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Studying 3D Subdomains of Proteins at the Nanometer Scale Using Fluorescence Spectroscopy

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Summary

Databases devoted to the crystal structure of proteins have dramatically increased in size during the last two decades. Moreover, X-ray and NMR technology studies have shown that proteins belonging to the same family generally share the same global 3D architecture. These results suggest that the need for experimental determination of protein structure will be reduced to those that are suspected to have sufficiently novel structures. Furthermore, NMR and other techniques have demonstrated that a protein in solution experiences constant random thermal motions that occur over large time scales, ranging from picoseconds to seconds and perhaps hours. Such changes may have important functional consequences, but identifying which changes are functionally relevant remains a difficult task even if this problem has been addressed both with experimental and computational methods. For that specific purpose, there is a need for methods allowing a fast and accurate monitoring of conformation changes (that occur at specific subdomains of proteins. Fluorescence resonance energy transfer (FRET) is a suitable tool for monitoring conformational changes at the nanoscale level. This chapter describes the various FRET methods that are used for monitoring the 3D sub-domain conformation of proteins in solution, in single living cells and at the single molecular level.

Key Words: Fluorescence spectroscopy; fluorescence resonance energy transfer; fluorescence lifetime imaging measurements; lifetime measurements; energy transfer; near-field scanning optical microscope; protein subdomain.

1. Introduction

Databases devoted to protein structures have dramatically increased in size during the last decade. Besides X-ray studies that have depicted the three-dimensional (3D) crystal structure of larger and larger molecules with amazing accuracy, nuclear magnetic resonance (NMR) and other techniques are used to monitor protein conformational changes in solution. A protein in solution is now considered as experiencing, within a stable equilibrium structure, constant random thermal motions that occur over time scales ranging from pico-

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seconds to seconds, and even hours. NMR methods are very attractive, for they can provide comprehensive information on movements occurring at different sites throughout the macromolecule. For instance, ^{15}N-NMR relaxation studies indicate that the movements of the backbone of a stable protein are generally restricted to the picosecond to nanosecond time scale ***(1)***. By contrast, side-chain dynamics, as revealed by ^{13}C or deuterium NMR relaxation methods, have indicated that protein hydrophobic cores are heterogeneously dynamic ***(2–4)***. Besides studies mainly devoted to folding and unfolding processes in proteins, recent articles deal with conformational changes related to protein functions (*see* **refs. *5–7*** and references therein). Both random thermal motions and the average conformation may be strongly modified when a protein experiences ligand or substrate binding, docking to another macromolecule, or phosphorylation. Such changes may have important functional consequences. Nevertheless, identifying which changes are functionally relevant is a difficult task. An even more fascinating challenge is to monitor structural rearrangement that might occur when a substrate is added to an enzyme ***(7–10)***.

Monitoring conformational changes may require the use of several techniques of investigation ***(11)***. Sometimes in association with NMR studies, differential scanning calorimetry, far-ultraviolet (UV) circular dichroism (CD), labeling with fluorescent markers, and site-directed spin labeling have been extensively used ***(11–14)***. The last two methods provide information on conformational changes only around specific amino acids, but this could be an advantage when monitoring 3D changes associated with enzyme activity. Furthermore, it is known that proteins belonging to the same family generally share the same global 3D architecture. It is thought that progress in protein structure modeling will reduce the need for experimental determination of the whole 3D structure of proteins, because structural description of all proteins in a family is possible when the structure of a single member is known ***(15)***. As a consequence, the interest in methods allowing easy monitoring of conformational change occurring at specific locations of the proteins must be reconsidered, especially when they can be used in vivo. One may argue that both fluorescence and spin labeling require slight modifications of the studied protein, but then this is also often the case for NMR studies.

Among the different techniques using fluorescence, fluorescence resonance energy transfer (FRET) is the best adapted for conformational change monitoring, although other methods have also been used. Of course, because of the high sensitivity of the fluorescence quantum yield to the microenvironment of the fluorescent molecule, other techniques are also available. Changes in fluorescence intensity or fluorescence lifetime have been used for monitoring changes in the polarity, hydrophobicity, and acidity of the environment of tryptophan(s) or fluorescent tags linked to proteins. For instance, the pH sensitivity of the fluorescence intensity of dansyl has been used for monitoring a

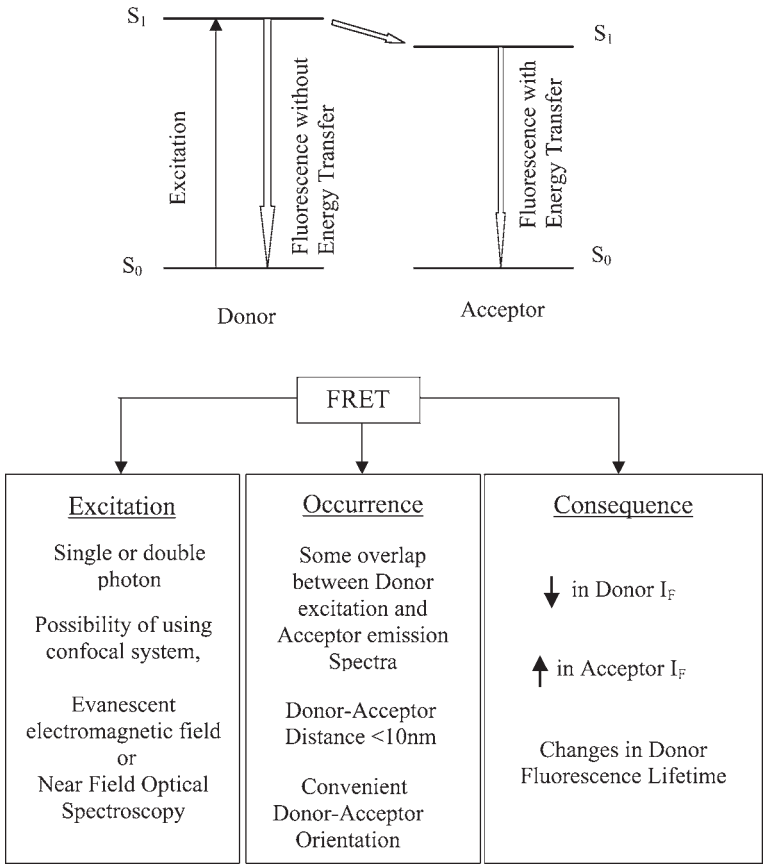

Fig. 1. Principle of FRET. I_F, fluorescence intensity.

proton transfer between a dansyl molecule bound to a protein and one of its endogenous amino acids (lysine) ***(11)***. However, FRET has the unique property of occurring only when the distance between the two involved molecules is <10 nm. Only FRET allows quantification of subtle conformational changes below the light diffraction limits. Furthermore, such a molecular ruler at the nanoscale can be easily used in living cells.

