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Synthesis and Interaction of Fluorescent Thapsigargin Derivatives with the Sarcoplasmic Reticulum ATPase Membrane-Bound Region

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Abstract

Fluorescent derivatives of thapsigargin (TG) were synthesized by replacing the C₈-butanoyl chain with a dansyl (DTG) or eosin (ETG) moiety. DTG and ETG retain the inhibitory effect of TG on the sarcoplasmic reticulum (SR) ATPase, displaying a 2 and 10 μ M K_i, respectively. Steady state and lifetime fluorescence measurements are consistent with energy transfer between tryptophanyl residues assigned to the ATPase membrane-bound region and DTG. This phenomenon exhibits saturation behavior, occurs in the presence of DTG concentrations producing ATPase inhibition, and is partially prevented by inhibitory concentrations of TG. Although long range conformational effects of TG binding affect the fluorescence properties of endogenous tryptophans as well as of a fluorescein 5'-isothiocyanate (FITC) label of the ATPase extramembranous region, no significant energy transfer was detected between DTG and the FITC label. It is concluded that the inhibitors partition within the membrane and the binding domain resides within or near the membrane-bound region of the ATPase.

Thapsigargin (TG) is a sesquiterpene lactone isolated from root extracts of *Thapsia garganica*. The initial extraction of TG from *Thapsia garganica* roots, its purification, and its chemical characterization were done by Christensen et al. (1982, 1988). TG interferes with cytosolic Ca²⁺ homeostasis (Thastrup et al., 1987 a,b), and a relatively extensive literature has developed on the functional consequences of this interference [for a review, see Inesi and Sagara (1994)]. The general biological relevance of the TG effect is related to the role of cytosolic Ca²⁺ as a second messenger.

The primary mechanism of the cellular effect of TG is a potent and specific inhibition of the transport ATPases which are responsible for maintaining intracellular Ca²⁺ stores (Sagara & Inesi, 1991; Lytton et al., 1991; Kijima et al., 1991; Campbell et al., 1991). The sarcoplasmic reticulum (SR) ATPase is a prominent member of the family of intracellular Ca²⁺ transport (SERCA) ATPases, as its natural abundance provides a convenient experimental system for functional and structural studies (Inesi et al., 1990). Total inhibition of the SR ATPase is produced by stoichiometric titration of the enzyme with TG. We have now synthesized two TG fluorescent derivatives which retain the inhibitory activity of TG, even though with a reduced affinity. We have then utilized the fluorescent properties of these analogs to study their interaction with the ATPase.

MATERIALS AND METHODS

SR vesicles, derived prevalently from longitudinal SR membranes, were obtained from rabbit leg muscle as previously described (Eletr & Inesi, 1972), and the protein concentration was determined by the method of Lowry et al. (1951). TG was obtained from LC Laboratories (Woburn, MA). FITC was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma.

ATPase Derivatization with Fluorescein 5'-Isothiocyanate (FITC).

A fresh 4 mM stock solution of FITC in DMF was first made. SR vesicles (1.0 mg of protein/mL) were then incubated for 10 min at 25 °C in the dark, in a reaction mixture containing 10 mM Tris, pH 9.2, 0.1 M KCl, 0.3 M sucrose, and 15 μ M FITC (taken from the fresh stock solution). The reaction was stopped by the addition of a 25-fold excess volume of cold neutral buffer (10% sucrose and 10.0 mM MOPS, pH 7.0). The mixture was then centrifuged at 70000*g* for 45 min at 4 °C, the pellet resuspended in cold medium as before, and the centrifugation repeated to remove nonreacted FITC. The stoichiometry of labeling was measured in the presence of 1% dodecyl sulfate and 0.1 N NaOH, using $8.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ as the extinction coefficient at 495 nm (Mitchinson et al., 1982), and found to be 1 FITC/catalytic site.

Organic Synthesis.

TG is a sesquiterpene lactone whose structure is shown in Figure 1.

(a) Selective Hydrolysis.—A specific feature of the TG molecule (Christensen et al., 1993) is that the ester bond at C₈ can be cleaved selectively by reacting TG in methanol with triethylamine for a few hours at room temperature. This reaction was carried out with 2 mg of TG dissolved 0.4 mL of methanol and 10 μ L of triethylamine and followed by HPLC. When this selective hydrolysis was completed, the solvent was evaporated in vacuo. The product, debutanoyl-TG (DBTG; **2** in Figure 1), was then purified by HPLC and used as a reactive species for further acylation.

