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Independent Flexible Motion of Submolecular Domains of the Ca^{2+},Mg^{2+}-ATPase of Sarcoplasmic Reticulum Measured by Time-Resolved Fluorescence Depolarization of Site-Specifically Attached Probes

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ABSTRACT: The Ca^{2+}-transporting ATPase of rabbit skeletal muscle sarcoplasmic reticulum was site-specifically labeled with either *N*-(1-anilinonaphth-4-yl)maleimide (ANM) or 5-[[(iodoacetamido)-ethyl]amino]naphthalene-1-sulfonate (IAEDANS), and the segmental motion of submolecular domains of the ATPase molecule was examined by means of time-resolved and steady-state fluorescence anisotropy measurements. The ANM-binding domain showed wobbling with a rotational relaxation time ϕ = 69 ns in the absence of free Ca^{2+} without any independent wobbling of the ANM moiety. The IAEDANS-binding domain showed a significantly slower wobbling with ϕ = 190 ns in the absence of Ca^{2+}. The present results demonstrated for the first time that the ATPase molecule is composed of distinct domains whose mobilities are considerably different from each other. The binding of Ca^{2+} to the transport site increased the segmental motion of ANM-labeled domain, leading to a ϕ value of 65 ns. Solubilization of the ANM-labeled SR membranes by deoxycholate led to a further increase in the segmental flexibility (ϕ = 48 ns in the absence of free Ca^{2+}), indicating that the mobility of the ANM-binding domain was considerably restricted through interaction with the membrane. The mobility of the ANM-binding domain of solubilized ATPase was also increased to some extent upon binding of Ca^{2+}.

Active Ca^{2+} transport across the sarcoplasmic reticulum (SR)[1] membranes is carried out by Ca^{2+},Mg^{2+}-ATPase (SR-ATPase), an intrinsic membrane protein of M_r 110000 whose amino acid sequence has recently been deduced from the nucleotide sequence of a cDNA clone (Brandl et al., 1986). The mechanism of coupling between ATP hydrolysis and the uphill movement of Ca^{2+} ions against the concentration gradient has long been a subject of intensive studies [for a recent

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[1] Abbreviations: ANM, *N*-(1-anilinonaphth-4-yl)maleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; E-P intermediate, enzyme-phosphate complex intermediate; IAEDANS, 5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonate; SR, sarcoplasmic reticulum; SR-ATPase, Ca^{2+},Mg^{2+}-ATPase of the sarcoplasmic reticulum; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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review, see Inesi (1985)]. Although the details of this coupling mechanism at the molecular level have not been well understood yet, there is little doubt that conformational transitions of the ATPase molecule underlie the process of gating and affinity change for Ca^{2+} ions and constitute the essence of the molecular mechanism of active transport.

In recent years, we and others have attempted to observe and characterize the conformational changes of SR-ATPase during the Ca^{2+}-transport cycle. These attempts include monitoring of the side-chain reactivities (Ikemoto et al., 1978; Murphy, 1978) and of the susceptibility to tryptic cleavage (Imamura et al., 1984) and the use of intrinsic (Dupont, 1976, 1978; Dupont & Le Maire, 1980; Champeil et al., 1986) and extrinsic spectral probes (Champeil et al., 1976; Coan & Inesi, 1977; Coan et al., 1979; Pick, 1981; Yasuoka-Yabe & Kawakita, 1983; Yasuoka-Yabe et al., 1983; Baba et al., 1986; Highsmith, 1986; Wakabayashi et al., 1986; Davidson & Berman, 1987; Suzuki et al., 1987). During the course of these studies we have developed procedures for the specific labeling of a limited number of SH groups of the ATPase molecule by several fluorescence and paramagnetic probes (Yasuoka-Yabe & Kawakita, 1983; Baba et al., 1986) and provided several lines of evidence indicating the conformational changes accompanying the binding of Ca^{2+} or nucleotides and the formation of E–P intermediate (Yasuoka-Yabe & Kawakita, 1983; Yasuoka-Yabe et al., 1983; Baba et al., 1986). Recently, these labeling sites were identified by peptide analysis (Saito-Nakatsuka et al., 1987; Yamashita & Kawakita, 1987). By using these sites as the targets of extrinsic probes, we would therefore be able to relate the observed changes with particular domains of the ATPase molecule.

