it is possible to detect conformational changes at a specific site in the catalytic process. On this line, Miki et al. (17) previously found that the fluorescence of \(N\text{-}(1\text{-anilinonaphthyl-1)}\text{maleimide attached to a specific thiol was enhanced by 3.6% upon addition of ATP. They suggested that this enhancement reflects conformational changes produced by EP formation. Subsequently, Kawakita and co-workers (19) demonstrated that the fluorescence of various maleimide-derivatives bound to the enzyme was reduced by 2.5% by addition of ATP. They further showed that the fluorescence of the bound EDANS increased significantly by addition of ATP in the presence of Ca\(^{2+}\) (20). However, in these studies, the ATP-induced fluorescence changes were too small for detailed kinetic and physicochemical analyses.

In the present experiment, 1 mol of readily reactive thiols per mol of the Ca\(^{2+}\)-ATPase of SRV has been labeled with I-EDANS without a loss of the catalytic activity under our conditions. The fluorescence of this label has decreased by

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‡ The abbreviations used are: EP, phosphoenzyme; SRV, sarcoplasmic reticulum vesicles; 1-EDANS, \(N\text{-Iodoacetyl-N'-(5-sulfo-1-naphthyl)}\text{ethylenediamine; EDANS, N-acetyl-N'-(5-sulfo-1-naphthyl)}\text{ethylenediamine; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; AMP-PCP, adenosine 5'-\((\beta,\gamma\text{-methylene})\text{triphosphate; AMP-PNP, adenosine 5'}-\((\beta,\gamma\text{-imido})\text{triphosphate.}\)
15% upon binding of ATP to the catalytic site provided the enzyme has been previously activated by Ca". This large change in the fluorescence intensity has allowed us to make kinetic measurements by the stopped-flow method. The results indicate that this fluorescence drop reflects a conformational change in the enzyme-ATP complex preceding EP formation.

**EXPERIMENTAL PROCEDURES**

Preparation of SRV—SRV were prepared from rabbit skeletal muscle as described previously (21) and stored in 1 M sucrose, 1 mM CaCl2, and 0.1 M Tris/HCl (pH 7.2) at -80 °C.

Chemical Modification of SRV with I-EDANS—SRV were labeled by iodination of SRV according to Vanderkooi et al. (22) with substantial modifications. All procedures were carried out at 4 °C. SRV (7.5 mg/ml) were preincubated with 100 μM N-ethylmaleimide in Buffer I (1 mM CaCl2, 2 mM ATP, 0.1 M sucrose, and 135 mM Tris/HCl (pH 8.5)) for 15 min, and the excess reagent was removed by gel filtration through a Sephadex G-50 column equilibrated with 1 mM CaCl2, 0.1 M sucrose, and 100 mM Tris/HCl (pH 7.2). Subsequently, the SRV (3 mg/ml) were incubated with 100 μM I-EDANS in Buffer I for 30 min (unless otherwise stated), and the excess reagent was removed by gel filtration through a Sephadex G-50 column equilibrated with 1 mM CaCl2, 0.1 M KCl, and 5 mM MOPS/Tris (pH 7.0). The resulting EDANS-SRV were collected by centrifugation and stored in 0.1 M KCl, 0.1 M MgCl2, and 0.1 M MOPS/Tris (pH 7.0) at -80 °C. For the control, SRV were subjected to all the procedures described above except the absence of N-ethylmaleimide and of I-EDANS (control SRV). The amount of EDANS bound to SRV was determined from the difference in absorbance between EDANS-SRV and control SRV in 10% SDS at pH 7.0 by using an extinction coefficient of 6.1 × 105 M⁻¹ cm⁻¹ at 337 nm (23).

Fluorescence Measurements—Steady-state fluorescence measurements were performed at 0 °C on a double-beam difference spectrofluorometer (Shimadzu, RF-505A) which was equipped with a cuvette stirrer and a cell holder connected with a controlled temperature bath. This apparatus was interfaced with a personal computer which was programmed to smooth, accumulate, and store the digitized data. The fluorescence intensity of EDANS-SRV in the sample cuvette was determined from the difference in absorbance between EDANS-SRV and control SRV in 10% SDS at pH 7.0. The resulting pyridylethylated peptides were analyzed by using a HPLC system (Pharmacia Biotechnology Inc., PepRPC HR 5/5, 0.5 x 20 mm) with a fluorescence detection at 515 nm and emission at 535 nm for 20 min, and the radioactivity was measured.