2. Methods

The basic fundamentals of FRET have been known for many years ***(16–18)***. FRET consists of a quantum mechanical process resulting in radiationless energy transfer from the first excited state of a fluorescent molecule, called the donor (D), to the first excited state of another fluorescent molecule, called the acceptor (A). As a consequence, under conditions that are given subsequently, excitation of the donor may induce the fluorescence of the acceptor (**Fig. 1**).

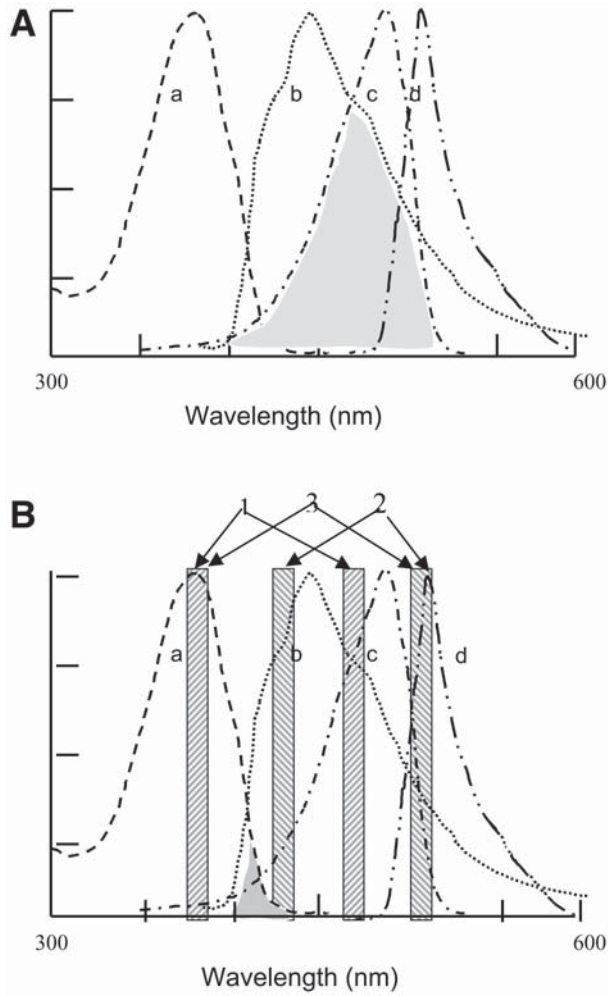

Fig. 2. FRET. **(A)** Example of overlap spectral region (in gray) required for FRET: a, donor absorption spectrum; b, acceptor absorption spectrum; c, donor emission spectrum; d, acceptor emission spectrum. **(B)** Minimal filter set required for FRET monitoring: 1, recording of donor fluorescence intensity; 2, recording of acceptor fluorescence intensity without FRET; 3, recording of FRET intensity.

For FRET to occur, the two fluorophores must be conveniently selected: the donor emission spectrum must significantly overlap the absorption spectrum of the acceptor, and the reabsorption of the fluorescence emitted by the donor must be minimal to avoid tedious data corrections (**Figs. 1**,**2**). Furthermore, FRET intensity is sensitive to the relative orientation of the transition dipoles

of the fluorophores. Then, because FRET has to compete with other deactivation processes of the donor excited state, the donor should have a high fluorescence quantum yield. Finally, the distance between D and A must be <10 nm, for FRET efficiency decreases with the sixth power of the distance between them. It is just this last property that makes FRET an invaluable tool for probing protein conformation and protein conformational changes. Moreover, both D and A must be insensitive to photobleaching when FRET is used for probing protein conformational changes, for that goal requires successive periods of irradiation.

Application of FRET techniques to biomedical research started quite recently ***(19,20)***. As a matter of fact, interpretation of FRET data on biological events is a difficult task for the following reasons. Observation is generally carried out on a population of biological molecules that are known to experience different 3D conformations even when sharing the same free-energy level. Thus, even if the time necessary for data collection is "ideally short," data will reflect the distribution of individual molecules in the same energy level, i.e., the relative weight of their different potential 3D conformations. Furthermore, technical limitations do not allow the collection of significant amounts of data within periods of time short enough compared to intracellular movements. Such a situation is common to every optical method of collecting information, making it difficult to assign an experimental value to a precise conformation. For these reasons, FRET is often used as an "all or nothing" method, separating the studied molecules into two subpopulations, that in which FRET occurs (D-A < 10 nm) and that in which FRET does not (D-A > 10 nm).

As indicated before, FRET can be used for experiments in solution with a conventional spectrofluorometer or under the microscope for studying biomolecules in living cells. In the latter case, owing to the concentration of light at the objective, the risk of photobleaching is highly increased. Thus, besides the use of direct single-photon irradiation, other methods have been developed. Multiphoton microscopy takes advantage of the existence of the "optical window," the wavelength domain between 700 and 1100 nm where the biological material is practically transparent to radiation ***(21,22)***. The simultaneous absorption of two photons at 800 nm may allow reaching the first excited state, since it is usually done with a single 400-nm photon. This occurs when two coherent photons "reach" the fluorescent target at the same time and requires the use of a high-energy, high-frequency (<100 fs) pulsed laser. The efficiency of two-photon excitation follows a power-squared relation and can be achieved only inside a tiny volume of the sample at a time. The whole cell is then not damaged by irradiation, and the risk of photobleaching is restricted to the tiny volume where the two photons collide. Nevertheless, some risk of photobleaching may remain for some fluorescent probes ***(23)***.

