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## FRET Study of a Trifluorophore-Labeled DNAzyme

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Received July 11, 2002

**Abstract:** A fluorescence resonance energy transfer (FRET) study of biomolecules typically employs two fluorophores. The increasing number of branches and complexity of biomolecules call for simultaneously monitoring structures and dynamics of several branches in a single system. Furthermore, despite recent studies that show DNAzymes can be a stable and cost-effective alternative to protein and ribozymes for pharmaceutical and biotechnological applications, no FRET study of DNAzymes has been reported. Here, we describe the FRET study of a trifluorophore-labeled "8-17" DNAzyme, in which each of the three branches is labeled with a different fluorophore. From the study, we found that the  $(\text{ratio})_A$  method that has been commonly used in dual-fluorophore-labeled systems is also applicable to trifluorophore-labeled systems. However, while both FRET efficiency and fluorophore-to-fluorophore distance can be used to measure FRET in dual-fluorophore-labeled systems, only the average distance should be used in trifluorophore-labeled systems. The ability to monitor all three branches in a single system allowed us to reveal new metal-ion-dependent conformational changes in the DNAzyme. The trifluorophore-labeled "8-17" DNAzyme has been found to adopt a two-step folding process in the presence of  $\text{Zn}^{2+}$ . Each step is induced by one  $\text{Zn}^{2+}$  binding, with apparent dissociation constants of 19  $\mu\text{M}$  and 260  $\mu\text{M}$  for binding the first and second  $\text{Zn}^{2+}$ , respectively. The trifluorophore FRET results are verified by a dual-labeled control experiment. The results demonstrated that the trifluorophore-labeled system is simple and yet powerful in studying complicated biomolecular structure and dynamics and is capable of revealing new sophisticated structural changes that may have functional implications.

## Introduction

FRET is a widely used technique for analyzing the structure and dynamics of biomolecules in solution.<sup>1–4</sup> It has been successfully applied to a variety of nucleic acid structural studies, such as the characterization of three-way<sup>5</sup> and four-way<sup>6</sup> DNA junction and the bending and kinking of DNA/RNA helices with bulged nucleotides.<sup>7</sup> These studies have laid a foundation for the investigation of ribozymes, such as the hammerhead<sup>8,9</sup> and hairpin ribozymes.<sup>10–12</sup> FRET is also a powerful tool for the study of the dynamics of nucleic acids, like ribozyme catalysis,<sup>13,14</sup> DNA and RNA folding,<sup>15,16</sup> metal-

ion-dependent nucleic acid tertiary structure and DNA protein interactions.<sup>2,17,18</sup>

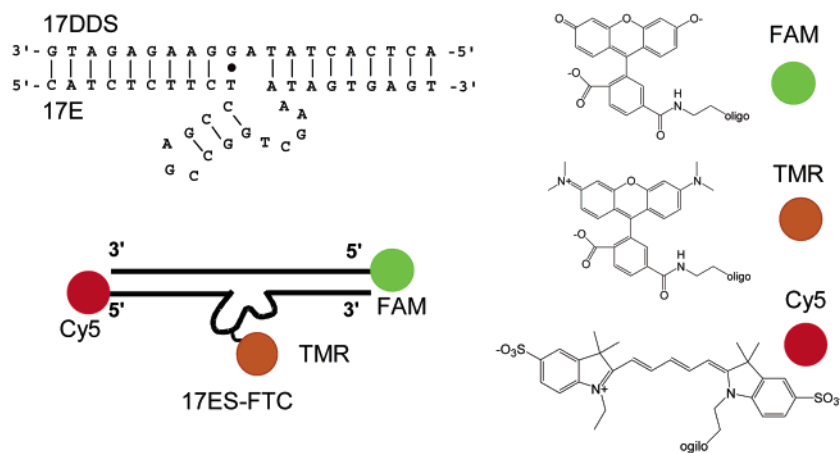
A typical FRET experiment involves labeling a molecule of interest with two fluorophores, a donor and an acceptor. By calculating the FRET efficiency, we can acquire the distance between the two fluorophores. From the change of FRET efficiency, the global conformational changes can be deduced. When the structure of the biomolecule is branched, such as in the three-way DNA junction or the hammerhead ribozyme, being able to monitor the relative movements of all the branches is preferred. The current approach to achieve this goal is by combinatorial labeling of each pair of arms in the biomolecule, obtaining information about each individual pair, and then combining all data together to deduce the global structure and its changes.

We are interested in exploring the FRET study of multi-branched biomolecules, where each branch of the molecules is labeled with a different fluorophore. There are several advan-

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**Figure 1.** Schematics of the trifluorophore-labeled “8-17” DNzyme system (called 17ES—FTC) that consists of a DNA enzyme strand (17E), a substrate strand (17DDS), and three fluorophores, FAM, TMR, and Cy5. The normal substrate strand (17DS) of “8-17” DNzyme contains all DNA except a ribonucleic acid linkage at the scissile position (between position 10 and 11). The substrate strand in this study, 17DDS, is an all DNA noncleavable analogue of the substrate strand. The 5' ends of 17E and 17DDS are labeled with Cy5 and FAM, respectively. In addition, the 16th base (cytosine) of 17E is attached with a TMR

tages of this kind of system. First, the ability to carry out FRET studies on a *single* system can significantly reduce the number of sample preparations. For example, when using current dual-label methods, to carry out FRET studies on a three-way DNA/RNA junction, three dual-labeled samples have to be made and purified. Up to six samples are required to investigate a four-way DNA/RNA junction. Second, being able to observe FRET between different branches of biomolecules may allow the simultaneous observations of structure and dynamics of all branches. Finally, a decrease in the number of sample preparations and FRET experiments also improves the consistency of the results from different experiments.

FRET from a single donor to multiple acceptors, or from multiple donors to a single acceptor, has been applied in protein studies, although these experiments utilized at most two different kinds of fluorophores. For example, energy transfer from a single donor (6-carboxyfluorescein or FAM) to multiple acceptors (6-carboxytetramethylrhodamin or TMR) has been used to study actin assembly.<sup>19</sup> Sawyer has quantitatively studied energy transfer from a FAM to two TMR fluorophores to calculate the distance between a DNA and ATP binding site in TyrR.<sup>20</sup> Energy transfer from multiple donors (tryptophans) to a single acceptor (colchicine) has been used to study the position of tryptophans in tubulin.<sup>21</sup> Depolarization due to homoenergy transfer between multiple FAM fluorophores (up to four) has been used to study melittin oligomerization.<sup>22</sup> Recently, a trifluorophore-labeled single stranded DNA for DNA sequencing purposes has been reported, although no quantitative energy transfer analysis has been performed.<sup>23</sup>

Among the biomolecules amenable to multifluorophore FRET studies, DNazymes are of great interest. Long considered as simply a genetic material, DNA was shown in 1994 to carry out catalytic functions<sup>24</sup> and thus became the newest member of the enzyme family after proteins and RNA. Since then, the DNA molecules (called DNazymes here; also called deoxyribozymes, DNA enzymes, or catalytic DNA elsewhere) have been shown to catalyze many of the same reactions as RNA or protein enzymes.<sup>25–28</sup> When compared with RNA and protein enzymes, DNazymes are relatively less expensive to produce and more stable to hydrolysis. Unlike proteins, most DNazymes can be denatured and renatured many times without losing

binding ability or activity. Therefore, DNazymes have shown great promise as antiviral agents,<sup>29</sup> biosensors for metal ions<sup>30</sup> and other organic molecules and biomolecules,<sup>31</sup> and DNA-based logic computation tools.<sup>32</sup> Since biochemical studies have shown that metal ions play essential roles in the structure and function of almost all DNazymes, including the “8-17” DNzyme,<sup>29,33–35</sup> it is surprising that few FRET studies of metal-ion-dependent structure and dynamics have been reported.