In the present study, we have extended the previous works and have analyzed the conformational change accompanying the binding of Ca^{2+} by observing the time-averaged and time-resolved fluorescence anisotropies which represent rotational diffusion of the fluorophore-binding portion of SR-ATPase. Phosphorescence and absorption anisotropy measurements of triplet probes such as (iodoacetamido)eosin and erythrosinyl isothiocyanate covalently attached to SR-ATPase are based on the same principle as fluorescence anisotropy measurements and have been utilized in studying rotational mobility of this protein in the SR membranes (Buerkli & Cherry, 1981; Speirs et al., 1983; Restall et al., 1985). In addition to the rotation of the whole molecule around the membrane normal axis with a rotational correlation time of about 40 μs, the triplet anisotropy decay suggested a flexible motion of a submolecular segment on a submicrosecond time scale (Buerkli & Cherry, 1981; Speirs et al., 1983; Restall et al., 1985). However, due to the disturbance by an intense prompt fluorescence, phosphorescence and absorption anisotropy measurements may not be sufficiently sensitive to molecular rotation on a 1–100-ns time scale. In contrast, the fluorescence anisotropy decay measurements are particularly sensitive to rotation on this time scale, on which a possible segmental rotation occurs. In this study we chose *N*-(1-anilinonaphth-4-yl)maleimide (ANM) and 5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonate (IAEDANS) as fluorescent probes. These probes are particularly valuable for the study of segmental motion of SR-ATPase, because they bind to distinct sites of the ATPase molecule which probably belong to different segments of the molecule and their respective binding sites are well-defined in the amino acid sequence of the ATPase peptides (Saito-Nakatsuka et al., 1987; Yamashita & Kawakita, 1987).

Materials and Methods

Materials. SR membranes were prepared from rabbit skeletal muscle as previously described (Kawakita et al., 1980). Site-specific labeling of the SR-ATPase by either ANM or IAEDANS was carried out as described in detail in our previous reports after a pretreatment with *N*-ethylmaleimide to minimize the labeling at nonspecific sites (Yasuoka-Yabe & Kawakita, 1983; Baba et al., 1986; Saito-Nakatsuka et al., 1987). When necessary, unbound fluorescent probes trapped by the membrane lipid were washed out with repeated centrifugation in the presence of bovine serum albumin. The fluorescent probes were purchased from Teika Pharmaceuticals Co., Toyama. Typically, 0.7–0.8 mol of either probe was bound per mole of ATPase, and the labeled SR membranes retained full Ca^{2+}-transporting activity. Radioactive phosphate and calcium were obtained from New England Nuclear. ATP was purchased from Oriental Yeast Industry Co. and deoxycholate from Nakarai Chemicals Co. All other chemicals were of the highest purity commercially available.

Biochemical Methods. The Ca^{2+}-transport, ATPase, and E–P-forming activities were measured as described previously (Kawakita et al., 1980; Yasuoka-Yabe & Kawakita, 1983). The concentration of free Ca^{2+} in the Ca^{2+}–EGTA buffer system was calculated by using a value of 1.26×10^{-6} M^{-1} for the apparent binding constant of the Ca^{2+}–EGTA complex at pH 7.0 (Ogawa, 1968). Protein was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

Fluorescence Decay Measurements and Analysis. For all the present time-resolved and steady-state fluorescence measurements, samples were suspended in a solution (pH 7.0) containing 40 mM TES, 0.1 M KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and an appropriate amount of EGTA to give a desired free Ca^{2+} concentration. Measurements with deoxycholate-solubilized samples were carried out in the same solution supplemented with 15% glycerol to protect the samples from inactivation.

The nanosecond pulse fluorometer used for fluorescence decay measurement was detailed elsewhere (Kawato et al., 1977). Briefly, the sample suspension was excited by a vertically polarized light of about 1-ns duration at wavelengths of 355 nm for ANM and 345 nm for IAEDANS. All the fluorescence above 420 nm for ANM or 490 nm for IAEDANS was collected through cutoff filters (SC-39 and SC-42 for ANM, or SC-39 and SC-49 for IAEDANS). The signals were analyzed by calculating the total fluorescence intensity $I_T(t)$ and the fluorescence anisotropy $r(t)$, given by

$$I_T(t) = I_V(t) + 2I_H(t) \quad (1)$$

$$r(t) = [I_V(t) - I_H(t)]/[I_V(t) + 2I_H(t)] \quad (2)$$

where $I_V(t)$ and $I_H(t)$, respectively, represent the intensities of vertically and horizontally polarized components at time t after the flash. The quantities $I_T(t)$ and $r(t)$ are related to $I_T^\delta(t)$ and $r^\delta(t)$, the response to an impulsive excitation expressed as $\delta(t)$ by the equations:

$$I_T(t) = \int_0^t g(t')\, I_T^\delta(t-t')\, dt' \quad (3)$$

$$r(t)\, I_T(t) = \int_0^t g(t')\, r^\delta(t-t')\, I_T^\delta(t-t')\, dt' \quad (4)$$

where $g(t)$ is the response function of the apparatus (Kawato et al., 1977). The fluorescence lifetime τ_i and the rotational relaxation time ϕ_j were determined by curve-fitting procedures,

