

Fluorescence Spectroscopy: A tool for Protein folding/unfolding Study

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Introduction

Principle of fluorescence

Fluorescence is the result of a three-stage process that occurs in certain molecules called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific stimulus. The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram) shown in Figure 1.

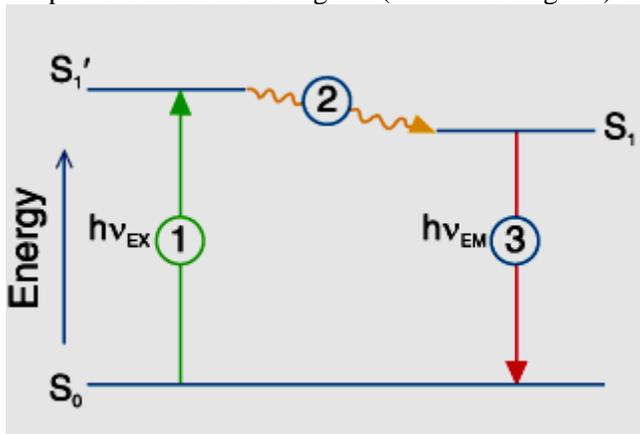


Figure 1.

Stage 1 : Excitation. A photon of energy $h\nu_{EX}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

Stage 2 : Excited-State Lifetime. The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (Stage 1) return to the ground state (S_0) by fluorescence emission. Other processes such as collisional quenching, Fluorescence Resonance Energy Transfer (FRET) and intersystem crossing may also depopulate S_1 . The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these processes occur.

Stage 3 : Fluorescence Emission. A photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state S_0 . Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon $h\nu_{EX}$. The difference in energy or wavelength represented by $(h\nu_{EX} - h\nu_{EM})$ is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

Fluorescence Spectra

The entire fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching), the same fluorophore can be repeatedly excited and detected. The fact that a single fluorophore can generate many thousands

of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques. For polyatomic molecules in solution, the discrete electronic transitions represented by $h\nu_{EX}$ and $h\nu_{EM}$ in Figure 1 are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected. With few exceptions, the fluorescence excitation spectrum of a single fluorophore species in dilute solution is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime, as illustrated in Figure 1. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength (Figure 2).

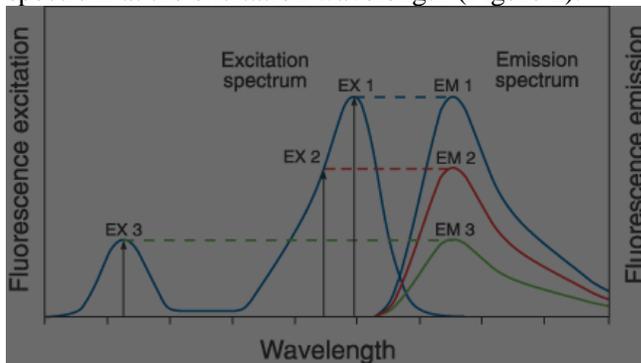


Figure 2.

Fluorescence Detection and Fluorescence Instrumentation

Four essential elements of fluorescence detection systems can be identified from the preceding discussion: (1) an excitation source, (2) a fluorophore, (3) wavelength filters to isolate emission photons from excitation photons and (4) a detector that registers emission photons and produces a recordable output, usually as an electrical signal or a photographic image. Regardless of the application, compatibility of these four elements is essential for optimizing fluorescence detection. Fluorescence instruments are primarily of four types, each providing distinctly different information:

Spectrofluorimeters and microplate readers measure the *average* properties of bulk (μL to mL) samples.

Fluorescence microscopes resolve fluorescence as a function of spatial coordinates in two or three dimensions for microscopic objects (less than ~ 0.1 mm diameter).

Fluorescence scanners, including microarray readers, resolve fluorescence as a function of spatial coordinates in two dimensions for macroscopic objects such as electrophoresis gels, blots and chromatograms.