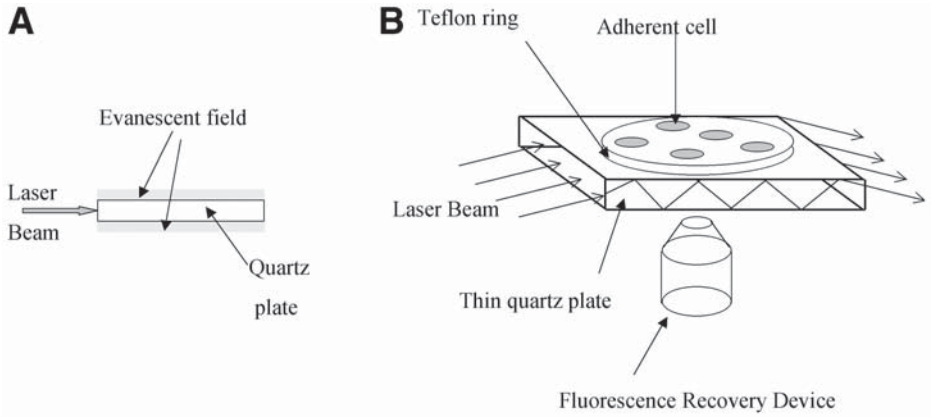

Fig. 3. Total internal reflection fluorescence microscopy. **(A)** Irradiation through the quartz plate used to support cells generates an evanescent field in thin volumes located on both sides of the plate. **(B)** Illustration of a device allowing grabbing of data from many cells at a time.

Because the source of potential photobleaching is the concentration of the excitation light through the objective, it has been suggested that cells be irradiated in another way. When cells are grown on a thin quartz plate, this plate can be used as a laser light guide inducing an evanescent electromagnetic field in the 0- to 100-nm range above the plate surface, a distance long enough to excite preferentially molecules inside the living cells ***(24)***. Moreover, this elegant technique allows the simultaneous collection of data on many individual cells (**Fig. 3**).

As indicated in **Fig. 1**, FRET affects the fluorescence intensity of both D and A and changes the lifetime of D. Methods that allow monitoring either fluorescence intensities or lifetime measurements can be used in quantification. When protein conformational changes are studied in solution, fluorescence spectroscopy can be used with conventional equipment (**Fig. 4**). The fluorescence spectrum resulting from a partial energy transfer will be a combination of the respective fluorescence spectra of the donor and the acceptor. The relative weight of these spectra will depend on the respective quantum yields of D and A for the wavelength used for excitation. The relative contribution of these spectra can be easily obtained even in the presence of other fluorophores or relatively noisy signals, using previously published methods of spectra resolution ***(25)***. By contrast, experiments performed on living cells require the use of excitation and emission filter sets (**Fig. 5**) ***(26)***. The use of three sets of filters is generally necessary, which is time-consuming ***(27)***. A simplification can be obtained by using fluorophores with minimal overlap of their respective excitation spectra and selecting excitation wavelengths out of this overlap

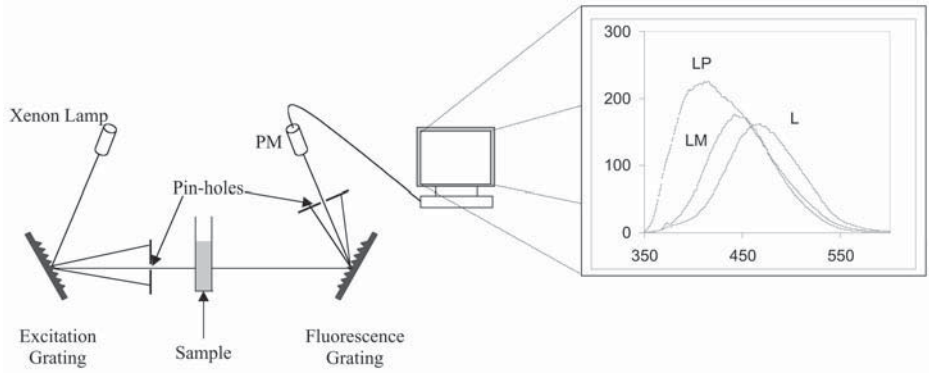

Fig. 4. Schematic drawing of a conventional spectrofluorometer (PM) photomultiplier. Respective fluorescence spectra of protein-bound (LP), cation-bound (LM), and free (L) Mag-indo 1 are shown.

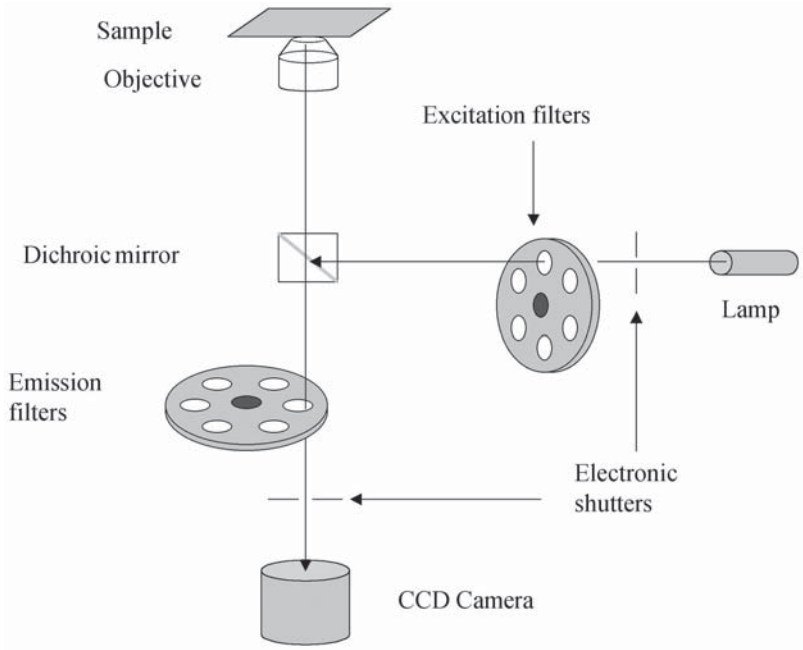

Fig. 5. Schematic drawing (not to scale) of a FRET multiwavelength fluorescence imaging microscope. The electronic shutters are necessary to minimize the cell irradiation resulting from illumination and data recording at different wavelengths. CCD, charge-coupled device.