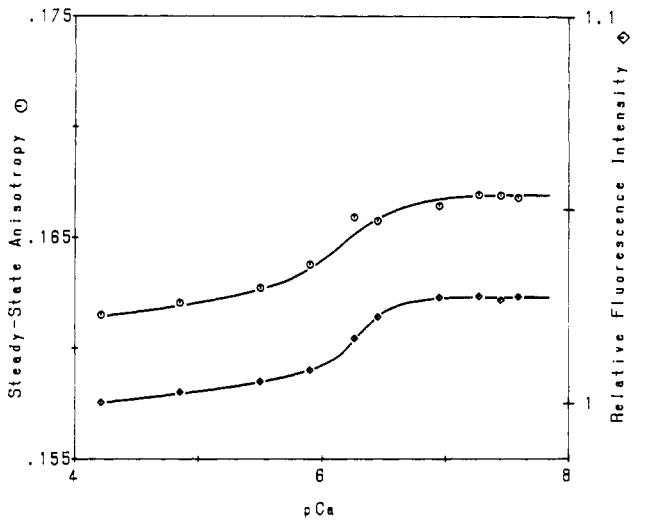

FIGURE 1: Ca^{2+} concentration dependence of steady-state fluorescence intensity and anisotropy of ANM-labeled SR membranes. SR membranes labeled with ANM at SH_N were suspended at a concentration of 1 mg/mL in 1 mL of the standard medium as described under Materials and Methods. Ca^{2+} concentration was adjusted by successive addition of appropriate amounts of EGTA in aliquots of 1 μL. Fluorescence intensity I_T^s (◇) and anisotropy r^s (○) were measured under constant illumination.

by assuming that $I_T^b(t)$ and $r^b(t)$ were expressed as sums of exponential functions:

$$I_T^b(t) = \sum_{i=1}^{n} I_i \exp(-t/\tau_i) \quad (5)$$

$$r^b(t) = \sum_{j=1}^{m} r_j \exp(-t/\phi_j) \quad (6)$$

The average lifetime is defined as

$$\langle \tau \rangle = \sum_{i=1}^{n} \alpha_i \tau_i \quad (7)$$

where

$$\alpha_i = I_i / \sum_{i=1}^{n} I_i \quad (8)$$

α_i ($i = 1, \ldots, n$) is an exponential fraction.

Steady-State Fluorescence Measurements. Steady-state fluorescence intensity I_T^s and anisotropy r^s were measured by using a Hitachi F-3000 spectrofluorometer equipped with a set of polarizer and analyzer. Excitation and emission wavelengths and the choices of filters were the same as in the fluorescence decay measurements. The excitation slit was set at 3 nm and the emission slit at 40 nm. The steady-state measurement is precise and quick compared with the time-resolved measurements. It was therefore used to obtain convincing evidence for a minor change in mobility of fluorescent probes, although complex molecular motions cannot be quantitatively analyzed with this measurement. Steady-state fluorescence anisotropy r^s is related to the time-resolved anisotropy and intensity by

$$r^s = \int_0^\infty r^b(t)\, I_T^b(t)\, dt \Big/ \int_0^\infty I_T^b(t)\, dt \quad (9)$$

RESULTS

Fluorescence Anisotropy and Intensity of ANM Bound to SR-ATPase. Steady-state fluorescence intensity I_T^s and anisotropy r^s of ANM covalently attached to the ATPase molecule were measured. Under appropriate conditions, ANM is assumed to specifically modify 0.5 mol/mol of ATPase each

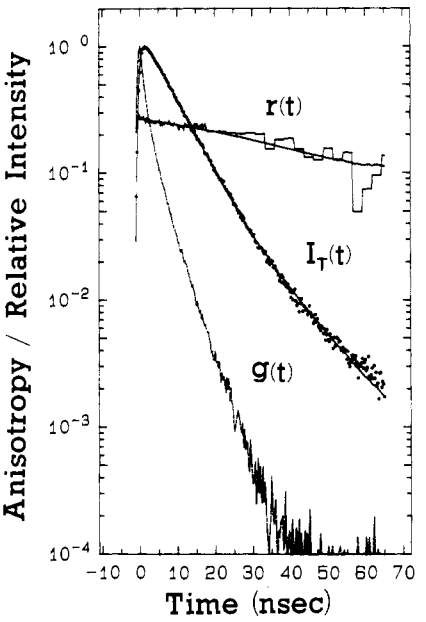

FIGURE 2: Time-resolved fluorescence intensity $I_T(t)$ and anisotropy $r(t)$ of ANM-labeled SR membranes. Measurements were carried out at 20 °C at a concentration of 0.25 mg of the labeled membranes/mL in a medium containing 0.03 μM free Ca^{2+}. $I_T(t)$: Dots are experimental data, and the solid line is the best fit curve by a triple-exponential approximation ($\alpha_1 = 0.594$, $\tau_1 = 0.7$ ns, $\alpha_2 = 0.354$, $\tau_2 = 5.0$ ns, $\alpha_3 = 0.052$, $\tau_3 = 12.9$ ns). $r(t)$: The zig-zag line is experimental data, and the solid line is the best fit curve by a monoexponential approximation. $g(t)$ (chain line) is the apparatus response function.