(b) Acylation of DBTG with N-Dansylglycine.—For this reaction we took advantage of the reactive =C₈—OH of DBTG. The reaction was carried out by preincubating 3.0 mg of DBTG in 0.3 mL of methylene chloride to which 1.8 mg of 4-(dimethylamino) pyridine and 3.5 mf of *N*-dansylglycine were added. After 5 min in ice, 2.0 mg of 1,3-dicyclohexylcarbodiimide was added. The reaction was followed by HPLC, and after a 24 h incubation at 4 °C, the dicyclohexylurea precipitate was filtered out, the solvent was evaporated in vacuo, and the resulting products were dissolved in diethyl ether. The organic phase was then washed with 1 M HCl and 1 M Na₂CO₃ in a cold room, and the final product, dansyl-debutanoyl-TG (DTG; **3** in Figure 1), was dried and concentrated in vacuo. Purification was obtained by HPLC.

(c) Derivatization with Eosine.—DBTG (2 mg) in 100 μ L of dimethylformamide was added to 2.8 mg of 4-(dimethylamino)pyridine and 4.6 mg of eosine in 200 μ L of dimethylformamide stirring on ice. After a 5 min incubation, 2 mg of

dicyclohexylcarbodiimide in 100 μ L of dimethylformamide was added. After 2 more hours of incubation on ice, the reaction was allowed to proceed at room temperature for 24 h with stirring. The reaction was then stopped by addition of 5 mL of water, and the precipitate was collected by centrifugation. The precipitate was extracted with 0.5 mL of methylene chloride, and the compound (ETG; **4** in Figure 1) was purified by HPLC.

HPLC was carried out using a Waters HPLC system equipped with a 1.8 mL sample injector loop, M510 and 6000 pumps, a 490E multiwavelength detector, an M680 gradient controller, and a 4 mm $\times$ 250 mm Alltech Lichrosorb C-18 reverse phase column preequilibrated with solvent B. Solvent A consisted of 0.5% acetic acid, and solvent B consisted of 0.5% acetic acid in methanol.

For DTG, a linear gradient was applied as follows: 70–80% B over 20 min, 80% B over 20 min, and finally 80–100% B over 10 min. Elution was monitored at 230 and 254 nm. Retention times for deutanoyl-TG and DTG were 17.4 and 25.2 min, respectively.

For ETG, a linear gradient was applied as follows: 70–90% solvent B over 40 min and then 90–100% solvent B over 5 min. Elution was monitored with absorption at 230 and 495 nm wavelengths. The retention time for ETG under these conditions was 41.4 min. The compound could also be detected by TLC on silica gel IB-F, and the R_f value was 0.35 of the developing system which consisted of methanol:chloroform (1:9).

NMR Spectroscopy.

Approximately 1 mg of TG, deutanoyl-TG, DTG or ETG was dissolved in 0.6 mL of deuterated chloroform or methanol, and proton NMR spectra were obtained on a Varian GEQE-300 or GN-500 MHz spectrometer. Temperature = 25 $^{\circ}$ C.

Fluorescence Spectroscopy.

Excitation and emission spectra were obtained with a JASCO FP777 spectrofluorometer, in media containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, and 50 μ M CaCl₂. Protein concentrations were kept below 0.05 optical density levels (i.e., <0.1 mg/mL) in order to minimize background scattering which was further reduced by monochromator selection of excitation and emission wavelengths.

Frequency domain data were obtained with a frequency domain fluorometer operating between 8 and 2000 MHz (Lakowicz et al., 1986a,b). The modulated excitation was provided by the harmonic content of a laser pulse train with a repetition rate of 3.75 MHz and a pulse width of 5 ps, from synchronously pumped and cavity dumped rhodamine 6G and pyridine dye laser. The dye laser was pumped with a mode-locked argon ion laser (Coherent, Innova 15). The dye laser output was frequency doubled to 292 nm for tryptophan, or 350 nm for DTG, with an angle-tuned KDP (potassium dihydrogen phosphate) crystal. For intensity decay measurements, magic angle polarizer orientations were used. The emitted light was observed through interference filters: 340 nm for tryptophan and 440 nm for DTG. Temperature control was set at 20 $^{\circ}$ C. The frequency domain data were analyzed by the method of nonlinear least squares (Gratton et al., 1984; Lakowicz et al., 1984).