Herein, we report the first FRET analysis of a trifluorophore-labeled “8-17” DNAzyme (Figure 1),<sup>29,33,34</sup> in which each of the three branches is labeled with a different fluorophore. The trifluorophore-labeled “8-17” DNAzyme is named 17ES–FTC. Equations for calculating FRET efficiency and its related distances between each pair of the three fluorophores have been derived. The ability to monitor all the three branches in a single system allowed us to reveal new metal-ion-dependent conformational changes in nucleic acid enzymes. The results presented here allowed for the first time a detailed study of metal-ion-dependent folding of the “8-17” DNAzyme. It also gives us an opportunity to compare the similarity and difference between the folding of DNAzymes with that of ribozymes.<sup>8–10,36</sup>

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## Experimental Section

**Oligonucleotides and Sample Preparations.** All DNA samples were purchased from Integrated DNA Technology Inc. and were purified by HPLC to ensure 100% labeling of fluorophores. Fluorophore-labeled DNazyme 17E and its substrate 17DDS were annealed in 50 mM trisacetate buffer, pH 7.2 with 25 mM NaCl, at 90 °C for 5 min, and the solution was subsequently cooled to 4 °C over a period of 1 h. The annealed duplex was then purified on a nondenaturing 16% polyacrylamide gel at 4 °C. The hybridized band was excised and recovered by the crush-and-soak method. The sample concentration was determined by monitoring electronic absorption at 260 nm, and the final concentration was diluted to ~200 nM.

**UV-vis Absorption Spectroscopy.** UV-vis spectra were obtained using a Hewlett-Packard 8453 spectrophotometer. The temperature was maintained at 4 °C to prevent melting of the duplex and to keep measurements consistent with those of the fluorescence experiments. The buffer was 50 mM trisacetate, pH 7.2 with 25 mM NaCl. By fitting singly labeled FAM, TMR, and Cy5 absorption spectra to the absorption spectra of 17ES-FTC, we can obtain the ratios of extinction coefficients of the three fluorophores in 17ES-FTC at wavelengths of interest. These values are needed for FRET efficiency calculations and are listed as follows:  $\epsilon^{\text{Cy5}}(560)/\epsilon^{\text{Cy5}}(647) = 0.096$ ,  $\epsilon^{\text{TMR}}(560)/\epsilon^{\text{Cy5}}(647) = 0.43$ ,  $\epsilon^{\text{TMR}}(490)/\epsilon^{\text{TMR}}(560) = 0.11$ ,  $\epsilon^{\text{FAM}}(490)/\epsilon^{\text{TMR}}(560) = 0.81$ ,  $\epsilon^{\text{Cy5}}(490)/\epsilon^{\text{Cy5}}(647) = 0$ ,  $\epsilon^{\text{FAM}}(490)/\epsilon^{\text{Cy5}}(647) = 0.35$ ,  $\epsilon^{\text{TMR}}(490)/\epsilon^{\text{Cy5}}(647) = 0.048$ .

**Fluorescence Spectroscopy.** Fluorescence emission spectra were recorded on an SLM 8000S fluorometer operating in photon counting mode and were corrected for lamp fluctuation and instrumental variations. Polarization artifacts were avoided by setting polarizers in "magic angle" conditions.<sup>37,38</sup> All spectra were collected at 4 °C. The FRET efficiency was calculated using the (ratio)<sub>A</sub> method (vide infra).<sup>1</sup> The emission spectra of FAM and TMR singly labeled DNA were collected for spectra fittings. The trifluorophore-labeled DNA, 17ES-FTC, was first excited at 490 nm. Emissions were collected from 500 to 700 nm. The second scan was taken by exciting TMR at 560 nm and collecting the emission spectrum from 570 to 700 nm. The third scan was taken by exciting Cy5 at 647 nm and collecting the emission spectrum from 650 to 700 nm. For the dual-fluorophore labeled DNazyme control experiment, the substrate was 5' labeled with a FAM and 3' labeled with a TMR, while no fluorophore was attached to the enzyme strand. The first emission spectrum was collected from 500 to 650 nm after exciting FAM at 490 nm. The second emissions spectrum was collected from 570 to 650 nm after exciting TMR at 560 nm. The bandwidth was set at 1 nm for both excitation and emission light. ZnCl<sub>2</sub> stock solution was titrated into the sample to initiate DNA folding. The total volume of ZnCl<sub>2</sub> added was less than 5% of the DNA sample volume.

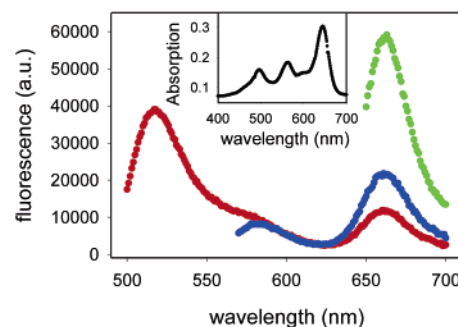
**From the Dual-Fluorophore (Ratio)<sub>A</sub> Method to the Trifluorophore (Ratio)<sub>A</sub> Method.** The distance-dependent interaction for FRET is the nonradiative dipole-dipole energy transfer between two fluorophores. When the donor (D) is excited, the nearby acceptor (A) can receive energy from the donor with efficiency  $E$  expressed as<sup>1,39,40</sup>

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (1)$$

$R$  is the donor-to-acceptor distance.  $R_0$  is defined by

$$R_0^6 = (8.785 \times 10^{23}) \times \Phi^D \times \kappa^2 \times \eta^{-4} \times J(\nu)^6 \text{ \AA}^6 \quad (2)$$

When  $R$  equals  $R_0$ ,  $E$  equals 0.5.  $\Phi^D$  is the quantum yield of the donor, and  $\kappa^2$  is the orientation factor for dipole coupling. When both



**Figure 2.** Electronic absorption (inset) and steady-state fluorescence spectra of 17ES-FTC. During the steady-state fluorescence experiment, three emission scans were collected for 17ES-FTC. The first scan was from 500 to 700 nm, with excitation fixed at 490 nm (red curve); the second scan was from 570 to 700 nm, with excitation fixed at 560 nm (blue curve), and the third scan was from 650 to 700 nm, with excitation fixed at 647 nm (green curve). All spectra were collected at 4 °C, and nitrogen was constantly pumped to avoid water condensation. The inset was the electronic absorption spectrum of 17ES-FTC at 4 °C.

the donor and the acceptor can rotate freely during the excited-state lifetime of the donor,  $\kappa^2$  has the average value of  $2/3$ .<sup>41</sup>  $\eta$  is the refractive index of the media.  $J(\nu)$  is the overlap integral of the fluorescence spectrum of donor and the absorption spectrum of the acceptor. Usually  $R_0$  is a constant. Thus,  $E$  is only a function of  $R$ . However, if  $R_0$  also changes (e.g., when  $\Phi^D$  changes), the relation between  $E$  and  $R$  is no longer straightforward. Corrections must be made on  $R_0$  to obtain the correct  $R$ .