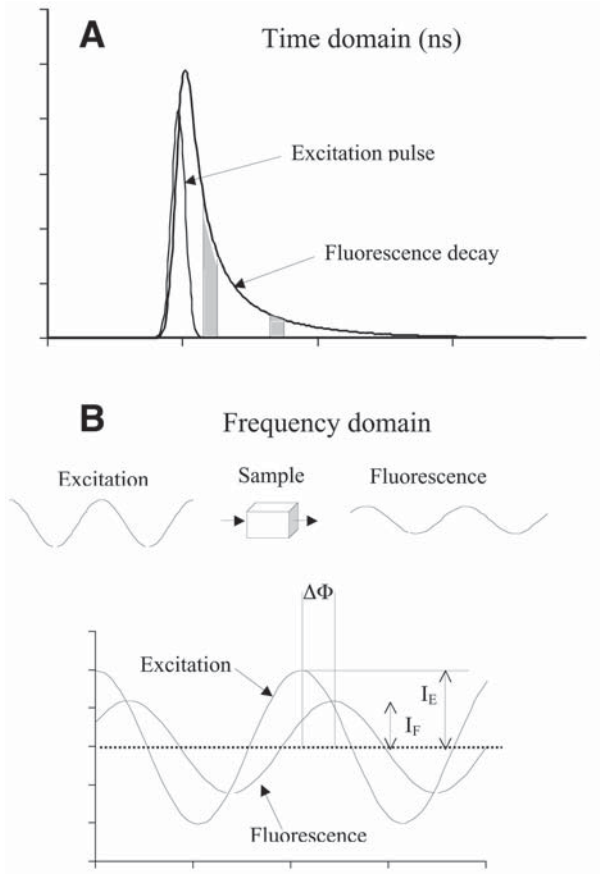

Fig. 6. Fluorescence lifetime imaging. **(A)** In the time domain method, a fast-gated camera is used to repeatedly record during very short periods of time (in gray) data from the multiexponential decay of the fluorescence (bold curve) excited by a very short excitation pulse (light curve). **(B)** In the frequency domain approach, measurable parameters are the phase shift ΔΦ and the relative reduction of amplitude of the fluorescence signal (I_F) compared with that of the excitation pulse (I_E).

range. As shown in **Fig. 2B**, the first set of filters will allow the monitoring of whole changes of the donor and acceptor fluorescence, respectively, and the second set will measure changes in the acceptor fluorescence resulting only from changes in the chemical microenvironment (without FRET). A combination of these data will allow monitoring of changes in FRET specifically.

Time life measurements can also be used to quantify FRET either in protein solution or in living cells. The fluorescence lifetime is independent of the concentration of the fluorophore, which is an important advantage because the

probe concentration is expected to vary inside a living cell. On the contrary, the lifetime strongly depends on molecular interactions between the probe and its chemical environment. Energy transfer itself is expected to influence the lifetime of the donor, for it competes with other deactivation processes of the donor excited state. Thus, a 3D conformational change inducing a change in energy transfer will affect in a complex way the donor lifetime. Nevertheless, fluorescence lifetime imaging has recently been extensively used for FRET monitoring ***(28–30)***. Two fundamentally different approaches have been used. In the time domain approach, the fluorescence lifetime (τ_f) is measured by excitation of the sample with a short pulse of light, much shorter than the fluorescence lifetime (**Fig. 6**). Such a method requires a camera capable of fast gating, allowing the collection of the fluorescence signal during precise short periods of time ***(31–33)***. The use of the time domain approach is thus limited by the difficulty of gating a camera fast enough to collect information during the lifetime of a fluorescence decay (**Fig. 6A**).

In the frequency domain approach, the sample is excited with sinusoidally modulated light. The optical angular frequency of the light is reciprocal to the fluorescence lifetime to be measured. As a result, the emitted fluorescence is sinusoidally modulated at the same frequency but with a reduction in the modulation depth, M, and shifted in phase, $\Delta\Phi$ (**Fig. 6B**). This technique requires a detector capable of high-frequency modulation. Parameters related to the respective intensities of the excitation pulse and the fluorescence signal, and to the phase shift $\Delta\phi$, are used to calculate two other parameters, the phase lifetime, τ_f, and the modulation lifetime, τ_M. These last lifetimes are equal only when the sample contains only one fluorescent species, which is never the case inside a cell, owing to molecular interactions. When more than one fluorescent species is present, the phase and modulation lifetimes differ. In such a situation, the true lifetime composition can be obtained by measuring the phase and modulation lifetimes over a range of frequencies and fitting the results to a set of dispersion relationships. Fluorescence lifetime imaging measurements (FLIMs) may become more usual in the near future owing to several advantages associated with spectral discrimination. Simultaneous readout of data and the requirement of only one dichroic and long-pass emission filter result in the use of a lower light dose and reduce the risk of photochemical damage to the cells. Recent articles have reported on progress in numerical analysis of data and on the feasibility of multiwavelength frequency domain FLIM ***(34–38)***.

3. Results

Among amino acids, only tryptophan experiences a significant fluorescence, and this fluorescence requires excitation wavelengths that cannot go through most microscope objectives. Thus, proteins must be previously tagged with

fluorescent chemicals to make it possible to study their conformational changes though fluorescence techniques. Depending on the protein and the actual goal, different tagging processes have been used. Examples are discussed next in some detail.