ATPase Activity.

Activity was measured by an enzymecoupled assay (Horgon et al., 1972) in a reaction mixture containing 180 mM KCl, 20 mM MOPS, pH 7.2, 5 mM MgCl₂, 50 μ M CaCl₂, 25 U of pyruvate kinase/mL, 25 U of lactic dehydrogenase/mL, 2 mM phospho(enol)pyruvate, 150 μ M NADH, 2 μ M ionophore A23187, and 10 μ g of SR protein/mL. The reaction was started by the addition of 0.2 mM ATP and monitored optically at a 340 nm wavelength.

RESULTS

Purification and Characterization of TG Derivatives.

The starting compound TG, the DBTG intermediate, and the final products DTG and ETG were subjected to HPLC to follow the progress of organic synthesis and insure their purity. A prominent and symmetrical elution peak, exhibiting distinct elution properties, was collected for each compound, and this material was used for synthetic work and/or spectroscopic and functional characterization. Retention times for DBTG, DTG, and ETG were 17.4, 25.2, and 41.4 min, respectively.

Structural identification of the products was obtained by proton NMR spectroscopy. Assignment of the TG spectral peaks was done according to Christensen et al. (1993), and the occurrence of derivatization was demonstrated by the appearance of dansyl or eosin moiety proton peaks.

Inhibitory Properties.

Measurements of ATPase activity were performed to determine the effect of structural modifications on the inhibitory properties of TG. We found that the inhibitor's concentration (total) required for half-maximal inhibition was shifted from 5 to 80 nM as the butanoyl group was removed and then to 1 and 10 μ M as the butanoyl group was replaced by the dansyl moiety or the eosin moiety, respectively (Figure 2).

Fluorescence Measurements.

Spectroscopic studies were then performed to explore the possibility of fluorescence energy transfer between bound inhibitor and reference fluorophores within the ATPase molecule. As reference fluorophores we chose the intrinsic tryptophans which reside prevalently within or near the membrane-bound ATPase region (MacLennan et al., 1985) and an FITC label covalently bound to Lys515 (Mitchinson et al., 1982) at the top of the ATPase extramembraneous region (Figure 3). The intrinsic tyrosine fluorescence is negligible in this protein, and in any event the tyrosine residues are not excited at 298 nm.

Pertinent overlaps of emission and absorption spectra indicate that, within the limits of appropriate distances, fluorescence energy transfer between tryptophanyl residues (donors) and the inhibitor's dansyl moiety (acceptor), or the inhibitor's dansyl moiety (donor) and the FITC label (acceptor), is possible and may be indicative of their proximity within the ATPase molecule (Bigelow & Inesi, 1992). Alternatively, energy transfer between the FITC label (donor) and ETG (acceptor) is possible, depending on the relative positions of the two fluorophores.

Excitation of Intrinsic Tryptophans.

Tryptophan fluorescence emission (solid line in Figure 4A) undergoes a sharp reduction upon addition of DTG. while a corresponding fluorescence signal is emitted by DTG upon tryptophan excitation at 292 nm. It should be pointed out that the fluorescence intensity of the same concentration of DTG (upon excitation at 292 nm) in the absence of SR corresponds to approximately 10% of that observed in the presence of SR.

The DTG concentration dependence of this effect manifests a saturation behavior (Figure 4B) inasmuch as increasing DTG above 10 μM produces proportionally smaller changes of fluorescence intensity. The micromolar concentration range corresponds to that required to produce maximal inhibition of ATPase function (Figure 2).

The observed reduction of tryptophan fluorescence may be due to conformational effects of bound DTG on the protein or to energy transfer from ATPase tryptophanyl residues to bound DTG. In fact, even binding of (nonfluorescent) TG reduces the fluorescence intensity (Figure 4A) of the Ca^{2+} ATPase through a conformational effect on the protein which is also manifested by an altered pattern of long range ATPase ordering (Sagara et al., 1992a; Mersol et al., 1995). We then performed additional experiments to study the frequency response of the intrinsic tryptophan fluorescence, which is expected to be sensitive to energy transfer resulting in a reduced tryptophan lifetime. In these experiments, increased demodulation, or an increased phase angle relative to the incident light, is observed as the frequency is increased.

