The ATP-induced Change of Tryptophan Fluorescence Reflects a Conformational Change upon Formation of ADP-sensitive Phosphoenzyme in the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase

**STOPPED-FLOW SPECTROFLUOROMETRY AND CONTINUOUS FLOW-RAPID QUENCHING METHOD**

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Satoshi Nakamura\(\dagger\), Hiroshi Suzuki\(\dagger\), and Tohru Kanazawa\(\dagger\)

*From the Departments of \(\dagger\)Biochemistry and \(\dagger\)Dermatology, Asahikawa Medical College, Asahikawa 078, Japan*

The ATP-induced change in the tryptophan fluorescence of the Ca\(^{2+}\)-ATPase was determined with sarcoplasmic reticulum vesicles at pH 7.0 in the presence of Ca\(^{2+}\) under various conditions by steady-state measurements and stopped-flow spectrofluorometry. Formation of the phosphoenzyme intermediate was also determined by the continuous flow-rapid quenching method. The steady-state fluorescence at 0°C decreased by 1.1% on addition of ATP, whereas no fluorescence change was induced by adenosine 5’-(β,γ-methylene)triphosphate (a nonhydrolysable ATP analog incapable of phosphorylating the enzyme). The time course of the ATP-induced fluorescence drop agreed well with that of the phosphoenzyme formation under all of the conditions tested, and the phosphoenzyme formed was largely sensitive to ADP. When phosphoenzyme isomerization from the ADP-sensitive form to the ADP-insensitive form was almost completely prevented by N-ethylmaleimide treatment, the time course of the ATP-induced fluorescence drop again agreed with that of the phosphoenzyme formation. These results show that the ATP-induced fluorescence drop occurs upon formation of the ADP-sensitive phosphoenzyme. The results further indicate that the tryptophan fluorescence of this enzyme is insensitive to the conformational change which was previously shown (Suzuki, H., Obara, M., Kuwayama, H., and Kanazawa, T. (1987) *J. Biol. Chem.* 262, 15448–15456) to occur upon formation of the calcium-enzyme-substrate complex. Thus, we conclude that the ATP-induced drop in the tryptophan fluorescence reflects a conformational change occurring upon formation of the ADP-sensitive phosphoenzyme.

The SR\(\dagger\) Ca\(^{2+}\)-ATPase is a 110-kDa protein which catalyzes ATP hydrolysis coupled to Ca\(^{2+}\) transport (1, 2). The primary structure of the protein has entirely been revealed, and the functional domains have been predicted from the primary structure (3, 4). 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 binding of Ca\(^{2+}\) to the Ca\(^{2+}\)-binding sites on the cytosolic side. The γ-phosphoryl group of ATP bound to the ATP-binding site is transferred to Asp-351 of the activated enzyme, and the affinity of the Ca\(^{2+}\)-binding sites is reduced, and the Ca\(^{2+}\) is released into the lumen.

The energy coupling in this active transport is believed to involve conformational changes, which may be essential for long range interactions between the ATP-binding site in the cytoplasmic domain and the Ca\(^{2+}\)-binding sites in the transmembrane domain (14, 15). Analysis of intrinsic tryptophan fluorescence provides a useful means of detecting the conformational changes in the individual step of the catalytic process. It was originally shown by Dupont and Leigh (16) that ATP binds to the Ca\(^{2+}\)-activated enzyme induces a tryptophan fluorescence drop by a few percent. Subsequently, Dupont *et al.* (17) suggested that this fluorescence drop is due to the formation of ADP-sensitive EP. However, in contrast to this hypothesis, Fernandez-Belda *et al.* (18), Andersen *et al.* (19), and Champeil *et al.* (20) presented data suggesting that the fluorescence drop may be attributed to the isomerization of EP from the ADP-sensitive form to the ADP-insensitive form. Thus, the previous reports on the assignment of this fluorescence drop to a specific reaction step are actually conflicting. For a better understanding of the energy coupling mechanism, it is necessary to reexamine the ATP-induced change in the intrinsic tryptophan fluorescence.

In this study, we have determined the ATP-induced change of tryptophan fluorescence in the presence of Ca\(^{2+}\) with SR vesicles under various conditions by the stopped-flow spectrofluorometry. We have also determined EP formation by the continuous flow-rapid quenching method and compared its time course with that of the ATP-induced fluorescence change. These results demonstrate that the ATP-induced fluorescence drop reflects a conformational change occurring upon formation of ADP-sensitive EP.