Determination of Ca" Uptake—Ca" uptake by EDANS-SRV (or control SRV) (20 μg/ml) was performed at 25 °C in 2 mM ATP, 0.1 mM CaCl2, 5 mM oxalate, 0.1 M MgCl2, 0.1 M KCl, and 50 mM MOPS/Tris (pH 7.0). At different times, an aliquot of the reaction mixture was filtered through a Millipore filter. The filter was washed with a buffer containing 10 mM CaCl2, 5 mM MgCl2, 0.12 M KCl, and 50 mM MOPS/Tris (pH 7.0), and the radioactivity remaining on the filter was measured. The ATPase reaction with EDANS-SRV (or control SRV) (4 μg/ml) was performed at 25 °C in 10 mM [γ-32P]ATP, 5 mM MgCl2, 5 mM oxalate, 0.1 M KCl, 50 mM MOPS and either 0.1 mM EGTA with 0.15 mM CaCl2 or 5 mM EGTA without CaCl2. The amount of 32P, liberated was determined as described previously (25).

Tryptic Digestion of EDANS-SRV—Tryptic digestion of EDANS-SRV (2 mg/ml) was carried out for various periods according to Stewart and MacLennan (26) at 32 °C in 0.1 M KCl, 1 M sucrose, and 20 mM MOPS/Tris (pH 7.0). The digestion was started by adding trypsin at a final concentration of 20 μg/ml, and terminated by adding soybean trypsin inhibitor (3 μg/μg trypsin).

Electrophoretic Analysis of Tryptic Fragments—The tryptic digest was subjected to SDS-polyacrylamide gel electrophoresis according to Weber and Osborn (27) by using 7.5% acrylamide gel. The gel was stained in 0.12% Coomassie Brilliant Blue R-250.

Thermolysin Digestion of EDANS-labeled Ca"-ATPase—EDANS-SRV were treated with deoxycholate by the method of Meissner and Fleischer (28). The treatment resulted in a partial purification of the EDANS-labeled Ca"-ATPase. The thermolysin digestion of this purified enzyme was performed essentially according to Saito-Nakatsuka et al. (29). Fifty mg of the EDANS-labeled enzyme was digested with 0.5 mg of thermolysin for 1 h at 46 °C in 5 ml of a medium containing 0.2 M NH4HCO3, 1 mM CaCl2, and 0.2 M sucrose. The reaction was stopped by adding 30 μl of 0.5 M EDTA (pH 8.0), and then formic acid was added to give 2.7%. The resulting suspension was centrifuged at 1000 × g for 20 min, and the clear supernatant was collected.

Purification of EDANS-peptides by Reversed Phase HPLC—Separation of EDANS-peptides from the above supernatant was performed by using a HPLC system (Pharmacia Biotechnology Inc., fast protein liquid chromatography) connected with a UV monitor (Pharmacia Biotechnology Inc., UV-M), a fluorescence monitor (Hitachi F-1000), and a fraction collector (Pharmacia Biotechnology Inc., FRAC-100).

**RESULTS**

Labeling of SRV with I-EDANS—The enzyme was rapidly labeled with I-EDANS during the initial 30-min period, and this was followed by much slower labeling (Fig. 1). In order to estimate the content of the catalytic site in SRV used, the amount of EP was determined with control SRV under opti-
Changes in the Steady-state Fluorescence Intensity of EDANS-SRV following Additions of Ca2+ and ATP—Effects of Ca2+ and ATP on the steady-state fluorescence level of EDANS-SRV were investigated at 0 °C (Fig. 2). When free Ca2+ in the medium was chelated by addition of EGTA, the fluorescence intensity decreased by 8%. The subsequent addition of CaCl2 to give 112 μM free Ca2+ entirely restored the initial level of fluorescence. When ATP was added to give 5 μM, the fluorescence intensity decreased by 15% and then gradually returned to the initial level. This spontaneous restoration is probably due to hydrolysis of added ATP. This is supported by the finding that the second addition of ATP to give a much higher concentration caused a prolonged fluorescence drop. Similar results showing the Ca2+-induced fluorescence enhancement and the ATP-induced fluorescence drop were obtained at 25 °C under otherwise the same conditions as above.

