J|A|C|S COMMUNICATIONS

Published on Web 05/06/2010

Mechanisms of Quenching of Alexa Fluorophores by Natural Amino Acids

Huimin Chen,[†] Syed S. Ahsan,[†] Mitk'El B. Santiago-Berrios,[‡] Hector D. Abruña,[§] and Watt W. Webb*,[†]

School of Applied and Engineering Physics, 212 Clark Hall, Cornell University, Ithaca, New York 14853, Department of Chemistry, University of Puerto Rico at Cayey, 205 Avenida Antonio R. Barceló, Cayey, 00736, Puerto Rico, and Department of Chemistry and Chemical Biology, 121 Baker Laboratory, Cornell University, Ithaca, New York 14853

Received February 2, 2010; E-mail: www2@cornell.edu

Quenching of fluorophores by the same proteins to which they are linked is a phenomenon that is neither well-known nor well-characterized. Fluorescence spectroscopy has become an important tool for studying protein conformations and protein−protein interactions. Extrinsic fluorescent molecules, with reactive side groups, are covalently attached to specific labeling sites within the proteins of interest. Changes in fluorescence intensity or lifetime report on changes in the fluorophore's local environment. Mechanisms such as Förster Resonance Energy Transfer (FRET) also allow for precise measurement of the distances between acceptor and donor fluorophores attached to various parts of a protein.[1]

Although proteins are usually assayed after labeling to ensure that the attached fluorophore(s) do not affect their intrinsic structure and function, the effect of the target protein itself on the attached fluorophore is not often considered when making quantitative measurements of fluorescence intensity. Here, we elucidate the quenching effects that the protein itself can have on the fluorophore attached to it, using members of the Alexa Fluor series of dyes as our model. Alexa Fluor dyes from Invitrogen are often used to label proteins, because they are more stable to photobleaching or conversions to the triplet states.[2] Their high quantum efficiencies make them suitable for single molecule studies where brightness is an important factor. Pairs like Alexa dyes 488/555 and Alexa dyes 488/594 are often used as conformational reporters in FRET experiments.

Spacers, such as a short saturated carbon chain, can be added between the fluorophore and the attachment site to mitigate unwanted interactions, but their flexibility still allows for contact with nearby amino acids. Indeed, fluorescence quenching as a result of contact with amino acids within the same molecule has been observed and exploited to report on changing conformational states[3,4] or intramolecular dynamics.[5−7] While the specific mechanism of quenching is unknown, it has been attributed to photo-induced electron transfer (PET), wherein a molecule (donor) excited by light transfers its excited state electron to another molecule (acceptor) when they are in close van der Waals contact.

In this communication, we identify amino acids that quench the fluorescence of Alexa dyes 488 (AL488), 555 (AL555), and 594 (AL594). We identify four quenchers of AL488: tryptophan (Trp), tyrosine (Tyr), histidine (His), and methionine (Met). We observe a combination of static and collisional mechanisms in the quenching of AL488.

Fluorescence emission scans and two-photon excitation lifetime measurements of AL488, AL555, and AL594 hydrazides were performed in both the presence and absence of the 20 naturally occurring L-amino acids (experimental details can be found in the Supporting Information). We identified Trp as a common quencher for all three fluorophores. We observed that Tyr, His, and Met also quench AL488 fluorescence. While Trp and Tyr are known to quench certain fluorophores,[5,8] the inclusion of His and Met was surprising. Fluorescence quenching can be broadly classified as arising from dynamic or static mechanisms.[9] (Theoretical background can be found in the Supporting Information.) Comparing the fluorescence intensity Stern−Volmer plots of all four amino acids (Figure 1), we observed that Trp and Tyr are strong quenchers of AL488 fluorescence, while His and Met are weak quenchers. The difference between the slopes of the lifetime and the intensity measurements indicate that quenching of AL488 by Trp has both static and dynamic components (Figure 2a), with a dynamic quenching rate of 3.5×10^9 M^{-1} s^{-1}. The predicted collision rate between AL488 and Trp in solution as calculated from the Smulochowski equation is 9.3×10^9 M^{-1} s^{-1}. The fraction of total collisions that result in fluorescence quenching is 0.379 ± 0.006. This value may indicate a dependence on the relative orientations (steric factor) of the rings of AL488 and Trp when they collide. This agrees with observations that the quenching of the oxazine-derived dye MR121 by Trp occurs through PET mediated by ring−ring interactions.[5] Trp and Tyr are also implicated in ultrafast electron transfer with riboflavin in flavoproteins.[10]