The FRET efficiency can be determined by using the (ratio)<sub>A</sub> method, developed by Clegg,<sup>1</sup> to minimize errors from sources such as instrumental noises and sample differences. In the (ratio)<sub>A</sub> method, the FRET spectra  $F(\lambda_{\text{em}}, \lambda_{\text{ex}}^D)$  (where  $\lambda_{\text{ex}}^D$  is the wavelength at which the donor is excited and  $\lambda_{\text{em}}$  is the emission wavelength) are fitted to the sum of two components. The first component is the emission spectra  $F(\lambda_{\text{em}}, \lambda_{\text{ex}}^A)$  of the dual-labeled sample excited at the FRET acceptor wavelength,  $\lambda_{\text{ex}}^A$ , where only the acceptor absorbs at this wavelength. The second component is the emission spectra  $F^D(\lambda_{\text{em}}, \lambda_{\text{ex}}^D)$  of another sample that is singly labeled with the donor and excited at donor absorption wavelength,  $\lambda_{\text{ex}}^D$ . Thus,

$$F(\lambda_{\text{em}}, \lambda_{\text{ex}}^D) = (\text{ratio})_A F(\lambda_{\text{em}}, \lambda_{\text{ex}}^A) + \alpha F^D(\lambda_{\text{em}}, \lambda_{\text{ex}}^D) \quad (3)$$

where (ratio)<sub>A</sub> and  $\alpha$  are the two weighting factors for the two components. Thus,

$$(\text{ratio})_A = \frac{F(\lambda_{\text{em}}, \lambda_{\text{ex}}^D) - \alpha F^D(\lambda_{\text{em}}, \lambda_{\text{ex}}^D)}{F(\lambda_{\text{em}}, \lambda_{\text{ex}}^A)} = \frac{\epsilon^A(\lambda_{\text{ex}}^D)}{\epsilon^A(\lambda_{\text{ex}}^A)} + d^+ E \frac{\epsilon^D(\lambda_{\text{ex}}^D)}{\epsilon^A(\lambda_{\text{ex}}^A)} \quad (4)$$

where  $\epsilon$  is the extinction coefficient of a fluorophore and  $d^+$  is the fraction of DNA labeled with donor. When donor labeling is 100%,  $d^+$  equals one.  $E$  can then be calculated from eq 4.

The concept of the dual-labeled (ratio)<sub>A</sub> method can be extended to the trifluorophore system. When the three fluorophores are considered two at a time, the simplest pair is TMR (donor) and Cy5 (acceptor), because when either of the two fluorophores is excited, FAM is not excited. In addition, there is no energy transfer from the two fluorophores to FAM. In this case, FAM can be ignored in FRET data analysis. The (ratio)<sub>A</sub> for this pair can be obtained by fitting the blue curve in Figure 2 to the sum of the green curve and a TMR singly labeled emission spectra. FRET efficiency can be

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calculated according to eq 4.

$$(\text{ratio})_A^{\text{TC}} = \frac{\epsilon^{\text{Cy5}}(560)}{\epsilon^{\text{Cy5}}(647)} + d_{\text{T}}^+ E_{\text{TC}} \frac{\epsilon^{\text{TMR}}(560)}{\epsilon^{\text{Cy5}}(647)} \quad (5)$$

The superscripts describe the name of the fluorophores, and the numbers in the parentheses indicate the corresponding wavelength. The FRET efficiency of the TMR–Cy5 pair, FAM–TMR pair, and FAM–Cy5 pair are represented by  $E_{\text{TC}}$ ,  $E_{\text{FT}}$ , and  $E_{\text{FC}}$ , respectively.  $d_{\text{T}}^+$  is the fraction of TMR labeling. Since both the substrate and enzyme strand are HPLC purified to ensure 100% fluorophore labeling, and the annealed duplex are purified by nondenaturing gel electrophoresis, all the DNA molecules in the system should be in the form described in Figure 1. Thus,  $d_{\text{T}}^+$  equals one.

When the FAM (donor) and TMR (acceptor) pair is considered, Cy5 can accept energy from both FAM and TMR. Thus, the effect of Cy5 is as a fluorescence quencher that decreases the quantum yield of both FAM and TMR. However, in the  $(\text{ratio})_A$  method, the quantum yield does not appear in the equation. Thus, by fitting the red curve in Figure 2 to the sum of the blue curve ( $\lambda_{\text{em}} = 570\text{--}650\text{ nm}$ ) and a FAM singly labeled emission spectra, we can acquire  $(\text{ratio})_A$  for the FAM–TMR pair. The FRET efficiency can be calculated based on eq 4.

$$(\text{ratio})_A^{\text{FT}} = \frac{\epsilon^{\text{TMR}}(490)}{\epsilon^{\text{TMR}}(560)} + d_{\text{F}}^+ E_{\text{FT}} \frac{\epsilon^{\text{FAM}}(490)}{\epsilon^{\text{TMR}}(560)} \quad (6)$$

$d_{\text{F}}^+$  is the fraction of FAM labeling. In our system, the pH is buffered at 7.2 to prevent the formation of the metal ion hydroxide. At this condition, the fluorescence of FAM was determined to be 93.4% of that at pH 8.3 (see Supporting Information) because of partial protonation of FAM. Thus, although every 17ES–FTC molecule has one FAM attached,  $d_{\text{F}}^+$  equals 0.934. TMR and Cy5 are pH insensitive fluorophores. No correction is needed for them in terms of pH changes.

Similar to the effect of protonation of FAM,  $\text{Zn}^{2+}$  might change the quantum yield of fluorophores by forming ground-state complexes with fluorophores. Thus, the  $d^+$  factor of the corresponding fluorophore could be affected. This effect was determined by titrating  $\text{Zn}^{2+}$  into FAM or TMR singly labeled DNA molecules under the same conditions of the trifluorophore experiment (see Supporting Information). Cy5 was always acting as an acceptor, and the quantum yield of an acceptor was not included in any of the calculations here. So, the effect of  $\text{Zn}^{2+}$  for Cy5 was not determined separately. For TMR, less than 2% fluorescence intensity change was observed up to 1 mM  $\text{Zn}^{2+}$ , the highest  $\text{Zn}^{2+}$  concentration used in this work. For FAM, only 5% intensity decrease was observed in the presence of 1 mM  $\text{Zn}^{2+}$ , and the quenching can be described by a Stern–Volmer plot. Therefore, this factor has little effect on the results described here.

