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STEADY-STATE AND TIME-RESOLVED FLUORESCENCE QUENCHING WITH TRANSITION METAL IONS AS SHORT- DISTANCE PROBES FOR PROTEIN CONFORMATION

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Abstract

A series of model dye-labeled histidine-containing peptides was used to investigate the nature of the quenching mechanism with Cu^{2+} and Ni^{2+} . The strong reduction in steady-state fluorescence was found to be unaccompanied by any noticeable changes in lifetime kinetics. This static nature of quenching is not consistent with the dynamic FRET phenomenon, which was assumed to dominate the quenching mechanism, and is likely caused by shorter range orbital coupling. Our results indicate that the FRET-like 6th power of distance dependence of quenching cannot be automatically assumed for transition metal ions, and time-resolved measurements should be used to distinguish various quenching mechanisms.

Keywords

static and dynamic fluorescence quenching; Förster resonance energy transfer; ground state complex; short-range orbital coupling

Understanding the molecular mechanisms of protein functioning requires accurate measurements of structural rearrangements that occur rapidly on a sub-nanometer scale. Recently, several spectroscopic approaches that utilize fluorescence quenching with transition metal ions have been suggested as aids in such studies. Their use has several potential advantages over regular FRET-based distance measurements: a weak dependence of resulting quenching on orientation, the ability to probe distances shorter than 20 Å, and the possibility of introducing chromophores without chemical labeling *via* genetically coded binding sites. Sandtner and co-workers had used lanthanide-based resonance energy transfer (LRET) between Tb^{3+} and Cu^{2+} chelated by genetically encoded tags to study short-range motions in the *Shaker* potassium channels *in vivo* (1). The dynamic nature of LRET was confirmed by the lifetime measurements. Taraska and coworkers (2, 3) suggested an extension of this approach, named “transition metal ion FRET,” to the quenching of organic dyes, such as fluorescein and bimane. In order to calibrate the distance dependence of quenching they designed a series of helical peptides in which a thiol-reactive dye was attached to a cysteine residue, while transition metal ions (Cu^{2+} or Ni^{2+}) were chelated by a

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pair of histidines placed at different positions in the sequence. In these studies, however, the dynamic nature of quenching was assumed, rather than demonstrated experimentally. Here we have used the same peptides (*e.g.*, Bi-C2H6H10 illustrated in Fig. 1) in time-resolved fluorescence measurements to demonstrate that the quenching is purely static and therefore not consistent with the FRET mechanism.

We have reproduced the published steady-state quenching results obtained with dye-labeled peptides at points of saturation with transition metal ions (Fig. 2A, B) and compared them to our original lifetime measurements (Fig. 2C, D). Samples containing 0.1 μ M of dye-labeled peptides were dissolved in the 1:1 v:v mixture of 2,2,2-trifluoroethanol and 520 mM NaCl/12 mM Hepes, pH 7.2 buffer as described in (2, 3). Steady-state fluorescence was measured using an SPEX Fluorolog FL3-22 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ) equipped with double-grating excitation and emission monochromators. The excitation wavelengths were set at 470 nm and 400 nm for fluorescein and bimane, respectively. Excitation slits were 1 nm; emission slits were 4 nm. Fluorescence decays were measured with a time-resolved fluorescence spectrometer FluoTime 200 (PicoQuant, Berlin, Germany) using a standard time-correlated single-photon counting scheme. Samples were excited at 373 nm by a sub-nanosecond pulsed diode laser (LDH 375, PicoQuant, Berlin, Germany) with a repetition rate of 10 MHz. Fluorescence emission of fluorescein-labeled peptides was detected at 519 nm and that of bimane-labeled ones at 500 nm. The other details of data collection and analysis were the same as described in (4).

As demonstrated by Taraska *et al.* (2, 3), titration with metal ions results in a sigmoidal change of intensity on the logarithm of concentration (Fig. 2, open symbols), which is consistent with progressive occupation of a di-histidine binding site with the metal ion. The overall level of quenching at saturation observed with either Fl-C2H6H10 (Fig. 2A) or Bi-C2H6H10 (Fig. 2B) is reproduced well in our experiments (solid symbols) for both Cu^{2+} (squares) and Ni^{2+} (circles). Despite the fact that the overall loss in intensity ranged from 40 to 80%, the time-resolved kinetics measured for quenched and unquenched samples were totally superimposable (Fig. 2C, D). Consistent with the loss of steady-state intensity, the time-resolved decay curves had to be collected for longer times to reach the same peak counts, yet no dynamic quenching was evident. The same lack of dynamic quenching was observed with Fl-C2H10H14 or Bi-C2H10H14 (not shown), in which histidines are located further from the dye and the overall intensity loss upon quenching is smaller.

