Biophysical Journal: Biophysical Letters

Fluorescence Quenching by TEMPO: A Sub-30 Å Single-Molecule Ruler

Peizhi Zhu, Jean-Pierre Clamme, and Ashok A. Deniz
Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

ABSTRACT A series of DNA molecules labeled with 5-carboxytetramethylrhodamine (5-TAMRA) and the small nitroxide radical TEMPO were synthesized and tested to investigate whether the intramolecular quenching efficiency can be used to measure short intramolecular distances in small ensemble and single-molecule experiments. In combination with distance calculations using molecular mechanics modeling, the experimental results from steady-state ensemble fluorescence and fluorescence correlation spectroscopy measurements both show an exponential decrease in the quenching rate constant with the dye-quencher distance in the 10–30 Å range. The results demonstrate that TEMPO-5-TAMRA fluorescence quenching is a promising method to measure short distance changes within single biomolecules.

Received for publication 18 July 2005 and in final form 13 September 2005.

Peizhi Zhu and Jean-Pierre Clamme contributed equally to this work.

Address reprints and inquiries to Ashok A. Deniz, Tel.: 858-784-9192; E-mail: deniz@scripps.edu.

The study of conformational distributions and dynamics of biomolecules has been revolutionized in recent years by single-molecule fluorescence resonance energy transfer (FRET), which is an efficient tool for measuring distances between 30 and 80 Å. In contrast, fewer examples of single-molecule studies have been reported for monitoring shorter distances in the sub-30 Å range. A general methodology with such a capability would be very useful for studying detailed molecular structures, such as those of protein and RNA secondary structures during folding, binding, and assembly. At the single-molecule level, fluorescence self-quenching has been used for protein folding studies (1), and fluorescence quenching of organic dyes by tryptophan or guanosine via photoinduced electron transfer has also been reported to measure short distances and fluctuations in peptides, proteins, and DNA (2–5). However, there has been no description so far of a method analogous to FRET, where dye and quencher are independently attached to points of interest on a biomolecule, allowing the distance between these points to be monitored.

TEMPO is a small organic nitroxide radical. Several studies have used its fluorescence quenching and electron paramagnetic resonance characteristics to obtain structural information for proteins and RNA, and have shown that its conjugation is well tolerated in these biomolecules (6,7). Furthermore, the distance dependence of the fluorescence quenching rate for a related nitroxide radical PROXYL has been studied by ensemble fluorescence spectroscopy, and shows an exponential distance dependence (8), providing additional support for TEMPO quenching as an attractive candidate for a short-range single-molecule ruler. Based on these features, in this work, we have used fluorescence correlation spectroscopy (FCS) to demonstrate intramolecular quenching by TEMPO of 5-carboxytetramethylrhodamine (5-TAMRA, a commonly used dye for single-molecule studies) as a sub-30 Å distance ruler for small ensemble/single-molecule measurements.

To this end, we labeled 29-mer DNA molecules with 5-TAMRA and TEMPO at different positions, producing a DNA series with different distances between the dye and quencher (sequence and constructs are shown in Fig. 1). To calculate theoretical distances between dye and quencher, we built a double-stranded DNA model (corresponding to the first 10 basepairs of the 29-mer sequence) using Hyperchem 7.51. We then used this model to optimize structures corresponding to each of our doubly labeled constructs using the BIO+(CHARMM) molecular mechanics force field, and calculated dye-quencher distances. Double labeling and distance calculation protocols are detailed in the Supplementary Material.

In our experiments, we first performed steady-state ensemble fluorescence measurements for each construct with and without the quencher. The quenching efficiencies (Q_{ens}) were calculated using the average fluorescence intensities for the DNA constructs with and without quencher (Supplementary Material). Q_{ens} varied from >50% at 10.5 Å, to close to zero at 47 Å. The quenching rate constants were calculated from Q_{ens} (Supplementary Material) and are plotted as a function of the theoretical quencher-dye distance (R) in Fig. 2, showing an exponential decrease with increasing distance (*solid line*, Fig. 2) in the 10–30 Å range, consistent with previous work (8,9,10).