For the FAM–Cy5 pair, special consideration has to be made. Different from the case of the other two FRET pairs, the acceptor Cy5 can accept energy from both FAM and TMR, even though only FAM is considered as a donor and TMR is considered as a quencher for FAM. To use the concept of  $(\text{ratio})_A$ , the red curve  $F(\lambda_{\text{em}}, 490)$  in Figure 2 is fitted to the sum of three components. The first component is the green curve, which is the emission spectrum of the trifluorophore-labeled DNA excited at 647 nm  $F(\lambda_{\text{em}}, 647)$  (only Cy5 absorbs at 647 nm). The second component is the emission spectra of a FAM singly labeled DNA  $F(\lambda_{\text{em}}, 490)$  excited at 490 nm. The third component is the emission spectra of a TMR singly labeled DNA  $F(\lambda_{\text{em}}, 560)$  excited at 560 nm. According to eq 4,

$$(\text{ratio})_A^{\text{FC}} = \frac{F(\lambda_{\text{em}}, 490) - \alpha F^{\text{FAM}}(\lambda_{\text{em}}, 490) - \beta F^{\text{TMR}}(\lambda_{\text{em}}, 560)}{F(\lambda_{\text{em}}, 647)} \quad (7)$$

$$= \frac{\epsilon^{\text{Cy5}}(490)}{\epsilon^{\text{Cy5}}(647)} + d_{\text{F}}^+ E_{\text{FC}} \frac{\epsilon^{\text{FAM}}(490)}{\epsilon^{\text{Cy5}}(647)} + d_{\text{T}}^+ E_{\text{TC}} \frac{\epsilon^{\text{TMR}}(490)}{\epsilon^{\text{Cy5}}(647)} + d_{\text{F}}^+ d_{\text{T}}^+ E_{\text{FT}} E_{\text{TC}} \frac{\epsilon^{\text{FAM}}(490)}{\epsilon^{\text{Cy5}}(647)} \quad (8)$$

$\beta$  is the weighting factor to fit the TMR contribution in the trifluorophore FRET spectra, similar to  $\alpha$ . When  $(\text{ratio})_A^{\text{FC}}$  is compared with eqs 5 and 6, it has more components. This is due to more sources that contribute to the emission of Cy5. In eq 8, the first component results from the direct excitation of Cy5 at 490 nm. Since Cy5 does not absorb at 490 nm, this part is zero. The second component results from the FRET from FAM to Cy5. The third component results from the FRET from the directly excited TMR to Cy5, and finally, the fourth component results from the FRET from FAM to Cy5 via TMR.  $d_{\text{F}}^+ d_{\text{T}}^+$  stands for the fraction of DNA labeled with all three fluorophores.

The three  $(\text{ratio})_A$  values were determined by fitting the fluorescence spectra. All the extinct coefficient ratios have been determined separately from absorption spectra. With these data, the three energy transfer efficiencies can be calculated from eqs 5, 6, and 8.

**Fluorophore-to-Fluorophore Distance Calculation from FRET Efficiency.** In a dual-labeled system, FRET efficiency ( $E$ ) is the most frequently used parameter to assess the folding of macromolecules. The larger the  $E$ , the shorter the fluorophore-to-fluorophore distance ( $R$ ), as long as  $R_0$  is kept constant. In a trifluorophore system, when a donor (FAM) can transfer energy to two acceptors (TMR and Cy5),  $R$  must be used to evaluate the folding. For example, when the FAM–TMR pair is considered, the presence of Cy5 decreases the quantum yield of FAM ( $\Phi^{\text{D}}$ ). From eq 2, the change of  $\Phi^{\text{D}}$  induces the change of  $R_0$ . Then,  $E$  is no longer a function of  $R$  only but a function of both  $R$  and  $R_0$ . Thus, when the FAM–TMR pair and the FAM–Cy5 pair are considered,  $R$  should be used to monitor the folding instead of  $E$ . For the TMR–Cy5 pair, since FAM does not interfere, both  $E$  and  $R$  can be used.

The donor quantum yield ( $\Phi^{\text{D}}$ ) in the expression of  $R_0$  (eq 2) is defined as the quantum yield of the donor (FAM) in the absence of the acceptor. When the FAM–TMR pair is considered, the quantum yield of the donor in the absence of the acceptor (TMR) will be less than that of free FAM, because of the energy transfer to Cy5. The extent of quenching depends on the distance between FAM and Cy5 and  $R_0$  for this pair, since the quenching is actually a FRET process from FAM to Cy5. According to eq 1, the decrease of quantum yield of FAM due to energy transfer to Cy5 is

$$\Delta\Phi = \frac{(R_0^{\text{FC}})^6}{(R_0^{\text{FC}})^6 + (R^{\text{FC}})^6} \quad (9)$$

From eq 2, in the presence of Cy5,  $R_0$  for the FAM–TMR pair has changed to

$$(R_0^{\text{FT}})^6 = (R_0^{\text{FT}})^6 \left( 1 - \frac{(R_0^{\text{FC}})^6}{(R_0^{\text{FC}})^6 + (R^{\text{FC}})^6} \right) \quad (10)$$

From eq 1,

$$E_{\text{FT}} = \frac{(R_0^{\text{FT}})^6}{(R_0^{\text{FT}})^6 + (R^{\text{FT}})^6} \quad (11)$$

$R_0^{\text{FT}}$  is the Förster's distance for the FAM–TMR pair when Cy5 is absent, and the value can be found in the literature or calculated from eq 2.  $R_0^{\text{FT}'}$  is the Förster's distance for the FAM–TMR pair when the quenching of FAM by Cy5 is taken into account. When the FAM–Cy5 pair is considered, very similar results can be acquired. The equations are listed as follows:

$$(R_0^{\text{FC}})^6 = (R_0^{\text{FC}})^6 \times \left(1 - \frac{(R_0^{\text{FT}})^6}{(R_0^{\text{FT}})^6 + (R^{\text{FT}})^6}\right) \quad (12)$$

$$E_{\text{FT}} = \frac{(R_0^{\text{FT}})^6}{(R_0^{\text{FT}})^6 + (R^{\text{FT}})^6} \quad (13)$$

From eqs 10 to 13, the distance can be determined and the results are listed as follows:

$$R^{\text{FT}} = R_0^{\text{FT}} \left( \frac{1 - E_{\text{FT}} - E_{\text{FC}}}{E_{\text{FT}}} \right)^{1/6} \quad (14)$$

$$R^{\text{FC}} = R_0^{\text{FC}} \left( \frac{1 - E_{\text{FT}} - E_{\text{FC}}}{E_{\text{FC}}} \right)^{1/6} \quad (15)$$

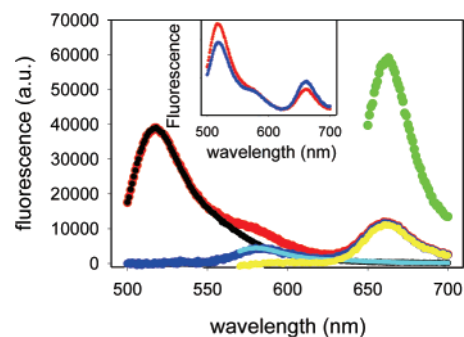
## Results

**Absorption and Steady-state Fluorescence Spectra of 17ES–FTC.** The absorption spectrum of the trifluorophore-labeled “8-17” DNazymes (17ES–FTC) is shown in the inset of Figure 2. Three absorption peaks at 490, 560, and 647 nm are observed, indicating the presence of FAM, TMR, and Cy5, respectively. The extinction coefficients of the fluorophores are determined according to procedures described in the Experimental Section.

To obtain quantitative FRET efficiency and distance information among the three fluorophores, 17ES–FTC is excited at 490, 560, and 647 nm (Figure 2). The excitation at 490 nm results in emissions at 518, 580, and 660 nm (Figure 2, red curve). The 518 nm peak is from the FAM emission. The 580 nm shoulder is attributable to the sum of the “red tail” emission of FAM, the emission from TMR, and FRET from FAM to TMR. The 660 nm peak is mainly a Cy5 emission resulted from energy transfer from both FAM and TMR, with a small fraction of the “red tail” emission from both FAM and TMR. With a 560 nm excitation, the influence of FAM can be ignored. A common TMR–Cy5 pair FRET spectrum is observed (Figure 2, blue curve). With a 647 nm excitation, only Cy5 emits at 660 nm (Figure 2, green curve).