Flow cytometers measure fluorescence per cell in a flowing stream, allowing subpopulations within a large sample to be identified and quantitated.

Other types of instrumentation that use fluorescence detection include capillary electrophoresis apparatus, DNA sequencers and microfluidic devices. Each type of instrument produces different measurement artifacts and makes different demands on the fluorescent probe. For example, although photobleaching is often a significant problem in fluorescence microscopy, it is not a major impediment in flow cytometry or DNA sequencers because the dwell time of individual cells or DNA molecules in the excitation beam is short.

Fluorescence Signals

Fluorescence intensity is quantitatively dependent on the same parameters as absorbance — defined by the Beer–Lambert law as the product of the molar extinction coefficient, optical path length and solute concentration — as well as on the fluorescence quantum yield of the dye and

the excitation source intensity and fluorescence collection efficiency of the instrument. In dilute solutions or suspensions, fluorescence intensity is linearly proportional to these parameters.

Protein folding

Protein folding is the reaction by which a protein adopts its native 3D structure (Fig. 3). The native structure is the functional state of the protein. Folding happens in several steps, in a simplistic manner, first is formation of the secondary structure (2D) followed by acquisition of the tertiary structure arrangement (3D), and sometime a further quaternary structure (4D) organization in the case of oligomeric complex proteins (Fig.3).

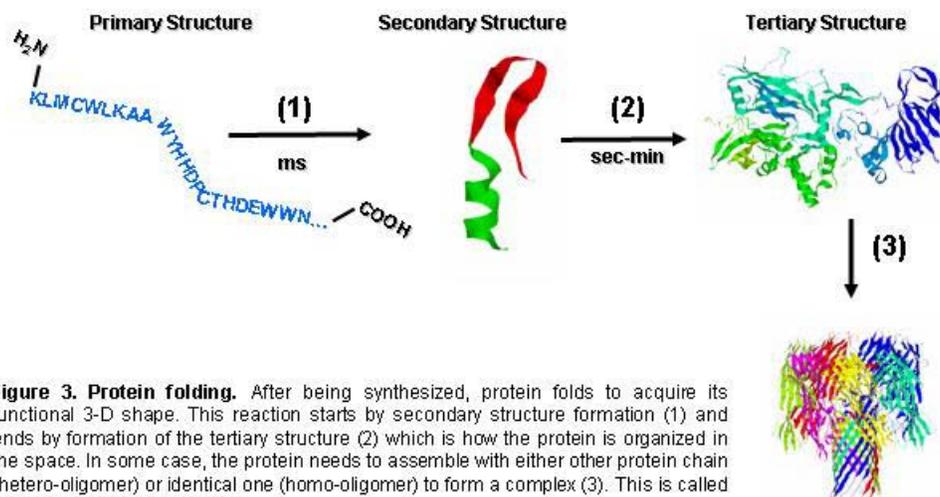


Figure 3. Protein folding. After being synthesized, protein folds to acquire its functional 3-D shape. This reaction starts by secondary structure formation (1) and ends by formation of the tertiary structure (2) which is how the protein is organized in the space. In some case, the protein needs to assemble with either other protein chain (hetero-oligomer) or identical one (homo-oligomer) to form a complex (3). This is called the quaternary structure (4D).

1D, 2D, 3D and 4D of proteins are defined in the biocomputer experiment. The 2D of a protein can be monitored by Circular Dichroism whereas the 3D structure can be tracked down using fluorescence spectroscopy, in particular intrinsic protein fluorescence. This is the subject of this practical.