**EXPERIMENTAL PROCEDURES**

*Preparation of SR Vesicles*—SR vesicles were prepared from rabbit skeletal muscle as described elsewhere (21) with two following modifications. First, α-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.* (22). Second, the suspension of SR vesicles in 0.6 M KCl and 5 mM Tris maleate (pH 6.5) was centrifuged at 15,000 × g for 20 min. The supernatant was centrifuged at 125,000 × g for 45 min, and the precipitate was homogenized in a buffer containing 0.1 mM CaCl\(_2\), 0.1 M KCl, 0.3 M sucrose, and 5 mM MOPS/Tris (pH 7.9).
The homogenate was washed twice by centrifugation with the same buffer. The SR vesicles thus obtained were stored at −80 °C. The Ca²⁺-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-32P-ATP, 5 mM MgCl₂, 0.5 mM CaCl₂, 0.4 mM EGTA, 0.1 mM KCl, and 20 mM MOPS/Tris (pH 7.0) was incubated for 5 min at 30 °C. The extent of phosphorylation site determined by γ-32P-ATP according to Barrabé et al. (23) was 3.81 ± 0.02 nmol/mg (n = 4).

Treatment of SR Vesicles with NEM—The SR vesicles were treated with NEM in the presence of AMP-PNP according to Kawakita et al. (24). The Ca²⁺-dependent ATPase activity was almost completely suppressed by this treatment, while the content of phosphorylation site was unaffected.

Steady-state Measurements of Tryptophan Fluorescence—The steady-state intensity of the tryptophan fluorescence of SR vesicles was measured on a computer-interfaced spectrofluorometer RF-5000 (Shimadzu, Japan), as described previously (25). The excitation and emission wavelengths were set to 290 and 338 nm, respectively. The slits of 1.5 and 5.0 nm in width were set in the excitation and emission light paths, respectively, unless otherwise specified.

Stopped-flow Measurements of Tryptophan Fluorescence—Rapid kinetic measurements of EP formation were made by using a continuous flow-rapid quenching system (RF-530) interfaced with a personal computer which was programmed to accumulate the photometric data. The excitation wavelength was 290 nm, and the emitted light was passed through a band-pass filter UV-D36A (Toshiba, Japan) which cut off the light below 308 nm and above 410 nm. The reaction was started by mixing equal volumes of solutions from two syringes, one containing SR vesicles and the other containing ATP. The measurement was repeated 600–1200 times, and the accumulated data were analyzed with the program RS1 (BBN Software Corp., Cambridge, MA) for nonlinear least squares fitting on a personal computer (IP/SM Corporation, Armonk, NY).

Determination of EP—Rapid kinetic measurements of EP formation were made by using a continuous flow-rapid quenching system (RF-530) (Unisoku, Japan), as described previously (25). The reaction was started by mixing equal volumes of solutions from two syringes, one containing SR vesicles and the other containing ATP or AMP-PCP. 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 ATP or AMP-PCP, and dissolved in 0.5 N NaOH containing 1% SDS, and the radioactivity was measured.

Miscellaneous Methods—Protein concentrations were determined by the method of Lowry et al. (26) with bovine serum albumin as a standard. Free Ca²⁺ concentrations were calculated as described elsewhere (27). Disodium ATP and amylase were purchased from Boehringer Mannheim. Monosodium ADP was obtained from Yamasa Biochemicals (Japan). AMP-PNP, AMP-PNP, and A23187 were from Sigma. Cr₆₀ was from Sigma Chemicals (Japan). γ-32P-ATP was purchased from Amercham Corp. The pH of solutions of ATP, ADP, and AMP-PNP was adjusted to 7.0 with Tris.