**Emission Spectra Fitting Using Trifluorophore (Ratio)<sub>A</sub> Method.** To extract quantitative energy transfer information from the spectra in Figure 2, spectra fitting based on the (ratio)<sub>A</sub> method has been applied. The fitting method has been described in detail in the Experimental Section. In summary, for the FAM–TMR pair and the TMR–Cy5 pair, since the acceptor receives energy from only one donor, the fitting method is identical to that of the dual-labeled system.<sup>1</sup> The main difference in the trifluorophore spectra fitting lies in the FAM–Cy5 pair. Since when FAM is excited, Cy5 can receive energy from both FAM and TMR. An example of spectra fitting for FAM–Cy5 pair is given here.

When excited at 490 nm, the fluorescence spectra of 17ES–FTC (Figure 3, red curve) contain three components. They are emissions from FAM, TMR, and Cy5, respectively. To acquire FRET efficiency from FAM to Cy5, we are interested only in



**Figure 3.** An example of emission spectra fitting using the trifluorophore (ratio)<sub>A</sub> method for the FAM–Cy5 pair (see the Results section for detail descriptions). The inset is the steady-state fluorescence spectra (excitation wavelength: 490 nm) for 17ES–FTC when  $\text{Zn}^{2+}$  concentrations are 0 (red curve) and 1 mM (blue curve), respectively. The decrease in the FAM (518 nm) intensity and increase in the Cy5 intensity (660 nm) indicate the folding of 17ES–FTC.

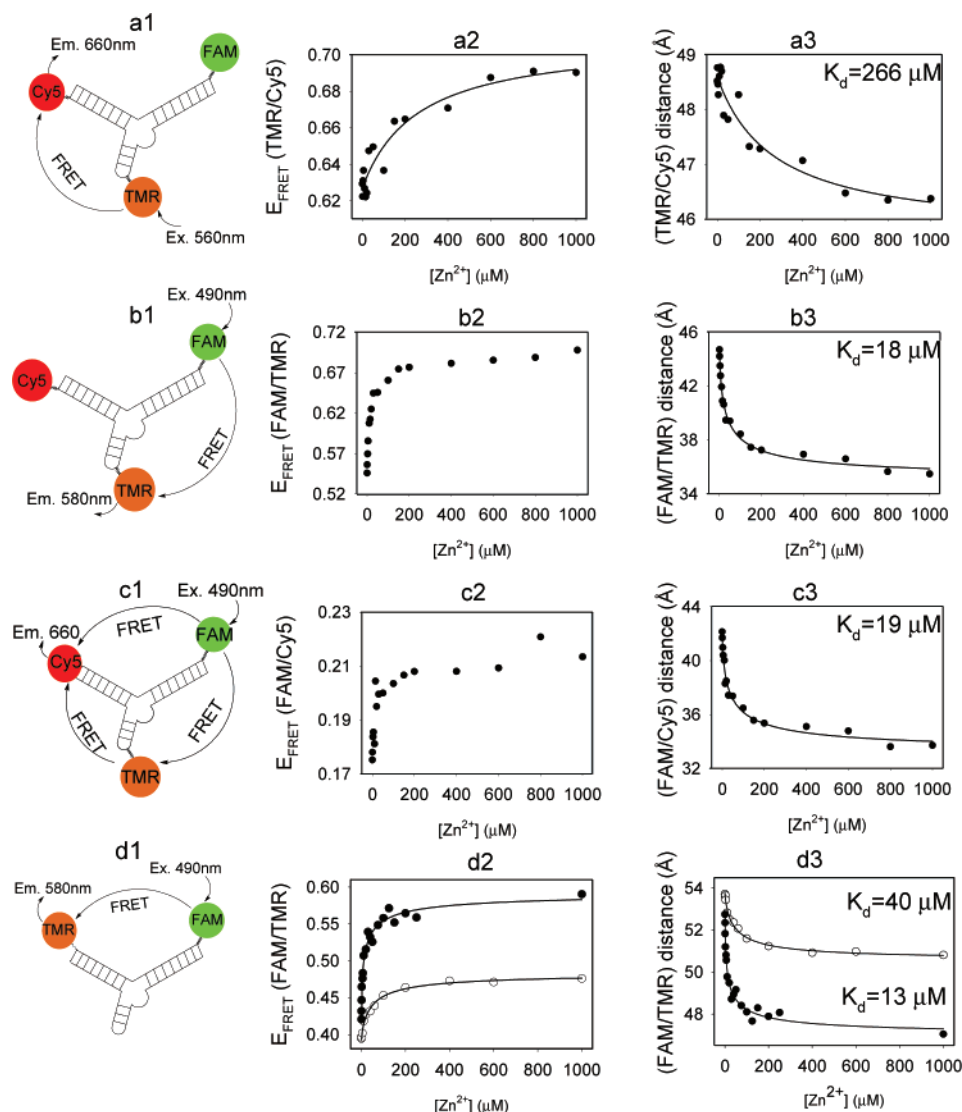
the sensitized Cy5 emission. Thus, the emissions from FAM and TMR have to be eliminated. The contribution of FAM emission is eliminated by fitting a FAM singly labeled emission spectrum (black curve) over  $\lambda_{\text{em}} = 500\text{--}540$  nm (only FAM emits in this range) to the red curve. The resulting difference spectrum is the blue curve, which shows only the emission peaks of TMR at 580 nm and Cy5 at 647 nm. A second fitting is performed to remove the TMR contribution by fitting a TMR singly labeled emission spectrum (cyan curve) over  $\lambda_{\text{em}} = 570\text{--}610$  nm (only TMR emits in this range) to the blue curve. The resulting difference spectrum is the yellow curve. Only the sensitized Cy5 emission at 660 nm is left. The ratio of this peak and the peak when 17ES–FTC is excited at 647 nm (green curve) is defined as the (ratio)<sub>A</sub> for the FAM and Cy5 pair (see eq 7). From eq 8, the FRET efficiency for this pair can be calculated.

The inset of Figure 3 shows fluorescence spectral changes in the absence (red curve) and presence (blue curve) of 1 mM  $\text{Zn}^{2+}$  when 17ES–FTC is excited at 490 nm. The change is mainly due to  $\text{Zn}^{2+}$ -induced folding of the DNzyme and indicates, like a dual-labeled system, a trifluorophore-labeled system can be very sensitive to environmental change too.

**Quantitative Analysis for the Folding of 17ES–FTC in the Presence of  $\text{Zn}^{2+}$ .** As an example to show the application of trifluorophore FRET, the  $\text{Zn}^{2+}$ -dependent 17ES–FTC folding is illustrated below. The equations for spectra fitting and calculations are derived in the Experimental Section, and only the results are presented here.