The fact that the quenching with transition metal ions reported here was found to be of a purely static nature is inconsistent with FRET's being the predominant quenching mechanism for these peptides. Normally, static quenching is associated with the formation of a non-emitting ground-state complex. A very efficient orbital overlap, however, can lead to an apparent static quenching (*i.e.*, one with a quenching rate too fast to be resolved by our lifetime experimental setup, $k \geq 10^{11} \text{ s}^{-1}$). Such fast rates will require a close proximity of dye and quencher and will have a distance dependence different from the six's-power specific to FRET. To investigate if such close proximities can be achieved in the studied peptides, we have created and analyzed their molecular models (Fig. 1). Depending on the orientation of the probe, the center-to-center distances between the dye and the quencher (relevant for FRET) range from 8 to 16 Å, which is comparable to the size of bimane (7.5 Å, long axis) or fluorescein (9.7 Å, long axis) dyes. As demonstrated by Wong and coworkers (5), such small separations cannot be adequately described by the Förster theory, developed in point-dipole approximation, and will inevitably lead to deviation from the sixth-power distance dependence of quenching. Moreover, the edge-to-edge distances (relevant to orbital overlap) can be quite short (even in the simplest of modeling illustrated in Fig 1, which ignores possible bending and distortions of chemical bonds), leading to efficient alternative quenching.

To illustrate what the lifetime kinetics would look like if the observed changes in the steady-state fluorescence were indeed due to FRET, we have simulated fluorescence decay curves assuming a dynamic quenching mechanism (see Fig. S1 of the supplemental material). These simulated curves look very different from the observed ones (Fig. 2). We have also estimated what the quenching efficiencies would be for the three conformations depicted in Fig. 1, under the assumptions of a FRET mechanism, and evaluated whether detecting them in a fluorescence lifetime experiment is realistic. Assuming the Förster distances (R_0) of 10 and 12 Å for bimane quenching with Ni^{2+} and Cu^{2+} , respectively (2), one can easily estimate the expected efficiencies (E) from the center-to-center distances (r), according to the classical Förster theory: $E = R_0^6 / (R_0^6 + r^6)$. For the closed conformation (Fig. 1, top panel), the calculated efficiencies are 0.80 and 0.92 for Ni^{2+} and Cu^{2+} , respectively. For the intermediate conformation (middle panel) the efficiencies are 0.55 and 0.74, while for the open conformation (bottom panel) they are 0.05 and 0.14. Given that the average decay time of bimane-labeled C2H6H10 in the absence of quenching (τ_0) is about 6 ns, one can estimate what the quenched decay times (τ) would have been if the quenching was caused by FRET: $\tau = \tau_0(1-E)$. For the closed conformation $\tau = 1.2$ and 0.5 ns (for Ni^{2+} and Cu^{2+} , respectively), for the intermediate conformation $\tau = 3.0$ and 1.6 ns, and for the open conformation $\tau = 5.7$ and 5.1 ns. All these lifetimes calculated under FRET assumptions are within the range of accurate detection, yet no change in lifetime kinetics is observed experimentally.

The data presented here indicate that a short-range quenching of fluorescence of organic dyes with transition metal ions can occur without the change in nanosecond time-resolved decay kinetics. This static nature of quenching is not consistent with the dynamic FRET phenomenon, which was assumed to dominate the quenching mechanism. The quenching is likely to be caused by shorter range orbital coupling or perhaps a ground state complex formation. Our data indicate that the FRET-like sixth-power of distance dependence of quenching cannot be automatically assumed for transition metal ions and that fluorescence lifetime measurements should be used to distinguish various quenching mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

FRET	Förster resonance energy transfer
Bi	bimane
Fl	fluorescein
IRF	instrumental response function
C2H6H10 and C2H10H14	peptides with the following sequences ACAAKHAAKHAAAACA and ACAAKAAKHAAAHKA, respectively
Fl-C2H6H10, Bi-C2H6H10; Fl-C2H10H14 and Bi-C2H10H14	corresponding peptides labeled with thiol-reactive Fl and Bi dyes at the cysteine residue C2

Figure 1.

Molecular models of a Bi-C2H6H10 peptide used in this study to investigate the mechanism of fluorescence quenching by transition metal ions (Cu^{2+} and Ni^{2+}). A bimane dye and an ideal α -helical model were built with Molden package (6). A bimane dye molecule attached to the cysteine residue is shown in blue, while the two histidines comprising a metal binding site are in red. Position of Cu^{2+} ion (green) and conformation of histidines was set based on X-ray structure of Cu^{2+} binding proteins (7). A starting configuration of the dye-labeled peptide with Cu^{2+} coordinated to the two histidine residues was initially energy-minimized with molecular mechanics MM+ force field available in Molden package. The three panels correspond to the three representative dihedral angles of the bimane linker. The center-to-center distances are the distances between the centers of bimane dye and metal ion. The edge-to-edge distances are the distances between the closest non-hydrogen atoms of the bimane and histidine rings.

Figure 2.

Steady-state (A, B) and time-resolved (C, D) quenching of fluorescein-labeled (A, C) and bimane-labeled (B, D) model peptide C2H6H10 using transition metal ions. Steady-state fluorescence titration with Cu^{2+} (squares) and Ni^{2+} (circles) results in substantial reduction of intensity (open symbols are published data of Taraska and coworkers (2, 3), closed symbols represent our measurements). Both time-resolved fluorescence decays (C, D) and steady-state intensities (A, B) were measured for the following: samples containing no quencher (F1, B1), samples with saturating concentrations of Ni^{2+} (F2, B2) and Cu^{2+} (F3, B3). Despite the substantial reduction in intensity in the presence of metal ions (F_M/F), time-resolved kinetics were superimposable (heavy solid, dashed and dotted curves in C and D). These results clearly indicate static nature of quenching, inconsistent with the originally proposed FRET mechanism.