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Application of tyrosine-tryptophan fluorescence resonance energy transfer in monitoring protein size changes

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ABSTRACT

Monitoring protein size changes has versatile applications in studying protein folding/unfolding, conformational rearrangements, and ligand binding. Traditionally, FRET has been used to obtain this information. However, the use of FRET often requires covalent attachment of exogenous fluorophores. Although intrinsic FRET also exists between tyrosine and tryptophan residues, it has been underused because of tyrosinate formation and spectroscopic overlap. Herein, we clarified the concern of tyrosinate formation and mathematically deconvoluted tyrosine/tryptophan fluorescence spectra. We define a new parameter called FirbY-W (fluorescence intensity ratio between tyrosine and tryptophan) to reflect protein sizes. We demonstrate its applications in studying protein unfolding using several model proteins. In all the cases, our method offers superior sensitivity, data quality, and robustness compared with traditional techniques. The unique power of our method is in its ability to detect elusive conformational changes of intrinsically disordered proteins (IDP). The lack of structure makes IDPs unsuitable for CD or tryptophan fluorescence characterization. Using histone mRNA stem-loop binding protein (SLBP) as an example of disordered proteins, we showed that our method is capable of detecting conformational changes caused by phosphorylation, which are effectively invisible for traditional spectroscopic methods. Our method can also be used to detect RNA binding of disordered proteins.

Introduction

Many biochemical questions, such as protein folding, ligand binding or conformational changes, can be ultimately simplified as measuring intra- and inter-atomic distances. One approach to measuring the desired distances is fluorescence resonance energy transfer (FRET). However, application of FRET to protein systems usually requires introducing exogenous donor/acceptor pairs [1]. This introduction is cumbersome and may introduce unnecessary artifacts into the system. Therefore, circular dichroism (CD), infrared spectroscopy, or tryptophan fluorescence are often used, each with shortcomings [2–6]. Specifically, CD and infrared are mainly sensitive to protein secondary structure but not tertiary structure. Tryptophan fluorescence intensity and emission maximum can be useful, assuming that folding or binding events perturb tryptophan microenvironments. The validity of this assumption, however, depends on the location of tryptophan residues. In addition, due to the fact that spectroscopic signals are proportional to sample concentration, small errors in pipetting may ultimately result in inaccurate measurements. This situation is further exacerbated by protein aggregation, which is not uncommon in many biochemical studies.

Moreover, studying intrinsically disordered proteins (IDPs) is challenging to most spectroscopic methods due to the lack of secondary or tertiary structure. IDPs account for over 30% of the human proteome and play versatile biological roles [7]. Despite being unstructured, IDPs do not sample all possible conformations stochastically. Instead, IDP conformation sampling is biased or can be shifted by phosphorylation or binding to their specific partners [8]. Detecting this elusive conformation redistribution is the key to understanding regulation of IDPs.

In this study, we developed a new method that makes use of protein tyrosine-tryptophan FRET to monitor protein size changes. Although tyrosine-tryptophan FRET was observed decades ago, it has remained

Abbreviations: FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; CD, circular dichroism; IDP, intrinsically disordered proteins; Nop9, nucleolar protein 9; Nop15, nucleolar protein 15; Nob1, 20S-pre-rRNA D-site endonuclease NOB1; SLBP, histone mRNA stem-loop binding protein; SRP19, signal recognition particle 19 kDa protein; DCL1, dicer-like protein 1; snRNP70, U1 small nuclear ribonucleoprotein 70 kDa; U1A, U1 small nuclear ribonucleoprotein A

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an underused method for measuring atomic distances for several reasons and the problems of using tyrosine fluorescence were summarized by Dr. Lakowicz [9,10]. One reason is that the tyrosine resonance energy is transferred to tryptophan residues, making tyrosine fluorescence invisible in most folded proteins. Another reason is the formation of tyrosinate which is the deprotonated product of the tyrosine sidechain hydroxyl group. Tyrosinate emission maximum is at 350 nm, where tryptophan fluoresces [11–13]. The final reason is that the spectral overlap between tyrosine and tryptophan further complicates the data interpretation. Herein we clarified the concern of tyrosinate and solved the problems in using tyrosine-tryptophan FRET. We used the fluorescence intensity ratio between tyrosine and tryptophan (FirbY-W) as an index of molecular size or folding status. Because tryptophan fluorescence intensity essentially serves as an internal control, FirbY-W is not affected by sample concentration. We showed that FirbY-W is extremely stable in tracking protein unfolding or binding compared to CD or tryptophan fluorescence. Moreover, our method is able to sensitively detect the elusive conformational changes of intrinsically disordered proteins, which usually escape the scrutiny of commonly used biophysical spectroscopic methods. We also demonstrated the application of our method in detecting binding events of intrinsically disordered proteins.