**1. Folding of the TMR, Cy5-Labeled Arms (TC Arms).** The  $\text{Zn}^{2+}$ -dependent folding of the TC arms can be displayed by either FRET efficiency ( $E$ ) changes (Figure 4a2) or average TMR-to-Cy5 distance ( $R$ ) changes (Figure 4a3). The two sets of data can be best fit to a  $\text{Zn}^{2+}$ -binding curve with Hill coefficients of 1.00 and 0.96, respectively, indicating one class of  $\text{Zn}^{2+}$  binding to the DNzyme. The one  $\text{Zn}^{2+}$ -binding curve fitting also results in apparent dissociation constants ( $K_d$ ) of 266  $\mu\text{M}$  and 260  $\mu\text{M}$ , respectively. This 6  $\mu\text{M}$  difference in  $K_d$  values when two different parameters are used is due to the nonlinear relation of  $E$  and  $R$  (see eq 1).  $R_0$  for the TMR and Cy5 pair is reported to be 53 Å.<sup>42</sup> Thus, the calculated TMR-to-Cy5 distance changed from 48.6 Å in the  $\text{Zn}^{2+}$ -free state (apo-state) to 45.7

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**Figure 4.** Quantitative results for the folding of 17ES-FTC in the presence of  $\text{Zn}^{2+}$ . The figures are organized into four groups. Group a is related to the folding of the TMR–Cy5 labeled arms. In part a1, TMR is excited at 560 nm and the sensitized Cy5 emission at 660 nm is monitored. Part a2 is the  $\text{Zn}^{2+}$ -dependent FRET efficiency changes for the TMR–Cy5 pair. Part a3 is the  $\text{Zn}^{2+}$ -dependent average distance change between TMR and Cy5. Group b is related to the folding of the FAM–TMR labeled arms. In part b1, FAM is excited at 490 nm and the sensitized TMR emission at 580 nm is monitored. Part b2 is the  $\text{Zn}^{2+}$ -dependent FRET efficiency changes for FAM–TMR pair. Part b3 is the  $\text{Zn}^{2+}$ -dependent average distance change between FAM and TMR. Group c is related to the folding of the FAM–Cy5 labeled arms. In part c1, FAM is excited at 490 nm and the sensitized Cy5 emission at 660 nm is monitored. Part c2 is the  $\text{Zn}^{2+}$ -dependent FRET efficiency changes for the FAM–Cy5 pair. c3 is the  $\text{Zn}^{2+}$ -dependent average distance change between FAM and Cy5. Group d is related to the folding of a dual-labeled DNAzyme as control experiments. In part d1, FAM is excited at 490 and sensitized TMR emission at 560 nm is monitored. Part d2 is the  $\text{Zn}^{2+}$ -dependent energy transfer efficiency change when the enzyme is 17E (●) and 17E–c (○). Part d3 is the  $\text{Zn}^{2+}$ -dependent FAM-to-TMR distance change when the enzyme is 17E (●) or 17E–c (○).

Å in the folded state, according to eq 1. For the  $\text{Zn}^{2+}$ -induced folding, a simple two-state folding model is assumed. When the DNAzyme does not bind to  $\text{Zn}^{2+}$ , the FRET efficiency is  $E_{\text{apo}}$  and the corresponding distance is  $R_{\text{apo}}$ . Upon  $\text{Zn}^{2+}$  binding, the parameters change to  $E_{\text{fold}}$  and  $R_{\text{fold}}$  because of the folding of the DNAzyme. The average value of  $E$  or  $R$ , which is obtained from experiment, shows the relative population of the DNAzyme in the folded state.

**2. Folding of the FAM, TMR-Labeled Arms (FT Arms).** For the FAM–TMR pair, the  $\text{Zn}^{2+}$ -dependent average FAM-to-TMR distance, instead of FRET efficiency, should be used to monitor the folding of FT arms (see the Experimental Section for explanations). For the sake of comparison, the  $\text{Zn}^{2+}$ -dependent FRET efficiency ( $E_{\text{FT}}$ ) changes for the FAM–TMR pair are plotted in Figure 4b2. Physically,  $E_{\text{FT}}$  is not related

directly to the  $\text{Zn}^{2+}$ -dependent folding of FT arms, so the data in Figure 4b2 are not fitted. To calculate the FAM-to-TMR distance (from eq 14), both  $E_{\text{FT}}$  and  $E_{\text{FC}}$  (FRET efficiency for FAM–Cy5 pair) are needed.  $R_0$  for the FAM–TMR pair is reported to be 50 Å.<sup>2</sup> The  $\text{Zn}^{2+}$ -dependent FAM-to-TMR distance is plotted in Figure 4b3, and the data are best fit with a Hill coefficient of 0.68 and an apparent dissociation constant  $K_d$  of 18  $\mu\text{M}$  for one  $\text{Zn}^{2+}$ -binding. From Figure 4b3, the distances between the two fluorophores are 44.4 Å for the apo state and 36 Å when one  $\text{Zn}^{2+}$  binds.

**3. Folding of the FAM, Cy5-Labeled Arms (FC Arms).** The data analysis for the FAM–Cy5 pair is similar to that in the FAM–TMR pair, except that Cy5 is now considered as an energy acceptor, while TMR is considered as a quencher to FAM. By fitting the spectra as illustrated in Figure 3, the (ratio)<sub>A</sub>



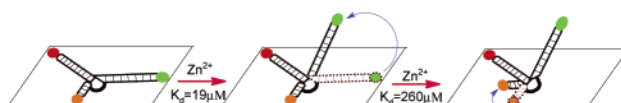
for the FAM–Cy5 pair can be acquired. From eq 8,  $E_{FC}$  can be calculated. The plot of  $E_{FC}$  versus  $Zn^{2+}$  concentration is shown in Figure 4c2.  $R_0$  for the FAM–Cy5 pair is calculated to be 39 Å under the current experimental conditions. The  $Zn^{2+}$ -dependent average FAM-to-TMR distance is plotted in Figure 4c3. The best-fitted curve is a Hill coefficient of 0.65 and a  $K_d$  of 19  $\mu M$  for one  $Zn^{2+}$  binding. The distances between FAM and Cy5 are 41.8 Å in the apo state and 34.2 Å in the folded state, according to the fitting.

**Verification of the Trifluorophore FRET Results by a Dual-Labeled DNzyme.** A dual-labeled DNzyme is used to verify the theory and results of trifluorophore FRET described above. In this case, the substrate is 5' labeled with a FAM and 3' labeled with a TMR (Figure 4d1). No fluorophore is attached to the enzyme strand. The  $Zn^{2+}$ -dependent distance change is plotted in Figure 4d3 (solid circle), from which a Hill coefficient of  $0.61 \pm 0.09$  and a  $K_d$  of  $13 \pm 4 \mu M$  can be obtained. The corresponding pair in the trifluorophore system is the FAM–Cy5 pair, which has a Hill coefficient of  $0.65 \pm 0.11$ , and a  $K_d$  of  $19 \pm 4 \mu M$ . Therefore, within the errors of the experiments, the result from the trifluorophore-labeled system is consistent with the that of the dual-labeled system. There is, however, about a 10 Å discrepancy in the absolute fluorophore-to-fluorophore distance. This may arise from the fact that, in the dual-labeled system, the acceptor (TMR) is on the substrate strand, while, for the trifluorophore-labeled system, the acceptor (Cy5) is on the enzyme strand. Since the diameter of a B-form DNA is  $\sim 20$  Å alone and the long carbon chain linked the fluorophores to DNA, the 10 Å difference is not unreasonable. This result supports the notion that FRET, in most cases, may not be used to accurately determine the absolute distance without knowledge of the exact positions of fluorophores on DNA.<sup>2</sup> It could, however, be used to accurately measure the relative distance changes such as metal-dependent DNzyme folding.