Dependence of the Ca2+-induced Fluorescence Enhancement on Ca2+ Concentration—The steady-state fluorescence level was determined at different Ca2+ concentrations (Fig. 3). The fluorescence intensity increased with increasing Ca2+ concentration above 0.3 μM, and this increase was almost saturated with 10 μM Ca2+. The Ca2+ concentration giving a half-maximum fluorescence enhancement was 1.8 μM, and the Hill coefficient was 1.9.

Kinetics of the Fluorescence Enhancement following Addi-

![Fig. 2. Changes in the steady-state fluorescence intensity of EDANS-SRV following additions of Ca2+ and ATP.](image-url)

![Fig. 3. Dependence of the Ca2+-induced fluorescence enhancement on Ca2+ concentration.](image-url)

![Fig. 4. Kinetics of the fluorescence enhancement following addition of Ca2+. EDANS-SRV (0.1 mg/ml) in a medium containing 1 mM EGTA, 10 mM MgCl2, 0.1 M KCl, and 20 mM MOPS/Tris (pH 7.0) were mixed with 1.08 mM CaCl2 in the same medium as above except that EGTA was absent. The free Ca2+ concentration after the mixing was 50 μM. For the control, EDANS-SRV were mixed with the medium without CaCl2, otherwise as above. The reaction was followed for 20 (A) or 0.5 (B) s.](image-url)
Drop on ATP Concentration—ATP was added to EDANS-SRV suspended in the presence of Ca2+ (Fig. 5A). After the addition, the fluorescence intensity decreased and then reached a steady-state level. The extent of this fluorescence drop increased with increasing ATP concentration, and it was saturated at a very low concentration of ATP (Fig. 5B). The ATP concentration giving a half-maximum fluorescence drop was about 0.2 \( \mu \text{M} \). The extent of the ATP-induced fluorescence drop remained constant in the range of ATP concentrations from 3 to 100 \( \mu \text{M} \) (the inset of Fig. 5B).

Dependence of the Initial Rate of the ATP-induced Fluorescence Drop on ATP Concentration—The initial rate of the ATP-induced fluorescence drop was determined at 0.05–3 \( \mu \text{M} \) ATP, as typically shown in the upper panel of Fig. 6A. The rate was proportional to the ATP concentration ranging from 0.05 to 3 \( \mu \text{M} \) (Fig. 6B). When ATP was higher than 3 \( \mu \text{M} \), the fluorescence drop was too fast to be entirely followed. The rate was not saturated even with 50 \( \mu \text{M} \) ATP (the lower panel of Fig. 6A).

Dependence of the ATP-induced Fluorescence Drop on Ca2+ Concentration—EDANS-SRV were mixed with 2 \( \mu \text{M} \) ATP at different Ca2+ concentrations by the stopped-flow method, and the extent of the fluorescence drop in the steady-state was determined in the same way as in Fig. 5A (Fig. 7). The fluorescence drop required Ca2+, and its extent increased with increasing Ca2+ concentration. The Ca2+ concentration dependence agreed with that of the Ca2+-induced fluorescence enhancement in the absence of ATP (cf. Fig. 3).

Kinetics of the Fluorescence Drop following Simultaneous Addition of Ca2+ and ATP—By using the stopped-flow method, Ca2+ and ATP were added simultaneously to EDANS-SRV suspended without Ca2+. When ATP was added with Ca2+ to give 1 \( \mu \text{M} \), the fluorescence increased to a small extent very rapidly and then decreased slowly (Fig. 8A, a). This initial increase probably corresponds to the first and rapid phase of the Ca2+-induced fluorescence enhancement in the absence of ATP (Fig. 8A, b). The subsequent slow drop almost coincided with the second and slow phase of the Ca2+-induced fluorescence enhancement (Fig. 8A, b). For the control, ATP was added to EDANS-SRV suspended in the presence of Ca2+ (Fig. 8B). In this control, the fluorescence intensity was initially on the high level and then decreased rapidly. This drop was much faster than the fluorescence drop following simultaneous addition of Ca2+ and ATP (compare Fig. 8A, a, with Fig. 8B).

When ATP was added with Ca2+ to give 3 \( \mu \text{M} \), the fluores-
extent of the ATP-induced fluorescence drop in the steady state is  
pend in the presence of Ca++. However, in contrast to EP formation (Fig. 9A). When ATP was reduced to 3 
/~), with that of EP formation. When ATP was added to give 100 
(114A) (Fig. 8B). The addition of Ca++ caused no detectable spectrum shift (spectrum 2). On the other hand, the addition of ATP in the presence of Ca++ caused a small but reproducible red shift which resulted in the maximum intensity at 481 nm (spectrum 3). The difference spectrum (Fig. 10B) between EDANS-SRV in the presence of Ca++ and EDANS-SRV in the absence of Ca++ again showed no detectable spectrum shift, as indicated by an upward peak at 479 nm (spectrum 1). In contrast, the difference spectrum between EDANS-SRV in the presence of ATP and EDANS-SRV in the absence of ATP revealed clearly the ATP-induced red shift which gave a downward peak at 469 nm (spectrum 2).