It is shown in Figure 5A that the frequency response is shifted to higher frequencies when DTG (acceptor) is added, due to the decrease tryptophan (donor) decay time as a consequence of energy transfer. The significant difference in the patterns obtained in the absence and presence of DTG (TG not present) is demonstrated by a very large increase in the goodness of fit parameter when the data from the two curves are cross-fitted. The presence of saturating concentrations of TG decreases but does not eliminate completely the effect of DTG (Figure 5B), suggesting that (a) the dansylated derivative and TG behave competitively and (b) the fluorescence energy transfer observed in the absence of TG is, at least in part, due to specific site occupancy by DTG.

When the data derived from the frequency response experiments were used to calculate model dependent parameters, a good fit was obtained with a double-exponential model (Table 1), indicating that the mean decay time decreased from 3.53 to 2.78 ns when DTG was added. Although participation of multiple tryptophans precludes precise estimates of distances, the high energy transfer efficiency (0.87) derived from the lifetime data indicates that most tryptophanyl residues reside within the 19 Å Foster distance from the fluorophore of DTG bound at the inhibitory site. When DTG was added in the presence of TG (1 μM), the mean decay time was reduced to 3.32 (rather than 2.78)ns.

It is noteworthy that addition of nonfluorescent 1 μM TG alone shifts the frequency response to a slightly lower frequency and higher decay times (Table 1), in parallel with a reduction of tryptophan fluorescence intensity (Figure 4A). This effect is related to redistribution of energy emission over time, due to the protein conformational change produced by TG

(Sagara et al., 1992a; Mersol et al., 1992). If one considers that DTG binding may produce a conformational effect analogous to that of TG, then the control for energy transfer between tryptophans and DTG in the absence of TG should be the fluorescence mean decay time of tryptophans in the presence of TG. In this case, transfer would be even more efficient than estimated above, as the mean lifetime would be reduced from 3.81 to 2.78 ns.

Excitation of DTG.

In another group of experiments we explored the possibility of energy transfer between DTG (donor) and the FITC label (acceptor) of the ATPase extramembraneous region. In emission spectra obtained with the DTG excitation wavelength of 330 nm, we found little evidence of energy transfer as the combined emission of DTG and the FITC label appears to be an approximate summation of the separate emission spectra of the two fluorophores (Figure 6). When we studied the frequency domain response, we found that the mean decay time of DTG underwent a very small reduction from 5.11 to 4.87 ns when the FITC label was present (Table 2). This small reduction, however, was also observed when the same experiment was repeated in the presence of TG (Table 2), suggesting that it may be due to nonspecific binding. It should be pointed out that the DTG mean decay time is increased from 5.11 to 6.23 ns when TG is added to a reaction mixture of DTG and SR which was not labeled with FITC. This is likely due to displacement of DTG from specific sites and relief of the influence of that environment on the DTG fluorescence characteristics.

The lack of significant energy transfer between bound DTG and the FITC label indicates that the distance between these fluorophores is larger than the pertinent 49 Å Förster distance. Since the distance between the FITC label and the closest membrane surface is approximately 50 Å (Teruel & Gomez-Fernandez, 1986; Gutierrez-Merino et al., 1987), it is unlikely that bound DTG resides within the extramembraneous region of the ATPase.

Excitation of the FITC Label.

The possibility of energy transfer between the FITC label and a fluorescent TG derivative was further explored by the use of ETG. As the fluorescence characteristics of FITC and ETG allow energy transfer from FITC (donor) to ETG (acceptor), we thought that this phenomenon can be studied favorably by exciting FITC which is present exclusively at the relevant site on the protein. However, the low affinity of the ATPase inhibitory sites for ETG (Figure 2) required exceedingly high concentrations of ETG. At these high concentrations, the high fluorescence intensity and the nonspecific binding of ETG precluded reliable measurements and analysis of FITC fluorescence. It should be pointed out that the FITC label fluorescence intensity is affected even by the (nonfluorescent) TG, likely due to conformational effects or a change in the spatial arrangement of ATPase molecules (Highsmith & Cohen, 1987; Vanderkooi et al., 1977; Mersol et al., 1995) triggered by TG binding.