3.1. Noncovalent Binding

It is well known that some chemicals have a preferential location inside specific pockets of the 3D protein structures. When fluorescent, they can be used for reporting conformational changes in and around these specific subdomains, for most of the fluorescence properties are sensitive to changes in the microchemical environment. That is specifically the case when the preferential location results from a noncovalent binding to a specific amino acid. Then, changes in protein conformation may influence fluorescent properties through either changes in the microchemical environment, a change in binding conditions, or even both at the same time. Sometimes, the binding of the exogenous fluorophore may induce some energy transfer from a neighboring tryptophan, which makes it possible to probe the interaction through FRET techniques.

Some years ago, it was demonstrated that Mag-indo 1, a fluorescent chemical initially used for probing intracellular calcium concentration, was able to interact with some proteins through a noncovalent binding with some histidine residue(s). Such a binding induces a specific shift in the fluorescence spectrum of Mag-indo 1, making it possible to quantify this binding using specific data treatments (**Fig. 4**) ***(25,26,39)***. The interaction of Mag-indo 1 with turkey egg white lysozyme (Tlys) in the absence of calcium has been studied using the synchronous fluorescence technique ***(40,41)***. This technique, in which both excitation and emission wavelengths are scanned simultaneously with a constant $\delta\lambda$, compresses the spectral information to a relatively narrow domain of wavelengths. This generally reduces the spectral overlap from various fluorescent components. In this study, it allows the simultaneous recording of synchronous fluorescence spectra both of free Mag-indo 1 and protein-bound Mag-indo 1 and of the protein itself. Moreover, a potential energy transfer from one of the protein tryptophans cannot affect the fluorescence intensity of the synchronous fluorescence spectrum of the protein-bound Mag-indo 1. As a consequence, the value of the dissociation constant characteristic of the interaction can be easily obtained. On the contrary, changes in the intensity of the protein fluorescence spectrum may reflect both a change in the chemical microenvironment of a tryptophan close to the interaction site and some energy transfer from this tryptophan to the protein-bound Mag-indo 1. The energy transfer resulting from the interaction can be monitored through changes in the intensity of the Mag-indo 1 excitation spectrum when excited in the range of

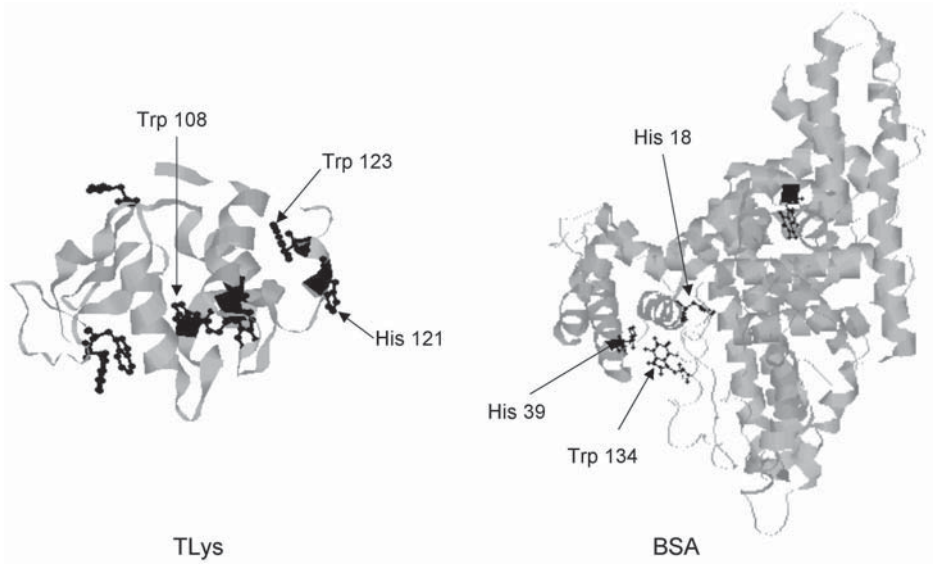

Fig. 7. Respective 3D conformations of TLys (**left**) and BSA (**right**). Chemical structures of tryptophan and histidine residues appears as balls and sticks.

wavelengths of tryptophan absorption ***(42)***. Furthermore, the amino acid sequence of Tlys has only two histidine residues, and only one of them, His 121, is located in the vicinity of one tryptophan in the stable 3D conformation. Thus, the existence of some energy transfer allows easy determination of the protein subdomain accessible through this interaction (**Fig. 7**).

Fluorescence techniques were also used to monitor conformational changes resulting from the binding of fluorescent substrates (4-methlyumbelliferyl chitobiosides or chitotriosides) to turkey or hen egg white lysozyme. In both cases, a quenching of the protein fluorescence was observed when the substrates were engulfed in the enzyme cleft, but no energy transfer was evidenced. This finding suggests that the quenching results only from a change in the chemical environment of one tryptophan, probably Trp 108, associated with the presence of the substrate inside the cleft (unpublished results).

Interactions of bovine serum albumin (BSA) and human serum albumin (HSA) with Mag-indo 1 have also been studied ***(42)***. These homologous proteins present two hydrophobic pockets but differ in their number of tryptophan residues: BSA has two tryptophans, one in each hydrophobic pocket, but HSA has only one, lacking the Trp 134 present in BSA. It was demonstrated that both proteins interact with Mag-indo 1 but that interaction induces a quenching of the protein fluorescence only for BSA. This quenching was associated with an energy transfer from BSA to Mag-indo 1 that parallels the protein-probe