Identification of the Tryptic Fragment Labeled with EDANS—After the SDS-polyacrylamide gel electrophoresis of the tryptic digest of EDANS-SRV, the gel was subjected to protein staining or fluorography (Fig. 11). The ATPase protein was cleaved to fragments A and B within 5 min after the start of digestion. Fragment A was cleaved slowly to subfragments A1 and A2 in further digestion. In contrast, fragment B was not cleaved significantly. These results agree with earlier observations (26, 34, 35). The fluorescence was found only on the protein band of the Ca++-ATPase before the digestion.  

When Ca++ or both Ca++ and ATP were added to control SRV in the sample cuvette (the air being in the reference cuvette), no change in the fluorescence intensity occurred at the wavelength ranging from 400 to 600 nm.
After the digestion, it migrated with fragment B on the gels. It is, therefore, evident that almost all of the bound EDANS was located on fragment B. However, there was a faint protein band with very weak fluorescence closely below the band of fragment B. Although the origin of this band is not clear, it is possible that this band is a degradation product of fragment B, as suggested by Dux et al. (36).

A protein band with a molecular mass similar to that of the Ca\(^{2+}\)-ATPase was found on the gels after 5- and 90-min digestions. Since this protein showed no fluorescence and its degradation was much slower than the appearance of fragments A and B, it is certain that this protein is not the Ca\(^{2+}\)-ATPase. It is possibly glycogen phosphorylase b, which may be a major contaminant in the SRV preparation as stated earlier (37). Two protein bands, the molecular masses of which were nearly equivalent to those of fragments A and B, were observed on the gel before the digestion. They are probably Ca\(^{2+}\)-binding proteins present in SRV, as shown previously (38).

**Purification of EDANS-peptides from the Thermolytic Digest of the EDANS-labeled Ca\(^{2+}\)-ATPase**—The EDANS-labeled Ca\(^{2+}\)-ATPase was purified from the EDANS-SRV by the deoxycholate treatment, and then partially digested with thermolysin. As estimated from the fluorescence intensity, about 90% of the bound EDANS in the digest was recovered in the supernatant after precipitation of the membranous remnants in 2.7% formic acid. The peptides in this supernatant were then analyzed by reversed phase HPLC with a CH\(_2\)CN gradient in 0.1% trifluoroacetic acid (Fig. 12A). The trace of the fluorescence in the chromatogram showed three minor peaks (Peak 1, Peak 2a, Peak 2b) and two major ones (Peak 2 and Peak 3). The amounts of the bound EDANS in Peaks 1, 2, 2a, 2b, and 3, respectively, accounted for 6.9, 37.4, 3.1, 2.4, and 42.0% of the total amount of the bound EDANS.
were obtained with the peptides from phosphate (pH 6.9) and 20 mM Na2SO4. The trace of the subsequent peptide sequencing, which showed that only one component of the EDANS-peptide in the chromatogram of the peptide from the thermolytic digest of the EDANS-labeled Ca2+-ATPase was applied to a PepRPC column and eluted with a CH3CN gradient in 0.1% trifluoroacetic acid. Three fractions indicated by arrows (→) at Peak 1, Peak 2, and Peak 3 were collected separately. In addition, Peaks 2a and 2b were pooled as indicated by an arrow (→). B, the fraction, Peak 2, obtained above was applied to the same column as in A and eluted with a CH3CN gradient in 5 mM sodium phosphate (pH 6.9) and 20 mM Na2SO4. The fraction indicated by an arrow (→) was collected. C, the fraction collected in B was applied to the same column as in A and eluted with a CH3CN gradient in 0.1% trifluoroacetic acid. The fraction indicated by an arrow (→) was collected.

### Table I

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<th>Amino Acid Sequences of Purified EDANS-Peptides</th>
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* X represents the EDANS-labeled cysteine residue which cannot be detected by the protein sequencer used.