DISCUSSION

Ca²⁺ binding and phosphoenzyme formation, together with their vectorial and interdependent reversals, are key functions of the Ca²⁺ ATPase cycle resulting in ATP

dependent Ca^{2+} translocation across the SR membrane in exchange for H^+ (Figure 7). Chemical (Bastide et al., 1973; Degani & Boyer, 1973; Sumbilla et al., 1971) and mutational analysis (Maruyama & MacLennan, 1988; Clarke et al., 1989) have shown that the phosphorylation reaction occurs in the extramembranous region of the ATPase while the Ca^{2+} -binding domain resides within the membrane-bound region. The phosphorylation domain and the Ca^{2+} -binding domain are separated by a distance of approximately 50 Å and are functionally coupled through a long range intramolecular linkage which is the basic mechanistic device of this enzyme. Both the Ca^{2+} -binding and phosphorylation functions of the ATPase are affected by interaction of 1 mol of the enzyme with 1 mol of thapsigargin (Sagara et al., 1992b) under conditions permitting the two reactions to occur independent of each other (i.e., in the absence of enzyme cycling). Since these two reactions occur at distant domains, it is difficult to infer the location of the TG-binding domain from studies of functional inhibition. For this reason we have undertaken the organic synthesis of fluorescent TG derivatives and tested their interaction with topological points of reference in the extramembranous and membrane-bound regions of the ATPase.

Although binding of nonfluorescent TG produces long range effects which are manifested by its influence on the fluorescence characteristics of tryptophan residues in the membrane-bound region and the FITC label in the extramembranous region of the ATPase (Table 1), the occurrence of energy transfer provides information on the binding of the TG derivatives. Our present experiments indicate that the inhibitors partition within the membrane and the binding domain resides within or near the membrane-bound region of the ATPase. This is consistent with previous experiments with chimeric constructs in which the large cytosolic loop (including the nucleotide and phosphorylation domains) of the Ca^{2+} ATPase was replaced by the corresponding segment of the Na^+, K^+ ATPase. Such chimeric proteins were found to be sensitive to Ca^{2+} and TG, suggesting that Ca^{2+} and TG binding occurs within the membrane-bound segment derived from the Ca^{2+} ATPase (Sumbilla et al., 1993). Further experiments with other chimeric constructs have indicated that the third membrane-spanning domain (M3) plays a major role in TG binding (Norregard et al., 1994). It should be pointed out that the absence of TG inhibition on chimeric proteins can be related to a direct or indirect effect of the chimeric construct on the TG-binding domain. On the other hand, fluorescence energy transfer demonstrates the physical proximity of the ATPase membrane-bound tryptophans and the fluorescent TG derivatives. Presumably, TG binding within the transmembrane region interferes with neighboring Ca^{2+} binding and stabilizes the enzyme in a dead-end complex retaining the Ca^{2+} free conformation (Wictome et al., 1992). This is also consistent with the observation that TG facilitates formation of bidimensional crystalline arrays (Sagara et al., 1992a) similar to those formed by the Ca^{2+} free enzyme (Dux & Martonosi, 1983).

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Figure 1:
Structures of thapsigargin (TG; **1**), deutanoylthapsigargin (DBTG; **2**), dansylthapsigargin (DTG; **3**), and eosinthapsigargin (ETG; **4**).

Figure 2:

Inhibition of ATPase activity by TG, DBTG, DTG, and ETG. Linear rates of ATPase activity were obtained in the presence of 10 μg of SR protein/mL of reaction mixture, under the condition described in the section on methods. Each experimental point in the figure corresponds to the slope obtained from several time points.

Figure 3:

Diagram of the SR ATPase amino acid sequence and its topology with respect to the membrane bilayer. The diagram is based on the original assignments by MacLennan et al. (1985) and Clarke et al. (1989). The major and minor extramembranous loops are likely to be folded, forming the cytosolic head of the enzyme. Transmembrane helices 4, 5, 6, and 8 (thick lines) contain the residues involved in Ca^{2+} binding and are likely to be clustered, forming a transmembrane channel. The positions of Lys515 (which is labeled with FITC) and the ATPase tryptophanyl residues are shown in the extramembranous and membrane-bound regions. Asp351, the residue undergoing phosphorylation, is also shown in the extramembranous region of the ATPase.