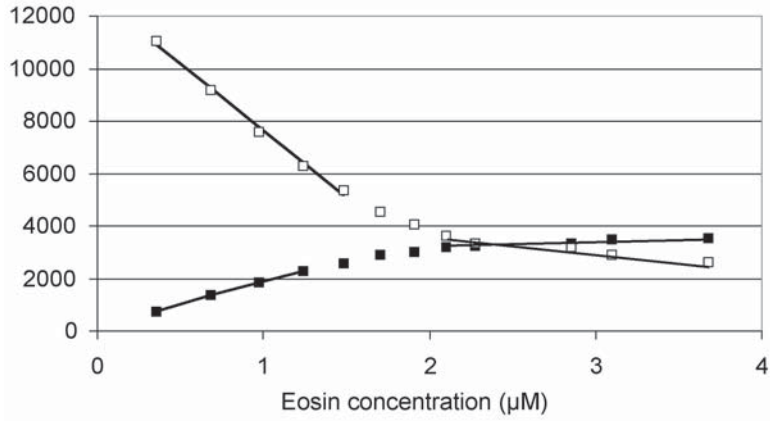

Fig. 8. Influence of increasing eosin concentrations on fluorescence intensity of BSA trytophans (□) and on fluorescence intensity of BSA-bound eosin (■). Fluorescence intensities on the *y*-axis are in arbitrary units. BSA concentration was 1.8 μ*M*.

binding. These results suggest that interaction takes place in the vicinity of Trp 134 and can be used for monitoring unfolding and refolding processes of this BSA subdomain in the presence of guanidine hydrochloride ***(43)***.

Recently, interactions of eosin with, respectively, BSA and HSA have been reconsidered from the conformational change point of view (J. M. Salmon and L. Bilia, personal communication 2001). The interaction of eosin with these proteins induces both an increase in intensity and a red shift in the eosin fluorescence spectrum that allows quantifying the interaction. The stoichiometry of the interaction is 1/1 for HSA and 1/2 for BSA. Moreover, this interaction induces a quenching of the fluorescence of both tryptophans of BSA (**Fig. 8**). Comparison with results obtained with HSA confirmed that the eosin interaction occurs with amino acid(s) located in the two hydrophobic pockets of BSA.

3.2. Tagging Protein With Variants of Green Fluorescent Protein

Green fluorescent protein (GFP) is a spontaneously fluorescent polypeptide of 27 kDa (238 amino acid residues) from the jellyfish *Aequorea victoria* that absorbs UV-blue light and emits in the green region of the spectrum ***(44)***. Its structure and potential uses for studying living cells have recently been reviewed in detail ***(45–50)***. The GFP chromophore results from a cyclization of three adjacent amino acids (S65, Y66, and G67) and the subsequent 1,2-dehydrogenation of the tyrosine ***(45–50)***. Although the chromophore by itself is able to absorb light, its fluorescent properties are associated with the presence of an 11-stranded β-barrel. GFP has been expressed in both bacteria and eukaryotic cells and has been produced by in vitro translation of the GFP mRNA. More-

over, GFP retains its fluorescence when fused to heterologous proteins on the N- and C-terminals, and this binding generally does not affect the functionality of the tagged protein ***(45,46,49)***. This leads the way for the use of GFP as an intracellular reporter even if the fluorescence intensity of the native GFP is not bright enough for most intracellular applications. Variants have been engineered that have different excitation and/or emission spectra that better match available light sources ***(46,48–50)***. The use of brighter mutants was also found necessary in the case of low expression levels in specific cellular microenvironments ***(46–50)***. Some of them, such as the cyan (ECFP) and yellow (EYFP) fluorescent protein, have been specifically engineered to match the FRET criteria.

Although the use of GFP and GFP-variants chimera have given amazing information on protein synthesis, translocation, and intracellular localization, their structure limits their use for monitoring conformational changes in proteins. Because of their own size, GFP variants may modify the kinetics of conformational changes when used for tagging small proteins. Furthermore, these variants are fused to the N- and/or C-terminals of the tagged protein, which is generally not the best location to be sensitive to conformational changes resulting from binding to other proteins or enzyme substrates. Thanks to recent progress in structural genomics, it has become easier to determine which kind of protein biological activities may change the distance between the N- and C-terminal tags above or below 10 nm ***(15)***.

Actually, GFP chimeras have demonstrated their potential for functional studies of proteins. This research field has been well covered by recent reviews (*see* **refs. *28***, ***46***, ***47***, ***51***, ***52***, and references therein). Intramolecular FRET was used to monitor protein activity especially for detecting UV-induced apoptosis and testing novel apoptosis inducers or inhibitors ***(53–57)***. All these studies were carried out with caspase fluorescent substrates that are labeled to show FRET when the substrate is intact. Caspase activity disrupts the substrate, inducing the disappearance of FRET. Unfortunately, this elegant method for monitoring enzyme activity is unable to give any information about potential associated conformational changes in the protein.

Phosphorylation is an efficient posttranscriptional way of modifying proteins to modulate protein functions. Different methods have been tested for monitoring phosphorylation through FRET methods. In special cases, phosphorylation induces significant conformational changes that can be evidenced by FRET methods if the peptide can be tagged at both ends by GFP mutants. In this scheme, chimeric reporters have been constructed based on a double-tagged appropriate substrate peptide sequence for a given protein kinase ***(58,59)***. In this approach, a flexible linker sequence and a phosphorylation recognition domain that binds the phosphorylated substrate domain were included

between two GFP variants. Thus, phosphorylation induces a huge conformational change that will change the distance and/or relative orientation between the GFP mutants. These changes will change the FRET conditions between the GFP mutants. In principle, this approach can be extended to any kind of kinases and the specific reporters can be expressed in both bacteria and mammalian cells ***(60,61)***. Nevertheless, although allowing efficient monitoring of the kinases' activity, this method does not give any information on the conformational changes experienced by kinases during the phosphorylation or dephosphorylation processes.

Intermolecular FRET techniques have also been used for monitoring an interaction between a nuclear receptor and its specific activator that is required for transcription of transiently transfected and chromosomally integrated reporter genes ***(62)***. High-resolution FRET microscopy was used for the study of the potential role of "lipid rafts" in the interaction between cholera toxin β-subunits and GPI-anchored proteins in the plasma membrane of HeLa cells ***(63)***. The interaction of epidermal growth factor receptor and Grb2 in living cells has also been monitored using FRET imaging ***(64)***. In addition, in a study by Janetopoulos et al. ***(65)***, receptor-mediated activation of heterotrimeric G-proteins was visualized in living cells by monitoring FRET between α- and β-subunits fused to cyan and yellow fluorescent proteins ***(65)***. Their study deserves special mention because the protocol used allowed the determination of both the kinetics and the sites of G-protein activation in cells and tissues. For that purpose, the subunits Gα2 and Gβ of *Dictyostelium discoideum* were respectively tagged with cyan and yellow fluorescent proteins. FRET intensity was used to monitor changes in the G-protein concentration, reporting both protein activity and associated conformational changes.