RESULTS AND DISCUSSION

Dependence of the Extent of Fluorescence Drop on ATP and AMP-PCP Concentrations—When ATP was added to SR vesicles in the presence of 5 mM MgCl₂ and 20 μM Ca²⁺, the tryptophan fluorescence decreased with increasing concentration of ATP (Fig. 1). The maximum fluorescence drop was 1.1%, and the ATP concentration giving a half-maximum fluorescence drop was 0.14 μM. A similar extent of the fluorescence drop was obtained by addition of ATP in the presence of 5 mM CaCl₂ without added MgCl₂, and the ATP concentration giving a half-maximum fluorescence drop was 0.08 μM. These high affinities for ATP in the ATP-induced fluorescence drop are consistent with previously reported affinities of the catalytic site of the SR Ca²⁺-ATPase for Mg-ATP (28-30) and Ca-ATP (31-33). These results indicate that this fluorescence drop is caused by binding of Mg-ATP or Ca-ATP to the catalytic site of this enzyme. The extent of this drop is comparable to that obtained with SR vesicles in other laboratories (16-19).

On the other hand, no fluorescence change was induced by addition of AMP-PNP (a nonhydrolyzable ATP analog which is incapable of phosphorylating the enzyme) (Fig. 1). This is in sharp contrast to our previous findings that the fluorescence of bound EDANS in EDANS-labeled SR vesicles decreased to a large extent (17–20%) upon addition of AMP-PNP under the same conditions as in Fig. 1 or in the presence of 10 mM MgCl₂ and 0.1 mM Ca²⁺ (25). Our results obtained earlier (25, 34, 35) demonstrated that this AMP-PNP-induced drop in the fluorescence of bound EDANS is attributed to a conformational change which occurs upon formation of the calcium-enzyme-substrate complex. Thus, the present results showing the lack of the AMP-PNP-induced change in the tryptophan fluorescence of this enzyme is insensitive to the conformational change occurring upon formation of the calcium-enzyme-substrate complex. It is, therefore, likely that the ATP-induced drop in the tryptophan fluorescence reflects a change in the conformation of the calcium-enzyme-substrate complex. This property is supported by the following results which were obtained by the stopped-flow spectrofluorometry and the continuous flow-rapid quenching method.

Coincidence between Mg-ATP-Induced Fluorescence Drop and EP Formation—The Mg-ATP-induced fluorescence drop and EP formation were followed in the presence of 5 mM MgCl₂ and 20 μM Ca²⁺ by the stopped-flow spectrofluorometry and the continuous flow-rapid quenching method, and the time course of the fluorescence drop was compared with that of EP formation. When ATP was added to give 0.5 μM, the fluorescence drop occurred coincidently with EP formation and was fitted well with a single exponential (Fig. 2). When ATP was increased to 1 μM at a final concentration, the fluorescence drop became about twice faster and again coincided with EP formation (data not shown).

Coincidence between Ca²⁺-ATP-Induced Fluorescence Drop and EP Formation—The time course of the Ca²⁺-ATP-induced fluorescence drop was compared with that of EP formation in the presence of a low or high concentration of CaCl₂ without added MgCl₂. When ATP was added to give 100 μM at 0°C in the presence of 0.25 mM CaCl₂, the fluorescence drop occurred coincidently with EP formation and was fitted well with a single exponential (Fig. 3A). When the measurement was per-
formed in the presence of 5 mM CaCl₂ under the otherwise same conditions as above, the fluorescence drop again coincided with EP formation (Fig. 3B). The ratio of the amplitude of the fluorescence drop to the amount of EP formed was almost the same as that in the presence of 0.25 mM CaCl₂, although the amplitude of the fluorescence drop and the amount of EP formed in the presence of 5 mM CaCl₂ were somewhat larger than those in the presence of 0.25 mM CaCl₂. Thus, the increase in the Ca²⁺ concentration from 0.25 to 5 mM had no significant effect on the kinetic and quantitative relationships between the fluorescence drop and EP formation. When the reaction temperature was raised to 15°C under the otherwise same conditions as in Fig. 3A, the fluorescence drop and EP formation became about four times faster (data not shown). Irrespective of this elevated reaction temperature, the fluorescence drop coincided with EP formation. When ATP was reduced to 0.2 µM at a final concentration in the same conditions as in Fig. 3B, the fluorescence drop and EP formation were markedly retarded (data not shown). Regardless of this reduced reaction rate, the fluorescence drop again coincided with EP formation.

Altogether, the present results (Figs. 2 and 3) demonstrate that the time course of the ATP-induced fluorescence drop agrees well with that of EP formation under all of the different conditions tested. The results further show that EP formed under the present conditions has largely been sensitive to ADP.