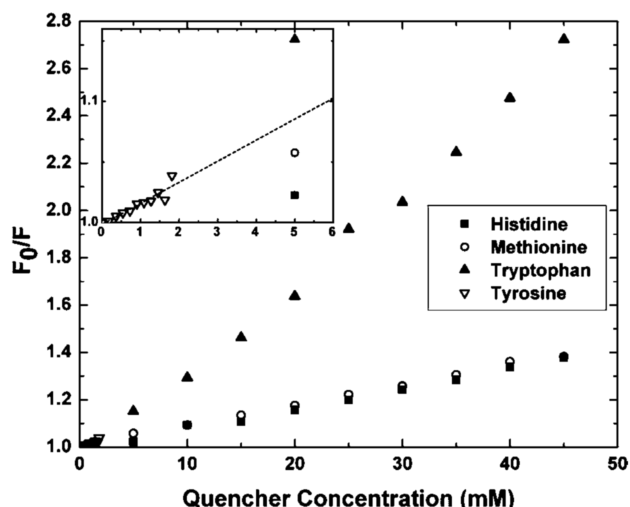

Figure 1. Comparison of quenching as measured by fluorescence intensity across four amino acids of interest. Tryptophan exhibits the strongest quenching effect, while histidine and methionine show similar quenching rates. Data for tyrosine is limited to 2 mM of quencher concentration due to its low solubility. The inset shows the tyrosine data at low concentrations with a line to show the slope relative to that of the other amino acids.

[†] School of Applied and Engineering Physics, Cornell University.
[‡] University of Puerto Rico at Cayey.
[§] Department of Chemistry and Chemical Biology, Cornell University.

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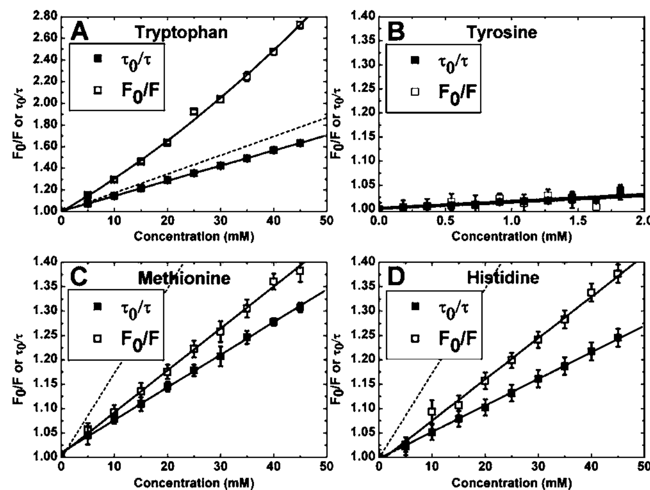

Figure 2. Stern−Volmer plots of Alexa488 quenched by (A) tryptophan, (B) tyrosine, (C) methionine, and (D) histidine. Open squares (□) represent total quenching detected by bulk fluorescence intensity measurements (static and dynamic quenching) while shaded squares (■) represent quenching detected by fluorescence lifetime measurements (dynamic quenching only). Solid lines represent linear fits to the data, except for the bulk fluorescence data of tryptophan, where the static quenching component is significant and causes a deviation from linearity. Note that data for tyrosine is limited to 2 mM of quencher concentration due to its low solubility. Dotted lines in A, C, and D show the tyrosine data from B for comparison.