3.3. Other Types of Covalent Binding

In 1995 it was demonstrated that lysine_{75} is the most reactive lysine residue in calmodulin and is therefore labeled extensively and selectively when calmodulin is treated with amine-reactive fluorophores ***(66–69)***. This labeling process was used to build up a fluorescent protein usable for visualizing the binding of Ca^{2+} to calmodulin, monitoring Ca^{2+} activation–dependent and –independent localization of calmodulin, and investigating the role of calmodulin in the control of mitotic transition ***(66,67,70)***. Moreover, this binding was found to be insensitive to enzyme digestion of the protein with chymotrypsin and trypsin, allowing a quite easy identification of the binding site through conventional methods of peptide characterization. Another potentially interesting method of chemically tagging protein with fluorescent markers is to use available cysteine residues that are generally limited in number in the protein sequence compared to the frequency of other amino acids.

The potential use of these chemical bindings will be enhanced if they are performed in the frame of a close scientific cooperation among scientists involved in the determination of the 3D structure of proteins and enzyme-substrate complexes. Scientists interested in the chemical tagging of proteins with small fluorescent molecules will take advantage of the recently published 3D structures for identification of the best candidate(s) among the amino acid sequence ***(71)***. When the fluorescent analog(s) has been synthesized, scientists may benefit from the powerful crystallographic, X-ray, and NMR techniques to check if the tag has induced some unwanted change in the 3D conformation.

Another exciting approach has been developed recently that consists of a chemical synthesis of a specific domain of a protein. This method, which takes full advantage of the 3D conformation protein databanks and progress in Proteomic, has been used for studying the unfolding of the peripheral subunit-binding domain of the dihydrolipoamide succinyltransferase (the E2 subunit) from the 2-oxoglutarate dehydrogenase multienzyme complex of *Escherichia coli* ***(11)***. The synthetic protein was tagged with a naphthyl-alanine at the NH_2 terminus and with a dansylated lysine at the COOH terminus to investigate the end-to-end distance variation during unfolding through FRET techniques. Differential scanning calorimetry and far-UV CD were also used in this study.

Compared with tagging through genomic protocols, chemical tagging may present some lack of specificity. Amino acids usable for chemical binding are generally not unique in the amino acid sequence of a protein. Thus, contrary to what happened with calmodulin, it can be expected that chemical tagging will result in different tagged proteins, and potentially multitagged proteins. Although this may require further purification to separate the tagged proteins, that may prove to be useful for probing conformational changes in different subdomains. In any case, chemical tagging seems an alternative to GFP tagging, allowing the use of far smaller fluorescent markers. It may provide researchers with fluorescent analogs that will match all the requirements for studying protein conformational changes in solution. These modified proteins should also be efficient for studying the efficacy of electroporation or any other process of assisted endocytosis ***(72–77)***.

4. What Next?

Whatever valuable information FRET methods yield on protein activity and conformational changes, this information is obtained from data that are generally collected from a molecular population. Because it is generally admitted that many 3D conformations may correspond to the same level of free energy, such data may as well reflect the heterogeneity of the molecular population or characterize a homogeneous intermediate conformation. For that reason, a quest for methods allowing the visualization of the conformational change of a

single molecule appears necessary, especially for monitoring intermediate conformations occurring during protein activity. Although the earlier optical methods were devoted to monitoring diffusion of macromolecules, recent developments are now focused on what is called "dynamic structural biology." In such a research field, methods allowing detection of changes of distance and/or orientation between two precise sites located on a macromolecule, or two different macromolecules, are required. Two optical methods, FRET and fluorescence polarization anisotropy (FPA), have been recently adapted for single-molecule detection ***(78)***. Whereas FRET efficiency depends on both the relative distance and the orientation of donor and acceptor, FPA efficiency depends only on changes in the orientation of one fluorophore. Thus, optimal experiments will consist of using both techniques at a time, a situation that is rarely met. On one hand, FPA requires only one fluorescent tag per macromolecule, which limits investigation of chemical synthesis. On the other hand, FPA consists of comparing the intensity of fluorescence polarized in the same direction as that of the excitation light (I_{par}) with that of the fluorescence polarized in a perpendicular direction (I_{perp}). Such a comparison may be difficult to quantify when the signal intensities are low and in the presence of a large background signal. However, when a fluorophore is either rigidly attached or tethered to a macromolecule, changes in FPA can be interpreted in terms of angular movements of that macromolecule, but bulk averaging may mask important information on dynamic changes in orientation.

Recording the fluorescence issued from a single molecule is not an easy task owing to the low-intensity signal. First of all, it is necessary to be sure that the recorded signal really comes from a unique molecule, which is generally achieved by using ultralow fluorophore concentrations and confocal microscopy. As an example, concentrations of fluorescent molecules are usually in the range of 10 to 30 pmol. Under these conditions, in a diffraction-limited excitation volume of about 0.5 fL, the probability of single-molecule occupancy is about 0.01, compared with the probability of simultaneous double-molecule occupancy of 5×10^{-5}. Thus, the signal intensity is generally far lower than that of the background, especially when measurements are performed with living cells, and must be extracted from this background. This can be achieved with single-photon-counting avalanche photodiodes, allowing detection of single-molecule photon bursts with integration times compatible with the rate of most biological events (<1 ms).