We next used FCS to investigate the properties of the fluorescence quenching of 5-TAMRA by TEMPO at a small ensemble/single-molecule level. FCS is based on the time correlation of fluorescence fluctuations observed in a sub-femtoliter volume of a sample solution. In this study, FCS

doi: 10.1529/biophysj.105.071027

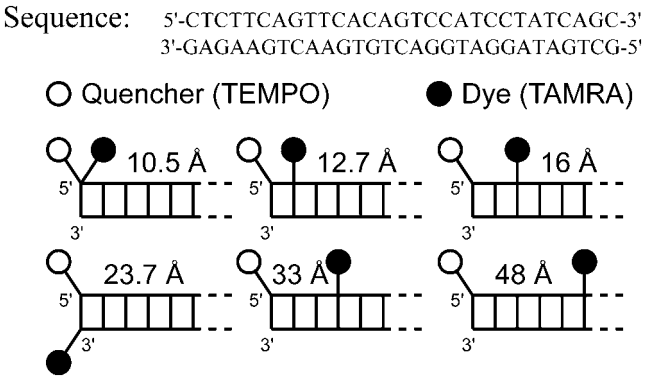

FIGURE 1 Sequences and dye/quencher labeling positions for the DNA constructs. Theoretical dye-quencher distances from molecular mechanics (see text and Supplementary Material) are also shown above each construct.

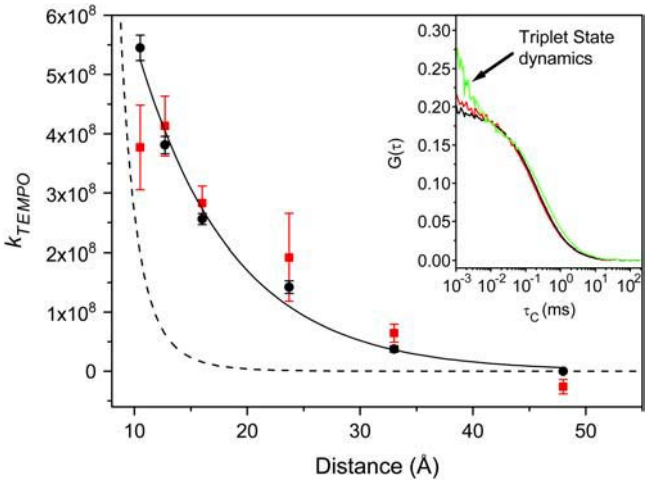

FIGURE 2 Distance dependence of TEMPO quenching rate constant (k_{TEMPO} (s^{-1})) measured by ensemble fluorescence (*black solid circle*) (*solid line* is single exponential decay fit) and FCS (*red solid square*). Dashed line is the quenching component expected due to FRET between TAMRA and TEMPO with R_0 = 8 Å. Inset shows FCS curves of DNA-TAMRA (*black*), DNA-TAMRA-TEMPO (*red*), and DNA-TAMRA in the presence of 10 mM iodide (*green*). Error bars are ±1 standard deviation.

data were recorded on a homebuilt setup (see Supplementary Material), averaged (10 files of 60 s each), and the correlation functions $G(\tau)$ analyzed to extract N, the average number of molecules in the excitation volume, and τ_D, the characteristic diffusion time of the particle (11,12). The average diffusion coefficient without ($D_T = 8.1\ (\pm 0.6) \times 10^{-11}\ m^2\ s^{-1}$) and with the quencher ($D_{TQ} = 7.8\ (\pm 0.5) \times 10^{-11}\ m^2\ s^{-1}$) are similar, showing that the addition of the quencher does not significantly alter the diffusional properties of the DNA. Our results also show that the number of molecules N (proportional to concentration) with the quencher is not significantly different from that without quencher (data not shown), thus excluding a quenching mechanism involving a long-lived (greater than the ~200 μs diffusion time) dark state, which would lower the number of fluorescent molecules detected in the focal volume. Moreover, no significant increase in the triplet state fraction was observed in the presence of the quencher (Fig. 2, *inset*, *black/red* curves with/without quencher, respectively). By comparison, quenching by iodide, known to occur by intersystem crossing to the triplet state, produces an FCS curve (Fig. 2, *inset*, *green*) displaying an additional fast decay. These results suggest that TAMRA quenching by TEMPO does not occur due to enhanced intersystem crossing of TAMRA to a dark triplet state.