ATP-induced Fluorescence Drop Is Attributed to Formation of ADP-sensitive EP—All the above findings indicate that the ATP-induced drop in the tryptophan fluorescence is closely related to EP formation. The data further suggest that this fluorescence drop occurs upon formation of ADP-sensitive EP, because EP formed under the present conditions has mostly been sensitive to ADP. To ascertain whether this idea is valid, SR vesicles were pretreated with NEM under the conditions where isomerization of EP from the ADP-sensitive form to the ADP-insensitive form should be prevented (24). In agreement with our previous observations (35), when ATP was added to untreated SR vesicles in the absence of K⁺ and presence of CₐEₘ, ADP-insensitive EP accumulated to a large extent (Fig. 4). In contrast, when ATP was added to the NEM-treated SR vesicles under the same conditions as above, no ADP-insensitive EP accumulated. The total amount of EP formed was unaffected by this NEM treatment. These findings show that the isomerization of EP from the ADP-sensitive form to the ADP-insensitive form was in fact almost completely prevented by the NEM treatment.

When ATP was added to give 1 µM to the NEM-treated vesicles in the absence of K⁺ and presence of CₐEₘ under the...
otherwise same conditions as in Fig. 2, the fluorescence drop occurred coincidentally with the formation of ADP-sensitive EP (Fig. 5). The rates of the fluorescence drop and EP formation were unaffected by this NEM treatment. These findings lead to the conclusion that the ATP-induced fluorescence drop is attributed to the formation of ADP-sensitive EP.

Possible Location of Tryptophans Responsible for the ATP-induced Fluorescence Drop—According to the predicted domain structure (3, 4), Trp-552 alone is located in the cytoplasmic domain, all of the other tryptophans (12 residues) being in or near the transmembrane domain. We previously showed that Cys-674 of the Ca$^{2+}$-ATPase is exclusively labeled with EDANS in the EDANS-labeled SR vesicles (34). This cysteiny residue is located near the ATP-binding site in the cytoplasmic domain (22, 36-45). Thus, the previously observed change in the fluorescence of bound EDANS upon formation of the calcium-enzyme-substrate complex is attributed to the conformational change in the vicinity of the ATP-binding site. In contrast, the fluorescence of Trp-552 is insensitive to this conformational change (cf. Fig. 1) although Trp-552 is also located near the ATP-binding site (22, 36-45).

It was previously suggested by Champeil and co-workers (20, 46) that a hydrophobic ionophore A23187 preferentially quenches the fluorescence of tryptophans located in or near the transmembrane domain and that these tryptophans are responsible for the well known Ca$^{2+}$-induced enhancement of the tryptophan fluorescence (16-18). In the present experiments (Fig. 6), 93-94% of the tryptophan fluorescence was quenched by 30 μM A23187 (center traces in A and B). The fluorescence drop (5.6%) occurred on addition of excess EGTA in the absence of A23187 (left trace in A), being in agreement with the observations reported previously (16-18). This EGTA-induced fluorescence drop was greatly reduced in the presence of A23187 (the drop was 1.2% of the fluorescence obtained before the addition of A23187) (right trace in A). These findings are in harmony with the above view of Champeil’s group, although the involvement of Trp-552 cannot entirely be excluded.

In agreement with the results given in Fig. 1, the fluorescence drop (0.9%) was induced by addition of ATP in the absence of A23187 (left trace in B). This fluorescence drop was almost completely inhibited by A23187: added previously (right trace in B). Earlier, we showed that ADP-sensitive EP selectively accumulates at the high A23187 concentration used in this experiment (47). It is possible that the observed A23187-induced fluorescence drop reflects a conformational change associated with Ca$^{2+}$ occlusion, which is coupled to formation of ADP-sensitive EP, at the high affinity Ca$^{2+}$-binding sites in the transmembrane domain.

Previously, on the basis of the kinetic analysis of EP formation from ATP, Petithory and Jencks (48) suggested the existence of a rate-limiting conformational change followed by rapid phosphoryl transfer from ATP to the enzyme. This conformational change predicted by Petithory and Jencks possibly corresponds to the ATP-induced fluorescence drop shown in the present experiments.

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**REFERENCES**

ATP-induced Tryptophan Fluorescence Change in SR Ca\(^{2+}\)-ATPase