Techniques of single-pair FRET (spFRET) and single-molecule fluorescence polarization anisotropy (smFPA) have been developed for observing the conformational fluctuations and catalytic activities of enzymes at single-molecule resolution. They were first used for studying conformational changes and interactions of Staphylococcal nuclease (SNase) ***(79)***. In this study,

intramolecular spFRET was measured between donor and acceptor fluorophores attached to single SNase protein immobilized on glass cover slips by means of hexahistidine tags. smFPA was performed with single-tagged SNase, and intermolecular spFRET was measured between donor-labeled SNase and acceptor-labeled DNA substrate. The use of spFRET has been extended to the study of conformational changes experienced by single molecules of chymotrypsin inhibitor 2 when denatured in solution ***(80)***. Although single molecules have been detected in single living cells using fluorescent chimera such as transferrin-tetramethylrhodamine, it seems that the spFRET technique has not yet been used in living cells ***(81)***. One reason for this situation may be that, in living cells, autofluorescence results in too large of a background signal. Nevertheless, in another recent study, lateral motion of lipid microdomains in native muscle cell membrane was imaged, which could be considered as a pilot application of single-molecule microscopy to a living cell ***(82)***. The behavior of membrane-bound proteins tagged with GFP variants has also been recently studied at the single-molecule level ***(83–85)***.

To our knowledge, the first biological application of smFPA was reported on the axial rotation of actin filaments sliding over myosin motor molecules fixed on a glass surface ***(86)***. The conformational states of the myosin motor from the same biological system were studied 1 yr later ***(87)***. Orientation dependence of single-fluorophore intensity was used for monitoring conformational changes in F1-ATPase in real time. The fluorophore used, Cy3, was attached to the central subunit of this molecule and revealed that the subunit rotates in discrete 120° steps, each step being driven by the hydrolysis of one adenosine triphosphate molecule ***(88)***. Although these experiments were performed with enzymes fixed on a glass surface, they lead the way to studies of rotations of molecular motors.

Obviously, the use of both spFRET and smFPA at the intracellular level requires an increase in signal/background ratio. This cannot result from an increase in the integration time, which must remain in the millisecond range to be consistent with the speed of intracellular events. Likewise, the acquisition rate has its own technical limitations. Thus, the most efficient way to increase the signal/background ratio is to decrease the background intensity, which can be achieved by decreasing the irradiated intracellular volume. Three optical techniques are now available for that purpose.

Owing to the marvelous background rejection in total internal reflection fluorescence microscopy, this technique seems perfectly suitable for detecting signals of low intensity. It has been recently used for monitoring intracellular events such as chemotactic signaling, nucleocytoplasmic transport of proteins, and actin filament turnover (*see* **ref. *89*** and references therein).

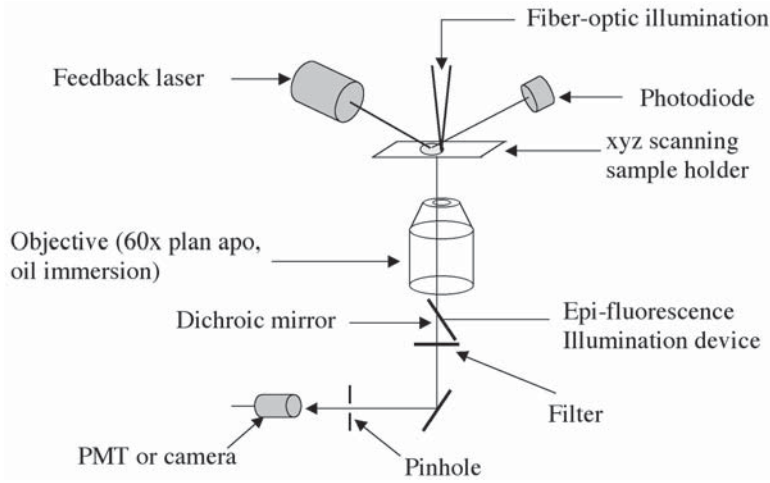

Fig. 9. Schematic diagram of a near-field scanning optical microscope. PMT, photomultiplier tube.

Near-field scanning optical microscopy (NSOM) allows the creation of a subwavelength light source in close proximity to the surface of a sample to generate optical images with a lateral optical resolution below the diffraction limit. The optical probe is a tapered optical fiber chemically etched into a conical tip and metallized with an opaque metal coating leaving a clear aperture of about 20 to 100 nm at its apex. Excitation light issued from the optical fiber creates a nanometer-size excitation light source. When the tip is maintained a few nanometers above the cell surface, only fluorescent molecules located in the near field under the tip are excited ***(90)***. Thus, besides the classic mechanism used for scanning the cell, a feedback mechanism is necessary for maintaining the tip at a fixed distance from the cell surface during the scanning process (**Fig. 9**). This technique is of particular interest for studies of plasma membrane proteins or for proteins located close to or linked with the plasma membrane ***(91–93)***.

As mentioned earlier, multiphoton microscopy requires that two coherent photons "reach" the fluorescent target at the same time, so this technique only delivers efficient irradiation inside a tiny volume of the cell. Furthermore, this technique is not limited to exploration of the vicinity of the plasma membrane. Improvements in both equipment and data treatment have resulted in a spatial resolution in the 10-nm range ***(93,94)***. An elegant association of NSOM with the two-photon technology that has been described could well be the best way to reduce the background signal ***(85)*** and makes spFRET and smFPA usable in intact living cells.

Besides potentially invaluable information in the biomedical domain, optical studies of individual molecules already provide information about the dynamics of photophysical processes of these fluorophores ***(95,96)***. The existence of unexpected long-lasting "dark states" populated through irradiation has been evidenced and correlated to the protonated state of the fluorophore. This is a striking example of potential perturbations of biomolecules induced by the used exploration technique. Optical studies of single fluorescent molecules might also be used to address the question of potential differences in quantum yield for molecules that populate the different conformations associated with the same value of the free-energy scale during the protein-folding pathway ***(97)***.

There is no doubt that fluorescence microscopy at the single-molecule level will bring new insights into the dynamics of cellular molecular biology. However, that will require a perfect knowledge of the biophysical and photophysical properties of the used fluorescent reporters. It is anticipated that studies performed on specific 3D subdomains will be necessary to achieve these goals.

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