DISCUSSION

The present results give strong support to the probability that the changes in the fluorescence intensity of the bound EDANS following additions of Ca2+ and ATP (Fig. 2) reflect conformational events in the vicinity of this probe during the catalytic process of the enzyme. The Ca2+ concentration dependence of the Ca2+-induced fluorescence enhancement (Fig. 3) agrees with that of Ca2+ binding to the high affinity sites of the enzyme (7, 39-42). This shows that the fluorescence enhancement is caused by Ca2+ binding to the high affinity sites. The Hill coefficient, being equivalent to 1.9, is compatible with the well-documented cooperativity in the Ca2+ binding to these sites (7, 41, 42) as well as in the Ca2+-induced enzyme activation (1-3, 43). The biphasic kinetics of this fluorescence enhancement (Fig. 4) is in harmony with the previously reported biphasic behavior of the Ca2+-induced enhancement of intrinsic fluorescence (7-9, 11). This finding suggests that a Ca2+-induced conformational change occurs in two sequential steps. This is also consistent with Dupont’s observation (7) which shows a sequential Ca2+ binding to two interacting sites. Thus, the present results add support to the current model (3, 6-11) in which one Ca2+ has fast access to a site with a low affinity and occupation of this site induces a slow conformational change resulting in an appearance of a second site with a high affinity.

The affinity for ATP in the ATP-induced fluorescence drop (Fig. 5) agrees with that in ATP hydrolysis as well as in EP formation (2, 11, 44, 45). It also agrees well with the affinity for ATP in the ATP-induced drop of intrinsic fluorescence in the EDANS-labeled cysteine residues upon the sequencing. The amino acid sequences of the peptides thus modified were determined by using a protein sequencer. The results showed that the sample of the purified peptide from each peak contained only one component and gave a single amino acid sequence (Table I).
the presence of Ca$^{2+}$ (10, 11). Actually, it is rather higher than the affinity for ATP in ATP binding to the catalytic site in the absence of Ca$^{2+}$ (39, 44, 46, 47). These findings show that the fluorescence drop in the present experiment is caused by ATP binding to the catalytic site.

The results (Fig. 7) demonstrate that Ca$^{2+}$ binding to the high affinity sites is essential for the fluorescence drop following ATP binding to the catalytic site. In addition, the data (Fig. 8A) show that the fluorescence drop following simultaneous addition of ATP and Ca$^{2+}$ is coincident with the second and slow phase of the Ca$^{2+}$-induced fluorescence enhancement and is much slower than the fluorescence drop following addition of ATP to EDANS-SRV preincubated with Ca$^{2+}$. These observations indicate that the second phase of the Ca$^{2+}$-induced conformational change may be prerequisite to the ATP-induced fluorescence drop. Furthermore, the results (Fig. 8A, b, and C) show that the fluorescence drop following simultaneous addition of ATP at a higher concentration and Ca$^{2+}$ is faster than the second phase of the Ca$^{2+}$-induced fluorescence enhancement. This finding suggests that the second phase of the Ca$^{2+}$-induced conformational change may be accelerated by ATP at high concentrations. This possible acceleration is consistent with the previous finding (8, 9) that EP formation following simultaneous addition of ATP and Ca$^{2+}$ is much faster than the Ca$^{2+}$-induced enhancement of intrinsic fluorescence in the absence of ATP. This is also compatible with the results from the kinetic analysis of EP formation (48, 49) showing the accelerating ATP effect on the Ca$^{2+}$-induced enzyme activation.

A comparison of the ATP-induced fluorescence drop with EP formation (Fig. 9) shows that the fluorescence drop represents a conformational change in the enzyme-ATP complex preceding EP formation. This conformational change is much faster than EP formation at a high ATP concentration (Fig. 9A), but it becomes rate-limiting for EP formation as the ATP concentration is reduced (Fig. 9, B and C). The occurrence of this conformational change in the enzyme substrate complex is further supported by the finding that AMP-PCP and AMP-PNP also induce a fluorescence drop almost to the same extent as ATP does. This conclusion is consistent with the earlier observations by Inesi and co-workers (50, 51) showing that AMP-PNP produces the Ca$^{2+}$-dependent change in the ESR spectrum of spin-labeled SRV.