Results

Tyrosinate does not form in commonly used buffers or salts

The formation of tyrosinate complicates the use of tyrosine fluorescence in spectroscopic measurements due to its spectral overlap with tryptophan emission. Tyrosinate fluorescence is observed in few proteins without tryptophan, such as bovine testes calmodulin, alpha-purothionin, and beta-purothionin [11–13]. Tyrosinate fluorescence is also observed at high pH (Fig. 1A and B) when the hydroxyl group of tyrosine is ionized, or in the presence of 2 M acetate [14,15]. Although acetate of this concentration is unlikely to be used in general, it is still unknown whether tyrosinate forms in commonly encountered biochemical solutions. To this end, we collected tyrosine fluorescence spectra in commonly used buffers (100 mM Citrate pH 5.5, 100 mM HEPES pH 7.0, 100 mM MES pH 6.0, 100 mM Phosphate pH 7.0, 100 mM Tris-HCl pH 7.5, 200 mM Arg/Glu pH 7.0), salts (4 M NaCl, 4 M KCl) or denaturants (8 M urea, 8 M guanidinium chloride) using Nop15 and U1A as model proteins (Fig. 1C and D). $Nop15^{81-191}$ and U1A have no tryptophan, but have 7 and 3 tyrosine residues, respectively. We determined that all the spectra collected for these two proteins at different conditions are essentially identical (Fig. 1C and D). No tyrosinate fluorescence (emission maximum around 350 nm) is observed at these conditions. Our results also clearly indicate that the shape of the tyrosine fluorescence spectrum is constant in the above solutions. Taken together, we conclude that tyrosinate fluorescence is not formed under often-used conditions.

Deconvolution of tyrosine and tryptophan fluorescence

Measuring pure tyrosine or tryptophan fluorescence intensities is difficult due to tyrosine and tryptophan spectral overlap. Although tyrosine fluoresces consistently at 302 nm, tryptophan fluorescence peak maximum varies between 320 nm and 355 nm depending on solvent accessibility (Fig. 2A) [16]. More specifically, the degree of tyrosine and tryptophan spectral overlap is dependent on the blue- or red-shift of tryptophan fluorescence in response to protein folding or unfolding. Therefore it is essential to deconvolute the overlapping spectra between tryptophan and tyrosine.

The fluorescence spectrum of Trp can be analytically expressed by the log-normal function (Equation (1)) [17–19]:

$$I(v) = I_m \cdot \exp\left[-\frac{ln2}{ln^2\rho}\cdot \ln^2\left(\frac{a-v}{a-v_m}\right)\right] \quad (1)$$

where v_m is the spectral maximum position; I_m is the maximal amplitude; v is the wave number. ρ is the band asymmetry parameter defined by $\rho = (v_m - v_-)/(v_+ - v_m)$. v_+ and v_- are the positions of the two half-maximal amplitudes; a is the function-limiting point position defined as $a = v_m + \frac{\rho\cdot(v_+ - v_-)}{\rho^2 - 1}$. v_m, v_+ and v_- define the shape of the tryptophan fluorescence spectrum and have a linear relationship. For tryptophan, the linear relationship is parameterized as:

$$v_+ = 0.831\cdot v_m + 7070\ (cm^{-1}) \quad (2)$$

$$v_- = 1.177\cdot v_m - 7780\ (cm^{-1}) \quad (3)$$

Currently, the analytical representation of the tyrosine fluorescence spectrum is still unknown. Finding an analytical equation will significantly simply our mathematical deconvolution process. The aforementioned log-normal function was initially proposed to describe the shape of absorption spectra of complex molecules and its mirror-symmetric form was later utilized to describe fluorescence spectra [20,21]. To test whether the log-normal function can be used to describe the tyrosine fluorescence spectrum, we parameterized Equations (2) and (3) to fit the experimental tyrosine spectra shown in Fig. 1, as the constant terms in these equations delineate the shape of the log-normal function. Using grid searching, we found that the following optimized empirical parameters to delineate the tyrosine fluorescence spectrum:

$$v_+ = 0.831\cdot v_m + 7028\ (cm^{-1}) \quad (4)$$

$$v_- = 1.177\cdot v_m - 8023\ (cm^{-1}) \quad (5)$$

As shown by Fig. 1, the tyrosine fluorescence peak maximum (302 nm) or its shape is not affected by buffer conditions or folding status of a protein. This suggests that Equations (1), (4) and (5) can be generalized to describe the tyrosine fluorescence spectra of other proteins.

For most proteins, tyrosine fluorescence has negligible influence on the tryptophan fluorescence emission maximum (340–350 nm). As exemplified by Fig. 2A, tyrosine fluorescence intensity at 340–350 nm is only 10–20% of its emission maximum for Nop15. Considering that tyrosine fluorescence is usually 10%–30% of that of tryptophan in a FRET spectrum, perturbation of tyrosine fluorescence on tryptophan emission maximum accounts for less than 5% of tryptophan maximum and is unable to shift the tryptophan emission maximum. This suggests that the pure tryptophan emission maximum can be accurately read from FRET spectra.

With the optimized parameters for tyrosine and tryptophan fluorescence spectra, the tyrosine emission maximum (302 nm), and the tryptophan emission maximum directly read from the FRET spectrum, we successfully deconvoluted the tyrosine-tryptophan fluorescence spectrum (Fig. 2B). The robustness of this deconvolution is bolstered by the negligible residual error. As expected, tryptophan fluorescence contributes to over 97% of its maximum and its contribution to the tyrosine region can be approached with reasonable accuracy by assuming tryptophan contributes 100% of the spectrum maximum (0.04042 versus 0.0416). This results in only a 0.5% error in estimation of tyrosine fluorescence intensity. A more thorough and precise deconvolution of tyrosine and tryptophan was carried out using the method of least squares (See the Method Section). However, we determined that there were no significant differences between the simplified (Equation (1)) and the more complex (least squares) methodologies.

Application of fluorescence intensity ratio between tyrosine and tryptophan (FirbY-W) to monitor unfolding event of structured proteins

Tyrosine fluorescence is usually invisible in folded proteins due the