Table I: Decay Parameters of Fluorescence Anisotropy and Intensity for ANM-Labeled SR-ATPase

$[Ca^{2+}]$ (μM)	deoxycholate (%)	ϕ^a (ns)	$\langle\tau\rangle^b$ (ns)
0.03		69 (0.5)[c]	3.3 (0.1)
1.0		65 (0.2)	3.3 (0.1)
0.03	0.2	43 (2)	4.5 (0.2)
1.0	0.2	41 (2)	4.6 (0.3)

[a] ϕ was obtained according to the monoexponential approximation of $r^b(t) = r(0) \exp(-t/\phi)$. [b] $\langle\tau\rangle$ was calculated according to eq 7 with $n = 3$ from triple-exponential approximation of $I_T^b(t) = I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2) + I_3 \exp(-t/\tau_3)$. [c] Numbers in parentheses are standard errors from several experiments.

of Cys344 and Cys364 (SH_N), which are likely located very close to each other, without inhibiting the activity of the Ca^{2+}-transport system (Yasuoka-Yabe & Kawakita, 1983; Saito-Nakatsuka et al., 1987). When ANM labeling of ATPase was examined fluorometrically in the presence of twice molar excess amount of ANM to ATPase, one SH_N (either Cys344 or Cys364) was observed to be rapidly reacted with ANM; however, the reactivity of a second SH_N was very low. The results suggest that the first ANM prevents binding of the next ANM within the same ATPase molecule, probably because two SH_N residues are close to each other. Under the present labeling condition of 0.7–0.8 ANM molecule/ATPase molecule, only one SH_N is likely to be labeled.

Fluorescence intensity I_T^s and anisotropy r^s at various Ca^{2+} concentrations are shown in Figure 1. The I_T^s decreased by 9%, reflecting the binding of Ca^{2+} in the range of concentration from 0.03 to 1 μM. This is essentially in accordance with our previous observation (Yasuoka-Yabe & Kawakita, 1983). Concomitantly with this decrease in I_T^s, r^s was also decreased by 2%. This indicates that the time-averaged mobility of ANM was increased upon Ca^{2+} binding.

Time-resolved fluorescence measurements were carried out in order to examine the effect of Ca^{2+} on the complex motion

of ANM bound to SR-ATPase, including a possible nanosecond independent wobbling of ANM and a segmental motion of the ANM-binding subdomain of SR-ATPase. The time-resolved fluorescence anisotropy $r(t)$ showed a monoexponential decay with a rotational relaxation time ϕ = 69 ns for Ca^{2+}-free SR-ATPase ([Ca^{2+}] = 0.03 μM) and ϕ = 65 ns, for Ca^{2+}-bound ATPase ([Ca^{2+}] = 1 μM) (Figure 2, Table I). No rapid independent wobbling of ANM with a ϕ value in the range from subnanosecond to a few nanoseconds was observed. This implies that ANM bound to SH_N is completely immobile within its binding pocket. This is in accordance with our previous observation that a maleimide spin label attached to SH_N was also strongly immobilized (Yasuoka-Yabe et al., 1983). The observed decays in $r(t)$ with ϕ = 69 ns and ϕ = 65 ns in the absence and presence of Ca^{2+}, respectively, are likely due to motion of a large submolecular domain to which ANM is firmly attached. The observed decrease in the ϕ value by 4 ns upon Ca^{2+} binding to SR-ATPase is small but definite and is consistent with the decrease in r^s upon binding of Ca^{2+}. The decrease in ϕ upon Ca^{2+} binding was reproducible over 10 independent experiments. The results indicate that the binding of Ca^{2+} causes an increase in the segmental mobility of the ANM-binding domain but does not affect the immobility of ANM at its binding site.

The total fluorescence intensity $I_T(t)$ showed a triple exponential decay for both Ca^{2+}-free and Ca^{2+}-bound SR-ATPase. The averaged lifetime $\langle\tau\rangle$ = 3.3 ns was unchanged upon binding of Ca^{2+} to ATPase (Table I). The longest component of the lifetime τ_3 was about 13 ns much longer than $\langle\tau\rangle$, supporting the reliability of the change in ϕ from 69 to 65 ns upon Ca^{2+} binding.

In order to examine possible effects of the lipid bilayer on the motion of ATPase, $r(t)$ of the ANM-labeled SR-ATPase was measured after solubilization of the membranes with deoxycholate. The steady-state fluorescence anisotropy r^s of the ANM-labeled SR-ATPase was gradually increased from 0.15 to 0.21 upon addition of increasing amounts of deoxycholate up to 2 mg/mL, in parallel with the clarification of the sample, and then leveled off (data not shown).

ATPase and E–P-forming activities of SR-ATPase remained unaffected by solubilization with 2 mg/mL deoxycholate in the presence of 15% glycerol, and these activities were stable even in the absence of Ca^{2+} at 20 °C for more than 4 h within which time-resolved experiments were completed.