In addition, to verify that the distance changes observed are not due to the nonspecific binding of  $Zn^{2+}$  to the phosphate backbone of the DNzyme, an inactive variant of the “8-17” DNzyme is used in the study. As shown in the previous study,<sup>29,33,34</sup> when the G•T wobble pair (10th base pair from the 5' of the enzyme strand, highlighted by a dot in Figure 1) was replaced by a G•C Watson–Crick base pair, the “8-17” DNzyme (named 17E–c) lost its activity completely. The  $Zn^{2+}$ -dependent FAM-to-TMR distance decrease of 17E–c is plotted as open circles in Figure 4d3. First, for 17E–c, the degree of folding is much less than that in 17E. Second, the apparent  $K_d$  is 40  $\mu M$  in the case of 17E–c, more than that for 17E (13  $\mu M$ ). These differences suggest that the folding of the DNzyme is sequence specific and not because of the non-specific interaction of  $Zn^{2+}$  with the DNA phosphate backbone. A similar effect of point mutation induced misfolding has also observed in the hammerhead ribozyme<sup>43</sup> and the hairpin ribozyme.<sup>12</sup>

## Discussion

**Trifluorophore-Labeled System.** Dual-labeled FRET has been commonly applied to study the folding and structure of many biomolecules.<sup>1–4</sup> Given the multiple-branched structure of many macromolecules, such as DNzymes, we find it is



**Figure 5.** Schematics of the two-step folding of 17ES–FTC in the presence of  $Zn^{2+}$ . Blue arrows indicate the arms that fold in the presence of  $Zn^{2+}$ . At low  $Zn^{2+}$  concentration, the arm labeled with FAM (green ball) folds toward the other two arms by binding one  $Zn^{2+}$ , with an apparent dissociation constant 19  $\mu M$ . The relative positions of the other two arms are not changed at low  $Zn^{2+}$  concentration. At higher  $Zn^{2+}$  concentration, a second  $Zn^{2+}$  binds to the DNzyme ( $K_d = 260 \mu M$ ), which induces the folding of the TMR (orange) and Cy5 (red) arms closer.

advantageous to study the structure and folding of all the branches at the same time. Using more than two fluorophores attached to different parts of the molecules, multiple sites monitoring can be realized. As shown in this study, the trifluorophore-labeled system can maintain the high sensitivity of the FRET technique for monitoring structure changes. A comparison of results from dual-labeled and trifluorophore-labeled systems indicated that the results for metal-dependent folding in the two systems are comparable. Moreover, the experiment results can be more consistent, and the amount of work can be reduced significantly using the multifluorophore labeling technique.

The presence of an additional fluorophore in the trifluorophore-labeled system, however, does complicate data analysis. As demonstrated in this study, the (ratio)<sub>A</sub> method that has been used successfully in the study of dual-labeled systems is equally effective in trifluorophore-labeled systems, where all the fluorophores can be different. To apply the (ratio)<sub>A</sub> method for FRET efficiency calculations, there should be sufficient separation in the absorption bands of the fluorophores, so that the acceptors can be excited separately without exciting the donors. For 17ES–FTC, the  $Zn^{2+}$ -dependent FRET efficiency ( $E$ ) and fluorophore-to-fluorophore distance ( $R$ ) changes are similar to those of a dual-label system (i.e.,  $E$  increases and  $R$  decreases) (Figure 4). However, this is not general. From eqs 14 and 15,  $R^{FT}$ ,  $R^{FC}$ ,  $E_{FT}$ , and  $E_{FC}$  are interrelated. For example, from  $E_{FT}$  alone, no conclusion can be drawn about  $R^{FT}$  without also knowing  $E_{FC}$ . Thus, when FRET with multiple acceptors present is considered,  $R$  should be used to evaluate folding, and not  $E$ . Finally, when  $E$  drops to very small values, small errors in  $E$  can bring relatively large variations in  $R$ . If the two fluorophores are separated by more than  $2R_0$  ( $E = \sim 0.016$ ),  $E$  is no longer sensitive to  $R$  changes. This is true for both dual-labeled and trifluorophore systems. Small FRET efficiency is more likely to occur in trifluorophore systems, since the overlap integral of the first fluorophore (FAM) and the third fluorophore (Cy5) is small. The small overlap integral gives a small  $R_0$  (see eq 2).

**Overall  $Zn^{2+}$ -Dependent Folding of 17ES–FTC.** The apparent dissociation constants are determined to be 18  $\mu M$  from the folding of the FT arms (Figure 4b3) and 19  $\mu M$  from the folding of the FC arms (Figure 4c3). The close similarity of the two dissociation constants reasonably indicates that the distance decrease between FAM and the other two fluorophores happens in the same process. The folding of the TC arms requires a much higher  $Zn^{2+}$  concentration ( $K_d = 260 \mu M$ ). Combining all the data, we propose a two-step folding scheme (see Figure 5). In the first step of folding, the distance between FAM and TMR decreases from 44.4 Å when 17ES–FTC is free of  $Zn^{2+}$  to 36 Å when it binds to one  $Zn^{2+}$ . The same process reduces the distance between FAM and Cy5 from 41.8

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to 34.2 Å. The  $K_d$  for the first  $\text{Zn}^{2+}$  binding is around 19  $\mu\text{M}$ . However, while the folding of the FT and FC arms are complete, the relative position of the TC arms has not changed much. The second step of folding is the folding of the TC arms. From Figure 4c3, the apparent dissociation constant for this process is 260  $\mu\text{M}$  by fitting the data to a one  $\text{Zn}^{2+}$  binding model, which is more than 1 order of magnitude higher than the  $\sim 19$   $\mu\text{M}$  for the first  $\text{Zn}^{2+}$  binding.

The structure of the “8-17” DNAzyme shares the most similarity with that of the hammerhead ribozyme. For example, both have the bulged three-way junction structure, can act as transcleaving enzymes, require divalent metal ions for the cleavage activity, and show interesting folding in the presence of divalent metal ions. By labeling the three branches of hammerhead ribozyme two at a time, with the FRET donor FAM and acceptor Cy3, Bassi et al. have observed two sequential  $\text{Mg}^{2+}$ -dependent conformation transitions,<sup>8</sup> where, at 500  $\mu\text{M}$   $\text{Mg}^{2+}$ , helices II folds away from helices III and at 10 mM  $\text{Mg}^{2+}$ , helices I folds away from helices III.

By using the trifluorophore-labeled system, the folding of the three arms of the DNAzyme in the presence of  $\text{Zn}^{2+}$  has been monitored simultaneously in this study. This scheme shares a similarity with  $\text{Mg}^{2+}$ -induced hammerhead ribozyme folding<sup>8</sup> in terms of a two-step folding process. However, there are two major differences, each of which is manifested in their metal-ion-dependent structures and catalytic mechanisms. The first difference is the metal-binding affinity. The dissociation constants for the  $\text{Zn}^{2+}$ -induced folding of “8-17” DNAzyme (19  $\mu\text{M}$  for the first step and 260  $\mu\text{M}$  for the second step) are  $\sim 4$ –5-fold stronger than those for the  $\text{Mg}^{2+}$ -induced folding of hammerhead ribozyme ( $\sim 110$   $\mu\text{M}$  for the first step and  $\sim 910$   $\mu\text{M}$  for the second step). The higher metal-binding affinity in the  $\text{Zn}^{2+}$ -induced folding of “8-17” DNAzyme is consistent with the higher metal-binding affinity measured in the  $\text{Zn}^{2+}$ -dependent catalytic reaction.<sup>34,44</sup> These results strongly suggest that metal-ion-dependent folding plays a critical role in the catalytic reaction of DNAzymes, just like in ribozymes.