Protein folding in cells (in vivo) is extremely complicated and differs depending on the organism. Folding is far more complicated in eukaryotic cells (for example: human cell) than in prokaryotic ones (bacteria). Due to this complexity, the study of protein folding is extremely limited in vivo. People have instead studied the folding of pure protein in vitro using mainly biophysical techniques. This means that the protein alone is studied outside of its normal environment. The first challenge is to isolate and to purify the protein of interest from the producing organism in enough quantity for these in vitro studies. The second challenge is to establish in vitro conditions at which the protein can be refolded in its native structure. In this context, the starting purified material is the native protein, therefore to study its folding into the native structure, one need to first unfold it (Fig. 4, step 1) to then followed its refolding (Fig. 4, step 2). The main problem being that there is no guarantee that such conditions exist in vitro, because in the cells this never happen, there are many partners involved to help the protein to fold. That is why most of the studies done in vitro are still limited to the unfolding of protein (Fig. 4, step 1). But one has to keep in mind that protein unfolding reaction is likely to differ from the mechanism of protein folding. Thus the extrapolation of protein folding based on protein unfolding is quite limited.

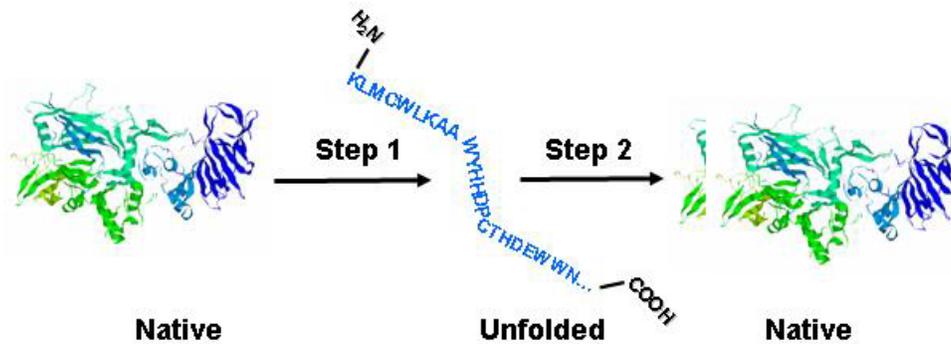


Figure 4. Protein folding in vitro. Because in vitro the starting material is the native form of the protein which is already folded, one needs to first unfold it (1) and then refold it (2) to study protein folding.

Trp/Tyr-fluorescence

There are three amino acids with intrinsic fluorescence properties, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) but only Tyrosine and tryptophan are used experimentally because their quantum yields (emitted photons/ excited photons) is high enough to give a good fluorescence signal. So this technique is limited to protein having either Trp or Tyr or both.

For an excitation wavelength of 280 nm, both Trp and Tyr will be excited. To selectively excite Trp only, 295 nm wavelength must be used.

Those residues can be used to follow protein folding because their fluorescence properties (quantum yields) are sensitive to their environment which changes when protein folds/unfolds. In the native folded state, trp and try are generally located within the core of the protein, whereas in a partially folded or unfolded state, they become exposed to solvent (Fig. 5A).

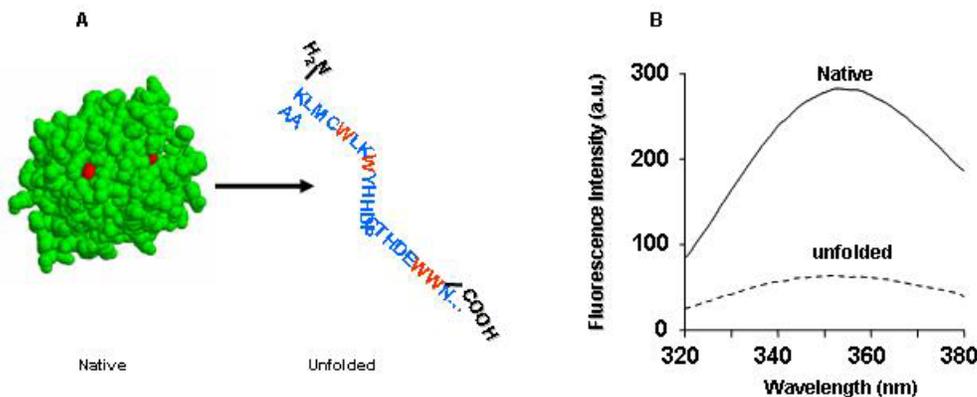


Figure 5. Protein unfolding as monitor by Trp-fluorescence. Trp are highlighted in red, (W: trp). **A.** In the native protein Trps are buried whereas in the unfolded state they are exposed. **B.** In the native state because Trps are buried and in a hydrophobic environment they have high fluorescence Intensity. In contrast when exposed to solvent (unfolded protein), their fluorescence intensity decrease.