A typical result of the time-resolved fluorescence anisotropy measurement at 0.03 μM Ca^{2+} (Ca^{2+}-free ATPase) for deoxycholate-solubilized ANM(SH_N)-labeled SR-ATPase shown in Figure 3 revealed a monoexponential decay of $r(t)$ with ϕ = 43 ns. The ϕ value was significantly shorter than that for the membrane-bound ATPase, suggesting that the mobility of the ANM-binding domain is considerably suppressed in intact membranes probably due to interactions with membrane lipids. No rapid independent wobbling of ANM was observed, and furthermore, $r(0)$ = 0.34 was almost the same as the limiting anisotropy r_0 = 0.365 for completely immobilized ANM (Kawato et al., 1980) within experimental error. This indicates that ANM is tightly fixed within the binding pocket. The observed lower $r(0)$ = 0.25 for ATPase in intact SR is probably due to a decrease in $r(0)$ caused by considerable light scattering of SR vesicles. It should be noted that light scattering does not alter the shape of $r(t)$ decay.

The results in the presence of 1 μM Ca^{2+} were qualitatively similar; i.e., $r(t)$ showed a monoexponential decay without rapid anisotropy decay due to the local probe wobbling. Although a small difference was noted between the ϕ value

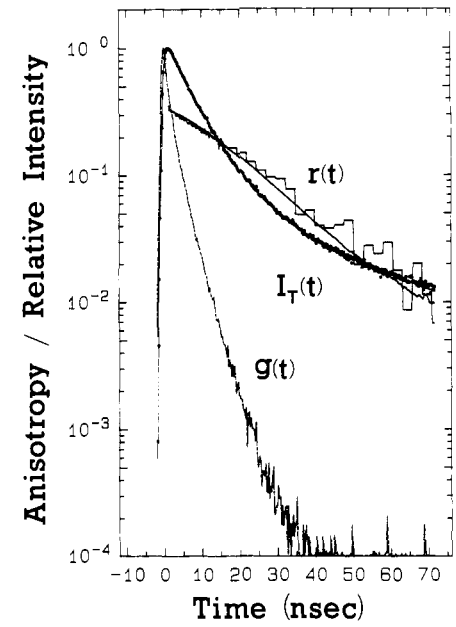

FIGURE 3: Time-resolved fluorescence intensity $I_T(t)$ and anisotropy $r(t)$ of the ANM-labeled SR-ATPase solubilized with deoxycholate. ANM-labeled SR membranes (0.25 mg/mL) in the standard medium supplemented with 15% glycerol were solubilized with 0.2% deoxycholate. Free Ca^{2+} concentration was 1.00 μM. $I_T(t)$: Dots are experimental data, and the solid line is the best fit curve by a triple-exponential approximation (α_1 = 0.722, τ_1 = 1.2 ns, α_2 = 0.253, τ_2 = 6.7 ns, α_3 = 0.025, τ_3 = 37.2 ns). $r(t)$: The zig-zag line is experimental data, and the solid line is the best fit curve by a monoexponential approximation. $g(t)$ (chain line) is the apparatus response function.

Table II: Steady-State Fluorescence Anisotropy of Deoxycholate-Solubilized ANM-Labeled SR-ATPase

expt	[Ca^{2+}] (μM)	r^s	I_T^s [a]
1	1.0	0.225 [b]	104.7
	0.03	0.228	107.8
2	1.0	0.228	111.4
	0.03	0.230	114.2

[a] The steady-state fluorescence intensity I_T^s is expressed in an arbitrary unit. [b] The values of r^s and I_T^s are the average from several samples. Standard error of r^s is 0.0005.

at 0.03 and 1 μM Ca^{2+} (Table I), this was within the range of experimental error and was by no means convincing by itself. Therefore, the effect of Ca^{2+} ions on the mobility of ANM bound to the deoxycholate-solubilized ATPase was examined by comparing the value of steady-state fluorescence anisotropy r^s at 0.03 and 1.0 μM Ca^{2+}. The results of typical experiments shown in Table II indicate that r^s decreased by 1% upon binding of Ca^{2+} to the ATPase molecule, and this change was reversible upon release of Ca^{2+} from ATPase by the addition of EDTA. This suggests that the Ca^{2+}-induced increase in the mobility of the ANM-binding domain is at least in part due to the conformational change of the ATPase molecule itself rather than the change in the interaction between the membrane and the ATPase peptide.