Figure 4:

Fluorescence emission of ATPase endogenous tryptophans and DTG upon tryptophan excitation (wavelength: 298 nm). The reaction mixture contained 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 50 μM CaCl₂, and 87 nM SR ATPase. A: The spectrum identified as “SR” corresponds to the control in the absence of DTG or TG. The spectra identified as “SR and DTG” and “SR and TG” were obtained in the presence of 1.2, μM DTG and 1.0 μM TG, respectively. B: Emission of tryptophan at 327 nm (O) and DTG at 501 nm (●) in the presence of various DTG concentrations upon tryptophan excitation at 298 nm.

Figure 5:

Frequency response of tryptophan fluorescence emission of SR vesicles (0.08 mg/mL) in the absence and presence of 3.5 μ M DTG (A). B: The experiment was repeated in the presence of 1 μ M TG.

Figure 6:

Fluorescence emission spectra of DTG and FITC-labeled ATPase upon DTG excitation (wavelength: 330 nm). The reaction mixture contained 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 50 μ M CaCl₂, and 0.13 μ M FITC-labeled ATPase, 3.5 μ M DTG, or 0.13 μ M FITC-labeled ATPase and 3.5 μ M DTG.

Figure 7:
 Simple reaction scheme for ATP utilization coupled to Ca^{2+} transport by the SR ATPase. Conformational changes (e.g., E1 to E2 and EP1 to EP2) of the ATPase protein following Ca^{2+} binding and/or phosphorylation (deMeis & Vianna, 1979) may be considered to occur in parallel with the chemical reactions outlined in the scheme.

Table 1:Multiexponential Analysis of Tryptophan Emission from SR ATPase with and without DTG and/or TG^a

sample	τ_i (ns)	α_i	f_i	χ_R^2	τ_{mean} (ns)
SR	1.187 $\pm$ 0.053	0.525	0.238		
	4.224 $\pm$ 0.088	0.475	0.762	2.34	3.502
SR + 3.5 μ M DTG	0.709 $\pm$ 0.028	0.721	0.336		
	3.739 $\pm$ 0.082	0.279	0.664	3.24	2.621
SR + 1 μ M TG	1.514 $\pm$ 0.090	0.490	0.249		
	4.578 $\pm$ 0.176	0.510	0.751	2.30	3.815
SR + 3.5 μ M DTG + 1 μ M TG	1.058 $\pm$ 0.017	0.718	0.348		
	4.546 $\pm$ 0.044	0.282	0.652	2.98	3.332

^aThe tryptophans were excited at 292 nm, and the emission was measured at 340 nm. The goodness of fit was calculated with $F=0.4^\circ$, $M=0.01$. τ_j is the decay time, α_j is the preexponential factor, and f_j is the fractional intensity, derived from $\tau_{\text{mean}} = \sum f_j \tau_j$, (Gryczynski et al., 1989) where τ_{mean} is the mean decay time calculated from the double-exponential model, χ_R^2 is the goodness of fit parameter. The asymptotic standard errors of fitted parameters are shown. These values are the average of results from two separate experiments, performed 3

Table 2:Multiexponential Analysis of DTG Emission with or without FITC and/or TG^a

sample	τ_i (ns)	a_i	f_i	χ^2_R	τ_{mean} (ns)
3.5 μM DTG + SR	1.040 ± 0.037	0.760	0.307		
	6.917 ± 0.188	0.240	0.693	6.40	5.110
3.5 μM DTG + FITC-SR	1.062 ± 0.041	0.738	0.309		
	6.575 ± 0.190	0.262	0.691	6.50	4.871
DTG + SR + 1 μM TG	1.263 ± 0.040	0.724	0.285		
	8.207 ± 0.205	0.276	0.715	5.45	6.228
DTG + FITC-SR + 1 μM TG	1.176 ± 0.041	0.720	0.286		
	7.266 ± 0.185	0.280	0.714	5.65	5.524

^aThe dansyl group was excited at 345 nm, and the emission was measured at 440 nm. The goodness of fit was calculated with $F=0.4^\circ$, $M=0.01$. τ_j is the decay time, a_i is the preexponential factor, and f_i is the fractional intensity, derived from $\tau_{\text{mean}} = \sum f_i \tau_i$ (Gryczynski et al., 1989) where τ_{mean} is the mean decay time calculated from the double-exponential model. χ^2_R is the goodness of fit parameter. The asymptotic standard errors of fitted parameters are shown.