To measure the feasibility of electron transfer between the species of interest, we performed cyclic voltammetry experiments. We observed a Trp oxidation peak at +0.82 V vs Ag/AgCl and an AL488 reduction peak at −0.68 V vs Ag/AgCl. We determined the free energy of the electron transfer reaction using the Rehm−Weller relation.[11] There is some uncertainty in these values, because the redox processes of the amino acids are chemically irreversible. However, a reasonable estimate of this effect would be no more than 100 mV. We observed that the electron transfer from Trp to AL488 is highly favorable, with a free energy of −1.15 eV. We note that approximately half of the total quenching by Trp arises from static mechanisms. We also calculated the static quenching constant K_S to be 15.1 M^{-1}. Static quenching often arises when the fluorophore and quencher take part in stacking interactions, thus forming a nonfluorescent complex.[9] This could explain the ubiquitous quenching of Alexa dyes by Trp.

Measurements of quenching by Tyr were limited by the low solubility of Tyr in aqueous solution. From fluorescence intensity measurements, we observed a strong quenching, comparable to Trp (Figure 1). The Stern−Volmer plots from lifetime and intensity measurements show the same slope (Figure 2b), implying that the quenching occurs entirely through a dynamic mechanism. Because phenylalanine (Phe) does not quench AL488 fluorescence, we surmise that quenching arises from PET between AL488 and the phenolic OH group on Tyr. Indeed, Tyr is known to be a good electron acceptor that undergoes PET.[8] We observed a reduction peak at −0.50 V vs Ag/AgCl, which gave a favorable free energy of −1.38 eV for electron transfer from Tyr to AL488.

We observed that Met quenches AL488 primarily by collisional quenching (Figure 2c), with a rate of 1.67×10^9 M^{-1} s^{-1}. The fraction of total collisions that results in quenching is 0.195 ± 0.008. The side chain of Met contains a sulfur atom embedded in a saturated hydrocarbon chain that is known to be susceptible to oxidation. Thus, we expect that quenching originates from PET from the reactive sulfur atom. Met displays an oxidation peak at +0.72 V vs Ag/AgCl. We observed that electron transfer from Met to AL488 is also feasible with a free energy of −1.21 eV.

The quenching of AL488 by His has both static and dynamic components (Figure 2d), with a dynamic quenching rate of 1.37×10^9 M^{-1} s^{-1}. The fraction of total collisions that result in quenching is 0.14 ± 0.01. The imidazole group of His is known to undergo both oxidation and reduction reactions, making PET a possible mechanism of collisional quenching. His has first oxidation peaks at −0.45 V and +0.53 V vs Ag/AgCl, with a favorable free energy of ∼ −1.40 eV for electron transfer to or from AL488. Similar to Trp, static quenching most likely occurs through stacking interactions between the imidazole ring of His and AL488.

Although it is often assumed that fluorophores do not interact with amino acids in the proteins they label, we show in this communication that the fluorescence of Alexa dyes 488, 555, and 594 is, in fact, quenched by interactions with Trp, Tyr, Met, and His residues through a combination of static and dynamic quenching mechanisms. Indeed, we observed strong dimming of AL488 when the fluorophore is attached less than three residues away from a Tyr residue (data not shown). In light of this finding, the potential effect of intramolecular quenching should be considered in the choice of labeling sites and in the interpretation of data that involves quantitative measurements of fluorescence intensity.

Acknowledgment. This research was supported by grants from the STC Program of the NSF under Agreement No. ECS-9876771 (H.C.), from NIH-NIBIB R01 EB006736 (W.W.W.), from the Cornell University Center for Materials Research funded by NSF Grant DMR 0520404, part of the NSF MRSEC Program, and technical support from the Provost's Academic Diversity Postdoctoral Fellowship Program at Cornell University (M.B.S.).

Supporting Information Available: Experimental details for fluorescence and electrochemical experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA100500K