The ATP-induced fluorescence drop in the present experiment can be reasonably explained in terms of the following scheme where $E$ and $E$-ATP denote the Ca$^{2+}$-activated enzyme and the encounter complex of $E$ and ATP, respectively.

\[
E + ATP \xrightarrow{(1)} E \cdot ATP \xrightarrow{(2)} E' \cdot ATP \xrightarrow{(3)} E' \cdot ADP \xrightarrow{(4)} E' + ADP
\]

conformational change (Step 2) following $E$-ATP formation (Step 1), and this conformational change is accompanied by the observed fluorescence drop. Possible conformational changes associated with $E' \cdot ADP$ formation (Step 3) and subsequent ADP liberation (Step 4) are not detected in the present experiment, since the fluorescence level after the initial drop remains constant during the further reaction in which Steps 3 and 4 should occur (21) (Figs. 5 and 9).

In this scheme, it is assumed that Step 1 is favorable to dissociation of $E$-ATP and that Step 2 is greatly favorable to formation of $E' \cdot$ ATP. These assumptions give an interpretation to the findings that the extent of the fluorescence drop is saturated with 3 $\mu$M ATP (Fig. 5) and that, in contrast, the rate of this drop is not saturated even with much higher ATP (Fig. 6). An alternative possibility is that Step 2 is accelerated by ATP binding to the putative regulatory site with a low affinity.

The ATP-induced red spectrum shift (Fig. 10) suggests that the bound EDANS is exposed to a more hydrophilic environment by the ATP-induced conformational change. Most of the labels are found on fragment B (Fig. 11) which includes the carboxyl terminus and the putative ATP-binding domain (4, 53, 54). In an attempt to understand the role of the ATP-induced conformational change in the catalytic process on the submolecular level, it is essential to locate the labeled site on the primary structure of the enzyme and to further investigate the steric relations between the bound EDANS and other sites, such as the fluorescein isothiocyanate binding site (4, 54), during the catalytic process.

From this point of view, the identification of the EDANS-labeled sites was attempted by sequencing the purified EDANS-peptides (Fig. 12 and Table I). From the established primary structure of the Ca$^{2+}$-ATPase (4), it is evident that the EDANS-labeled site in the thermolytic peptides from Peak 2 and Peak 3 is Cys$^{74}$ which is located on tryptic fragment B. On the other hand, the EDANS-labeled site in the thermolytic peptide from Peak 1 is possibly Cys$^{70}$ because the dipeptide, Ala$^{69}$-Cys$^{70}$, could be produced from a peptide, Ala$^{66}$-Ala$^{69}$-Cys$^{70}$-Ile$^{71}$, by the thermolytic cleavage. However, it should be noted that Cys$^{70}$ is probably buried within the membrane as deduced from the primary structure of this enzyme (4). Other sites, such as Cys$^{71}$ in -Ala$^{67}$-Cys$^{71}$-Asn$^{72}$-, Cys$^{76}$ in -Ala$^{69}$-Cys$^{69}$-Arg$^{71}$-, and Cys$^{76}$ in -Ala$^{67}$-Cys$^{71}$-Cys$^{76}$- are also possible candidates for the labeled site in the EDANS-peptide from Peak 1, although the substrate specificity of thermolysin is unfavorable to these possibilities.

The EDANS bound to Cys$^{71}$ accounts for at least 76% of the total amount of the bound EDANS in the EDANS-labeled enzyme, as estimated from the total amount of the bound EDANS present in Peaks 2, 2a, 2b, and 3 (Fig. 12A). It seems, therefore, likely that the observed large extent of the ATP-induced fluorescence drop could be derived from the change in the microenvironment of the EDANS bound to this Cys$^{71}$. This Cys$^{71}$ is also a promising candidate for the EDANS-labeled site responsible for the Ca$^{2+}$-induced fluorescence enhancement. However, other possible sites cannot be entirely excluded because the extent of the Ca$^{2+}$-induced fluorescence change is rather small.

Recently, Kawakita and co-workers (20) reported an ATP-induced fluorescence change with I-EDANS-labeled SRV. In sharp contrast to our results, they observed an increase rather than a decrease in the fluorescence intensity. The amplitude of this fluorescence enhancement varied for different preparations, ranging from 2.5 to 9%, for unknown reasons. Furthermore, no fluorescence change was caused by addition of AMP-PNP. Their conclusion is that the fluorescence change occurs upon ADP liberation from $E' \cdot$ADP complex, being quite different from ours. In spite of the pronounced difference in the fluorescence behavior between their experiment and ours, Yamashita and Kawakita (55) found that the EDANS-labeled site was also Cys$^{71}$. This discrepancy remains unsolved at present.

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