Fluorescence Intensity and Anisotropy of IAEDANS Bound to SR-ATPase. To examine whether the local environment and the segmental mobility of the dye-binding domains differ depending on the nature of a particular probe and the attachment site, IAEDANS was chosen as the second fluorescent probe directed to a distinct site. IAEDANS is covalently attached to Cys674 of the ATPase molecule (Yamashita & Kawakita, 1987). The steady-state fluorescence intensity I_T^s and anisotropy r^s of IAEDANS were measured at various Ca^{2+}

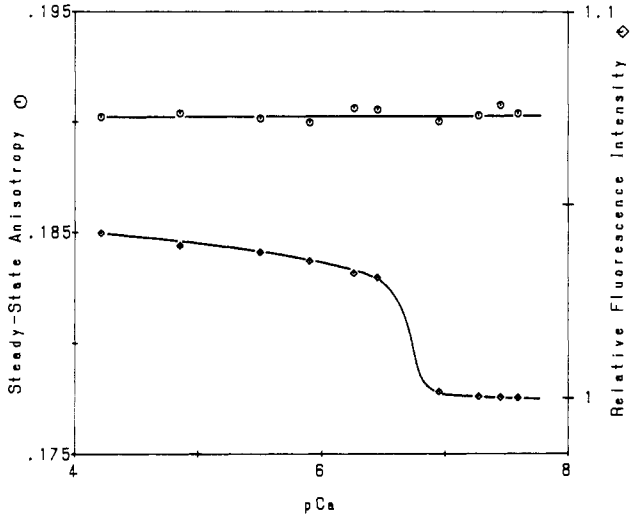

FIGURE 4: Ca^{2+} concentration dependence of steady-state fluorescence intensity and anisotropy of IAEDANS-labeled SR membranes. SR membranes labeled with IAEDANS at Cys674 were suspended at a concentration of 1 mg/mL. Other details are as in Figure 1. (◇) Fluorescence intensity I_T^s; (○) fluorescence anisotropy r^s.

Table III: Decay Parameters of Fluorescence Anisotropy and Intensity for IAEDANS-Labeled SR-ATPase

$[Ca^{2+}]$ (μM)	ϕ^a (ns)	$\langle\tau\rangle^b$ (ns)
0.03	192 (3)[c]	8.5 (0.4)
1.0	189 (2)	8.9 (0.4)

[a] ϕ was obtained according to the monoexponential approximation of $r^s(t) = r(0)\exp(-t/\phi)$. [b] $\langle\tau\rangle$ was calculated according to eq 7 with $n = 2$ from double-exponential approximation of $I_T^s(t) = I_1\exp(-t/\tau_1) + I_2\exp(-t/\tau_2)$. [c] Numbers in parentheses are standard errors from several experiments.

concentrations. As shown in Figure 4, I_T^s increased by 3% when the Ca^{2+} concentration was increased from 0.03 to 1 μM in accordance with our previous observation (Baba et al., 1986). The change occurred at the same concentration of Ca^{2+} as that of I_T^s for the ANM-labeled SR-ATPase, although the direction of the change due to the conformational change was opposite. This indicates that the environment of IAEDANS was also changed due to the conformational change of SR-ATPase caused by Ca^{2+} binding to the transport sites. However, r^s did not change significantly over the range of Ca^{2+} concentration between 0.01 and 0.1 mM. The conformational change caused by Ca^{2+} ions does not seem to affect the time-averaged mobility of the probe.

Molecular motion of the bound fluorophore was investigated in further detail through time-resolved fluorescence anisotropy measurements. Figure 5 shows a typical result obtained with Ca^{2+}-free SR-ATPase ($[Ca^{2+}] = 0.03$ μM). Fluorescence anisotropy showed a monoexponential decay which is characterized by rotational relaxation time $\phi = 189$ ns. Almost the same $r(t)$ curve showing monoexponential decay with $\phi = 192$ ns was obtained for Ca^{2+}-bound ATPase at $[Ca^{2+}] = 1$ μM (Table III). Because of the relatively shorter lifetime of IAEDANS ($\langle\tau\rangle = 8.5$–9 ns) as compared with the much longer ϕ value, the observed difference in ϕ values between 189 and 192 ns is not convincing within experimental error. Therefore, $r(t)$ did not seem to change significantly upon Ca^{2+} binding to the ATPase. This interpretation is consistent with the observation that r^s scarcely changed regardless of whether Ca^{2+} was present or absent (see Figure 4). When the membrane was solubilized by deoxycholate, the ϕ value of IAEDANS remarkably decreased from 189 to 116 ns by about 70 ns, which may suggest that the IAEDANS-labeled domain