To evaluate the quenching observed by FCS, the average count rate (I) for each construct was recorded and used to calculate the brightness or count rate per molecule (η) defined as $\eta = I/N$. The quenching rate constants were then derived from the quenching efficiencies (derived from η for constructs with and without quencher; see Supplementary Material). This quenching rate constant is plotted as a function of the dye-quencher distance in Fig. 2, also showing an exponential decay in good agreement with the ensemble data. Finally, Fig. 2 also shows the expected quenching based on a FRET mechanism and an R_0 of 8 Å (Supplementary Material), observed to drop below 5% above 13 Å. A comparison with the experimental data shows that a FRET mechanism of quenching cannot be making a significant contribution to the observed quenching above ~13 Å.

Our FCS distance dependence results show that TAMRA fluorescence quenching by TEMPO can be used to measure sub-30 Å distance changes with ~5 Å resolution in biomolecules at single-molecule resolution. The results also rule out a predominant role of FRET or intersystem crossing in the mechanism of the quenching process. Further technical refinements will include improving accuracy by using single-molecule fluorescence lifetime measurements as well as monitoring long time trajectories on surface immobilized molecules. We believe that this single-molecule distance measurement technique will complement FRET for measuring localized conformational changes for biomolecules during their folding, assembly, and function, including monitoring conformational fluctuations using FCS measurements (5).

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

ACKNOWLEDGMENTS

We thank J. C. Van der Schans for assistance with synthesis of the labeled constructs.

This work was supported by a grant from the National Institutes of Health (grant. No. GM066833).

REFERENCES and FOOTNOTES

1. Zhuang, X., T. Ha, H. D. Kim, T. Centner, S. Labeit, and S. Chu. 2000. Fluorescence quenching: a tool for single-molecule protein-folding study. *Proc. Natl. Acad. Sci. USA*. 97:14241–14244.
2. Edman, L., U. Mets, and R. Rigler. 1996. Conformational transitions monitored for single molecules in solution. *Proc. Natl. Acad. Sci. USA*. 93:6710–6715.
3. Yang, H., G. B. Luo, P. Karnchanaphanurach, T. M. Louie, I. Rech, S. Cova, L. Y. Xun, and X. S. Xie. 2003. Protein conformational dynamics probed by single-molecule electron transfer. *Science*. 302:262–266.
4. Neuweiler, H., A. Schulz, M. Bohmer, J. Enderlein, and M. Sauer. 2003. Measurement of submicrosecond intramolecular contact formation in peptides at the single-molecule level. *J. Am. Chem. Soc*. 125: 5324–5330.
5. Chattopadhyay, K., E. L. Elson, and C. Frieden. 2005. The kinetics of conformational fluctuations in an unfolded protein measured by fluorescence methods. *Proc. Natl. Acad. Sci. USA*. 102:2385–2389.
6. Hubbell, W. L., D. S. Cafiso, and C. Altenbach. 2000. Identifying conformational changes with site-directed spin labeling. *Nat. Struct. Biol*. 7:735–739.
7. Kim, N. K., A. Murali, and V. J. DeRose. 2004. A distance ruler for RNA using EPR and site-directed spin labeling. *Chem. Biol*. 11:939–948.
8. Matko, J., K. Ohki, and M. Edidin. 1992. Luminescence quenching by nitroxide spin labels in aqueous-solution: studies on the mechanism of quenching. *Biochemistry*. 31:703–711.
9. Zelent, B., J. Kusba, I. Gryczynski, M. L. Johnson, and J. R. Lakowicz. 1996. Distance-dependent fluorescence quenching of p-bis[2-(5-phenyl-oxazolyl)]benzene by various quenchers. *J. Phys. Chem.* 100:18592–18602.
10. Rae, M., A. Fedorov, and M. N. Berberan-Santos. 2003. Fluorescence quenching with exponential distance dependence: application to the external heavy-atom effect. *J. Chem. Phys*. 119:2223–2231.
11. Rigler, R., Ü. Mets, J. Widengren, and P. Kask. 1993. Fluorescence correlation spectroscopy with high count rate and low background. *Eur. Biophys. J*. 22:169–175.
12. Thompson, N. 1991. Fluorescence correlation spectroscopy. *In* Topics in Fluorescence Spectroscopy. R. Lakowicz, editor. Plenum Press, New York, NY. 337–378.