The second difference between the two systems is that, in the hammerhead ribozyme, upon binding to  $\text{Mg}^{2+}$ , certain branches (e.g., branch I and II in the second step) fold closer to each other, while others (e.g., I and III in the second step) move apart.<sup>8</sup> In contrast, all branches of the “8-17” fold closer upon binding to  $\text{Zn}^{2+}$  and form a more compact structure.

The difference in the metal-ion-dependent folding patterns between the “8-17” DNAzymes and hammerhead ribozymes may have functional implications. It is believed that the resting state conformation of hammerhead ribozymes is not necessarily the active form of the enzyme, and a conformational switch that allows a closer interaction of two branches may be necessary to form the active form of the hammerhead ribozyme.<sup>45,46</sup> This energy cost may explain the relative low catalytic rate of  $\sim 1$   $\text{min}^{-1}$  in the hammerhead ribozyme.<sup>47</sup> On the other hand, the  $\text{Zn}^{2+}$ -dependent catalytic rate of the “8-17” DNAzyme is much higher ( $\sim 50$   $\text{min}^{-1}$ ) under similar conditions.<sup>34</sup> While many factors may contribute to the high catalytic rate, including  $\text{Zn}^{2+}$

being a better Lewis acid and possessing a lower  $\text{p}K_a$  of metal-bound water, it is tempting to suggest that the more compact structure resulted from the  $\text{Zn}^{2+}$ -induced folding may help eliminate or at least minimize the need for further conformational changes for catalytic function. Further studies, including X-ray crystallography, are needed to support this hypothesis.

**Could More Than Three Fluorophores Be Used in a FRET Study?** After demonstration of the effectiveness of the trifluorophore system, the next logical question is whether a system with more than three different fluorophores could work. In principle, as long as there is adequate separation of absorption peaks of fluorophores, the (ratio)<sub>A</sub> method can still be applied, no matter how many different fluorophores are labeled. For example, the two by two combination of four different fluorophores will give 6 donor–acceptor pairs (Figure 6). With four excitations, four FRET spectra can be obtained. When the four spectra are combined two at a time, six equations can be established so that the six FRET efficiencies can be calculated, just like what has been accomplished in the trifluorophore system. The advantage of the (ratio)<sub>A</sub> method is that the FRET efficiency can be determined accurately without considering the change of  $R_0$ . With the FRET efficiency between each fluorophore pair, the corrected  $R_0$  can be expressed to calculate distance  $R$ . The equations for distance calculation are listed according to the scheme in Figure 6.

$$R_{12} = (R_0)_{12} \times \left( \frac{1 - E_{12} - E_{13} - E_{14}}{E_{12}} \right)^{1/6}$$

$$R_{13} = (R_0)_{13} \times \left( \frac{1 - E_{12} - E_{13} - E_{14}}{E_{13}} \right)^{1/6}$$

$$R_{14} = (R_0)_{14} \times \left( \frac{1 - E_{12} - E_{13} - E_{14}}{E_{14}} \right)^{1/6}$$

$$R_{23} = (R_0)_{23} \times \left( \frac{1 - E_{23} - E_{24}}{E_{23}} \right)^{1/6}$$

$$R_{24} = (R_0)_{24} \times \left( \frac{1 - E_{23} - E_{24}}{E_{24}} \right)^{1/6}$$

$$R_{34} = (R_0)_{34} \times \left( \frac{1 - E_{34}}{E_{34}} \right)^{1/6}$$

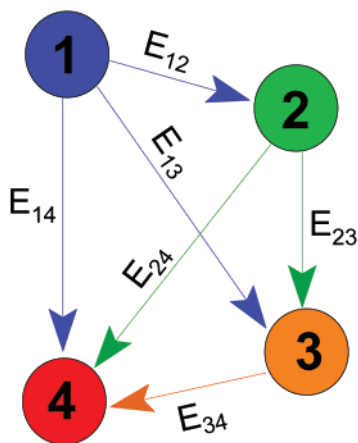
The above equations can be generalized to an even higher number of fluorophore-labeled systems. A potential problem is that the increasing number of accumulated errors may decrease the accuracy of data. Another practical problem for a multi-fluorophore-labeled system is whether the first donor can transfer energy to the last acceptor in the system. FRET requires overlap between the emission spectra of the donor and the absorption spectra of the acceptor. For four fluorophores with adequate separation in absorption, the overlap integral between the first and the last fluorophore may be very small, which in turn gives small  $R_0$ . In the extreme case of zero overlap, no energy is transferred in this pair and the distance information of this pair is lost. Despite this problem, FRET efficiency and distance information can still be obtained from other pairs with observable amounts of energy transfer.

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**Figure 6.** Schematics of a tetrafluorophore system. Fluorophore 1, 2, 3, and 4 have an increasing absorption wavelength maximum. Fluorophore 1 can transfer energy to fluorophore 2, 3, and 4. Fluorophore 2 can transfer energy to fluorophore 3 and 4. Fluorophore 3 can transfer energy to fluorophore 4. When the four fluorophores two by two are considered, there are six FRET pairs.

## Conclusions

We have demonstrated for the first time the use of a trifluorophore-labeled system in the FRET study of a DNzyme where three different fluorophores are attached to each branch of the molecule. Equations for calculating the FRET efficiency and average fluorophore-to-fluorophore distance have been derived, and its effectiveness in elucidating the metal-ion-dependent structure and folding of the DNzyme has been established. It was also further verified by a comparison of results between dual-labeled and trifluorophore-labeled systems.

From the study, a  $\text{Zn}^{2+}$ -induced two-step folding process was observed in the *in vitro* selected “8-17” DNzyme and its substrate duplex. The enzyme–substrate folds into a more compact structure that may be critical for enzyme catalysis. In principle, this methodology can be applied to multifluorophore-labeled systems (i.e., a tetrafluorophore system for four-way DNA junctions). The multifluorophore-labeled system will find increasing applications in the FRET study of complex systems, where structural changes of several branches of the same molecule need to be observed simultaneously.

**Acknowledgment.** We thank Professor Robert Clegg for advice and helpful discussions. This material is based upon work supported by the Natural and Accelerated Bioremediation Research (NABIR) program, Biological and Environmental Research (BER), U.S. Department of Energy (DEFG02-01-ER63179), and Nanoscale Science and Engineering Initiative of the National Science Foundation (DMR-0117792). The experiments reported in this paper were performed at the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois at Urbana-Champaign (UIUC). The LFD is supported jointly by the Division of Research Resources of the National Institutes of Health (PHS 5 P41-RRO3155) and UIUC.

**Supporting Information Available:** Plot of FAM fluorescence quenching by protonation, Stern–Volmer plot of FAM quenching by  $\text{Zn}^{2+}$ , and Stern–Volmer plot of TMR quenching by  $\text{Zn}^{2+}$ . This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA027647Z