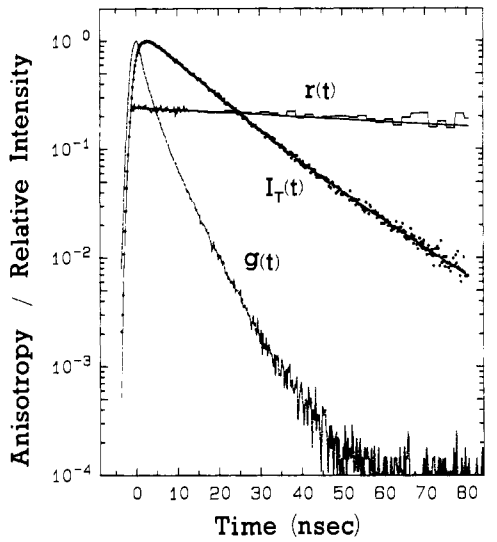

FIGURE 5: Time-resolved fluorescence intensity $I_T(t)$ and anisotropy $r(t)$ of IAEDANS-labeled SR membranes. Measurements were carried out as in Figure 2 except that IAEDANS-labeled SR membranes were used. $I_T(t)$: Dots are experimental data, and the solid line is the best fit curve by a double-exponential approximation ($\alpha_1 = 0.650$, $\tau_1 = 2.6$ ns, $\alpha_2 = 0.350$, $\tau_2 = 14.8$ ns). $r(t)$: The zig-zag line is experimental data, and the solid line is the best fit curve by a monoexponential approximation. $g(t)$ (chain line) is the apparatus response function.

is immobilized by the membrane.

Discussion

In this article segmental motion of the SR-ATPase and its conformational changes induced by the binding of Ca^{2+} ions were analyzed by means of fluorescence anisotropy measurements.

Restricted Wobbling of Submolecular Domains of SR-ATPase. Results of recent studies with triplet anisotropy measurements on rotational diffusion of the ATPase molecules in the SR membranes imply a rapid segmental motion of the molecule in the submicrosecond time range in addition to an indication of the rotation of the whole ATPase molecule in the range of 50–100 μs (Buerkli & Cherry, 1981; Speirs et al., 1983; Restall et al., 1985). However, since these experiments were performed under rather unphysiological conditions in a viscous medium such as 50% sucrose or 60% glycerol to reduce tumbling of the SR vesicles, examination of rotational diffusion in a medium of more physiological composition was necessary to obtain conclusive evidence for a segmental motion of ATPase. Furthermore, absorption and phosphorescence anisotropy measurements may not be sensitive enough to analyze the rotation on a 1–100-ns time range quantitatively because of the disturbance by an intense prompt fluorescence of triplet probes. These difficulties are obviated in the present study by using nanosecond pulse fluorometry with which we can measure the rotational diffusion of the SR-ATPase labeled by a fluorophore in a medium of more physiological composition. The single photon counting technique in nanosecond pulse fluorometry is particularly sensitive to rotational motion in the time range of 1–500 ns which covers the possible subdomain wobbling of SR-ATPase. The binding sites of fluorophores ANM and IAEDANS used in the present study are highly specific and well-defined and are likely situated in distinct submolecular domains of SR-ATPase. IAEDANS binds solely to Cys674 (Yamashita & Kawakita, 1987) which lies in the "nucleotide-binding" domain (Brandl et al., 1986), whereas ANM most likely modifies SH_N (Cys344/Cys364) (Yasuoka-Yabe & Kawakita, 1983; Saito-Nakatsuka et al.,

1987) which seems to be close to each other and also to Asp351, the phosphorylation site (Bastide et al., 1973). It should be emphasized that the precise knowledge of the labeling site is essential to analyze the behavior of extrinsic probes. It should be noted that only one SH_N (Cys344 or Cys364) and not both cysteine residues was labeled. The observed decay in $r(t)$ or ANM-labeled ATPase cannot be affected by a possible energy transfer between ANM molecules on one ATPase molecule. If energy transfer takes place between ANM molecules, the initial anisotropy r_0 would decrease significantly, because energy transfer takes place within a picosecond time range, which is within a dead time for the present measurements, and does not change the observed slow ϕ value of about 40–70 ns. However, the observed initial anisotropy $r_0 = 0.25$ for ANM is even higher than $r_0 = 0.24$ for IAEDANS, excluding a possibility of energy transfer between ANM molecules. ANM is expected to bind to the same site as that of NEM, because the selective NEM binding (Saito-Nakatsuka et al., 1987) prevented the ANM labeling to SR-ATPase.

We can reasonably expect the rotational motion of subdomains in SR-ATPase labeled with ANM or IAEDANS to be a restricted wobbling. For direct demonstration of an angularly restricted wobbling for the ANM- and IAEDANS-binding domains, it is necessary to show that the fluorescence anisotropy reaches a time-independent anisotropy r_∞. Although we did not show r_∞ directly in the present experiment because of a limitation by the shorter fluorescence lifetime of the probes, it was proved by absorption and phosphorescence anisotropy measurements of triplet probes bound to the ATPase molecule that the rotation of the whole ATPase molecule is anisotropic and the ATPase never rotates across the membrane (Buerkli & Cherry, 1981; Speirs et al., 1983; Restall et al., 1985). If we assume that the restricted wobbling of a dye-carring domain is adequately approximated by the "wobbling-in-cone model" (Kinosita et al., 1977) in which a subdomain randomly wobbles within a cone of half-angle θ_c, $r^b(t)$ can be described by

$$r^b(t) = (r_0 - r_\infty) \exp(-t/\phi) + r_\infty \quad (10)$$

where r_0 is the limiting anisotropy when a fluorophore is completely immobile and r_∞ represents the residual anisotropy, which is related to the cone angle θ_c of the wobbling by