In a hydrophobic environment (buried within the core of the protein), Tyr and Trp have a high quantum yield and therefore a high fluorescence intensity (Fig. 5B). In contrast in a hydrophilic environment (exposed to solvent) their quantum yield decreases leading to low fluorescence intensity (Fig. 5B). For Trp residue, there is strong stoke shifts dependent on the solvent, meaning that the maximum emission wavelength of Trp will differ depending on the Trp environment.

There are several means to unfold a protein based on the disturbance of the weak interactions that maintains the protein folded (hydrogen bonding, electrostatic interactions, hydrophobic

interactions). The most common ways of unfolding a protein are chaotropic agents (urea, guanidinium hydrochloride), the change of pH (acid, base) or the rise of temperature. It is possible to study either steady state or kinetic of protein unfolding. For example, the protein is unfolded by increasing temperature, so at each temperature the protein undergoes unfolding and reach an equilibrium state corresponds to a partially folded or fully unfolded state depending on the conditions (Fig. 6A). For each temperature, the fluorescence emission of Trp or Tyr is measured and compare to that of the native protein (Fig. 6B).

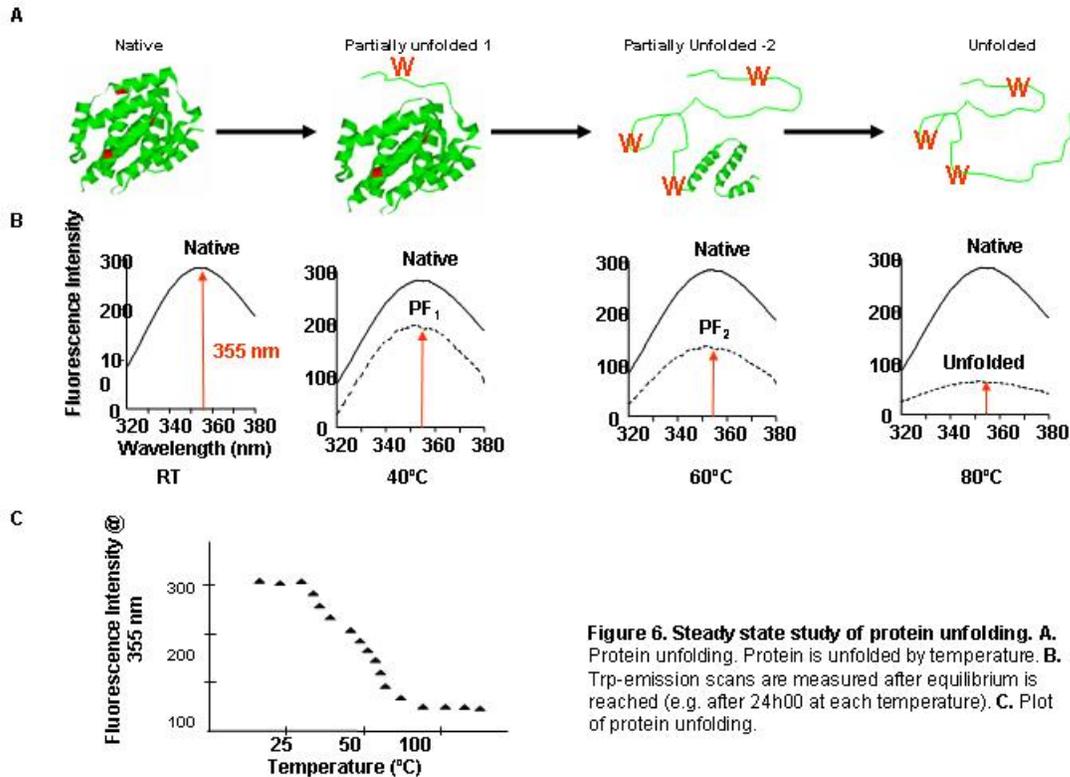


Figure 6. Steady state study of protein unfolding. A. Protein unfolding. Protein is unfolded by temperature. **B.** Trp-emission scans are measured after equilibrium is reached (e.g. after 24h00 at each temperature). **C.** Plot of protein unfolding.

Fluorescence intensity (FI) will change upon unfolding as well as the maximum emission wavelength (λ_{max}) if Trp is used as a probe. Following the change of this parameter (FI or λ_{max}) the unfolding curve is generated by plotting $FI=f(\text{temperature})$ or $\lambda_{max}=f(\text{temperature})$ (Fig. 6C). Those kinds of study are steady state studies. For kinetic studies, the protein is put at one temperature and its unfolding reaction is followed in time. Here again the change in either FI or λ_{max} is measured but in time (Fig. 7).