$$r_\infty/r_0 = [(1/2) \cos \theta_c(1 + \cos \theta_c)]^2 \quad (11)$$

The analysis of the experimental data in terms of the model has provided direct evidence for a segmental motion on 50–200 ns and has further revealed that the distinct submolecular domains of SR-ATPase have different intrinsic mobilities. It was shown that both ANM and IAEDANS were strongly immobilized at their binding sites. Because of the absence of complex independent probe wobbling, detailed and precise analysis of the mobility of submolecular domains was enabled, and a striking difference in the segmental motion of the respective dye-binding domains was demonstrated. The ANM-binding domain wobbles faster with a ϕ value of approximately 65–70 ns in a wider angular range with $\theta_c \geq 42°$, whereas the wobbling of the IAEDANS-binding domain occurs slowly with a ϕ value of about 190 ns in a narrower angular range with $\theta_c \geq 29°$. The lower limit of cone angle θ_c was calculated by substituting values of r(65 ns) for ANM and r(85 ns) for IAEDANS, which are upper limits for the true r_∞, instead of r_∞ in eq 11. These findings illustrate the fine resolution of this method and its usefulness in analyzing the protein conformation. To our knowledge this is the first example that the different mobility of distinct domains within a single membrane protein has been definitely demonstrated.

If we assume the viscosity of the medium surrounding the ANM-binding domain to be the same as that of water, i.e., 0.01 P, the diameter of the ANM-binding domain can be calculated to be 7 nm from $\phi = 69$ ns. A polypeptide of this size would correspond to the whole cytoplasmic domain of SR-ATPase. However, this is likely an overestimate since the viscosity of the medium surrounding the ANM-binding domain may be much higher than that of water due to possible interactions with membranes. The decrease in the rotational relaxation time by about 20 ns upon solubilization implies that the mobility of the ANM-binding domain is significantly restricted by interaction with the membrane. In addition, it should be noted that the rotating unit observed in the present fluorescence anisotropy measurements is, in effect, a portion of the ATPase peptide with solvation water. In this context, it may be noted that myosin head S1 which may be approximated as an ellipsoid with major and minor axes of 13 and 7 nm (Wakabayashi & Toyoshima, 1981), respectively, behaves in aqueous solution as a sphere with a diameter approximately the same as its major axis, as revealed by fluorescence anisotropy measurements (Kinosita et al., 1984). These considerations lead us to the conclusion that the ANM-binding domain likely represents a portion of the cytoplasmic domain. Another part of the cytoplasmic domain, to which IAEDANS was bound, showed even slower segmental motion possibly through restriction imposed by interaction with other part of the molecule and/or the membranes.

Ca^{2+}-Induced Conformational Change in SR-ATPase. Ca^{2+}-dependent conformational changes have been examined in a number of earlier studies including our own (Imamura et al., 1984; Dupont, 1976, 1980; Champeil et al., 1976; Pick, 1981; Yasuoka-Yabe & Kawakita, 1983; Yasuoka-Yabe et al., 1983; Baba et al., 1986; Suzuki et al., 1987; Guillain et al., 1980; Verjovski-Almeida & Silva, 1981), but the physical nature of these changes has not been sufficiently well characterized so far. The phosphorescence anisotropy study on the rotational diffusion of the erythrosin-labeled SR-ATPase in 60% glycerol solution provided indirect evidence suggesting a change in the segmental motion of ATPase molecule in response to the increase in Ca^{2+} concentration from 0.01 μM to 0.55 mM (Restall et al., 1985).

Distinct submolecular domains of the SR-ATPase were affected differently by the binding of Ca^{2+}. The segmental motion of the ANM-binding domain was increased upon Ca^{2+} binding, whereas that of the IAEDANS-binding domain was not. Mobility of the ANM-binding segment of the SR-ATPase was increased significantly upon binding of Ca^{2+} to the high-affinity transport site, in both the membrane-bound and solubilized states. This indicates that the increased mobility of the ANM-binding domain upon Ca^{2+} binding is at least in part caused by conformational change of the ATPase molecule itself, although additional contribution due to a decrease in interaction with the membrane may not be excluded. On the basis of time-resolved X-ray diffraction studies, Blasie et al. (1985) suggested that the transition from E^{Ca} to $E\text{–}P^{Ca}$ is accompanied by a significant redistribution of the protein mass from the extravesicular surface to the lipid hydrocarbon core. Such a change, due to an alteration either of protein conformation or of lipid–protein interactions, may significantly affect the segmental motion of the SR-ATPase. In this respect it would be interesting to examine a possible change in mobility of submolecular domains of SR-ATPase accompanying the steps such as ATP binding, E–P formation, and decomposition. A preliminary result suggests that the binding of AMP-P-

(NH)P tends to restrict the mobility of ANM bound to the ATPase molecule.

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