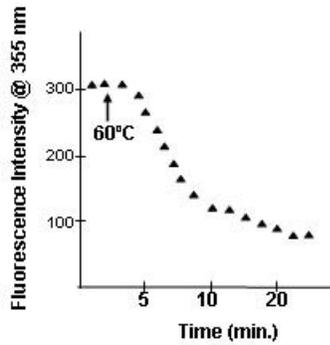


Figure 7. Protein unfolding kinetic. The unfolding reaction is monitored by collecting the fluorescence intensity at 355 nm.

Other fluorescence

As mentioned, the previous technique is limited to protein containing Trp or Tyr residues. But proteins can be chemically labeled with fluorescence probes (Fic, alexa red...). In addition, some proteins contain other fluorophores such as NADH or FAD. It is also possible to use probes which bind specifically to protein hydrophobic surfaces (ANS, TNS). Those surfaces are hidden in a native protein but exposed in partially folded or fully unfolded proteins.

Experimental Procedure

USE THE CUVETTE WITH GREAT CARE. IT IS VERY FRAGILE AND VERY EXPENSIVE.

Material available

BSA stock 12 mg/ml
 Lysozyme stock 3 mg/ml
 Buffer: 1×PBS
 Acid: 0.1M HCL/KCL pH 1
 Cuvette
 Cuvette holder
 Eppendorf tubes
 Pipettes

Solution preparation

1. BSA: 25 µl of BSA 12 mg/ml + 2975 µl of 1×PBS in an eppendorf tube.
2. BSA + acid : 25 µl of BSA 12 mg/ml + 2975 µl of 0.1M HCL/KCL pH 1 in an eppendorf tube.
3. lysozyme: 100 µl of lysozyme 3 mg/ml + 2900 µl of 1×PBS in an eppendorf tube.
4. lysozyme + acid : 100 µl of lysozyme 3 mg/ml + 2900 µl of 0.1M HCL/KCL pH 1 in an eppendorf tube.

Switch on the fluorimeter

- Double Click “Scan”

- Setup parameters as: $\lambda_{ex}=280$ nm, $\lambda_{em}=300-400$ nm, slit width (emission and excitation)=5, medium speed. Auto-store/ storage: on prompt at start, auto-convert: Ascii (csv) with log
- Clean cuvette with 1*DI water, 2* ethanol, 3* DI water. Dry the cuvette with kimweeps
- Add buffer 1×PBS in the cuvette and press “zero”
- Add the sample to another cuvette and start measurement.
- Print all the spectra

Clean cuvette between each sample as indicated above

Results analysis

1. Plot and describe how the fluorescence spectrum is changed when acid is added to BSA and lysozyme, respectively
2. If above changes are different for BSA and lysozyme, explain the reason
3. If the student has done CD experiment, combine the result from CD to explain question 2