3 Fluorophores

Fluorescence probes represent the most important area of fluorescence spectroscopy. The wavelength and time resolution required of the instruments is determined by the spectral properties of the fluorophores. Furthermore, the information available from the experiments is determined by the properties of the probes. Only probes with non-zero anisotropies can be used to measure rotational diffusion, and the lifetime of the fluorophore must be comparable to the timescale of interest in the experiment. Only probes that are sensitive to pH can be used to measure pH. And only probes with reasonably long excitation and emission wavelengths can be used in tissues, which display autofluorescence at short excitation wavelengths.

Thousands of fluorescent probes are known, and it is not practical to describe them all. This chapter contains an overview of the various types of fluorophores, their spectral properties, and applications. Fluorophores can be broadly divided into two main classes—intrinsic and extrinsic. Intrinsic fluorophores are those that occur naturally. These include the aromatic amino acids, NADH, flavins, derivatives of pyridoxyl, and chlorophyll. Extrinsic fluorophores are added to the sample to provide fluorescence when none exists, or to change the spectral properties of the sample. Extrinsic fluorophores include dansyl, fluorescein, rhodamine, and numerous other substances.

3.1. INTRINSIC OR NATURAL FLUOROPHORES

Intrinsic protein fluorescence originates with the aromatic amino acids[1–3] tryptophan (trp), tyrosine (tyr), and phenylalanine (phe) (Figure 3.1). The indole groups of tryptophan residues are the dominant source of UV absorbance and emission in proteins. Tyrosine has a quantum yield similar to tryptophan (Table 3.1), but its emission spectrum is more narrowly distributed on the wavelength scale (Figure 3.2). This gives the impression of a higher quantum yield for tyrosine. In native proteins the emission of tyrosine is often quenched, which may be due to its interaction with the peptide chain or energy transfer to tryptophan. Denaturation of proteins frequently results in increased tyrosine emission. Like phenol, the Pk_A of tyrosine decreases dramatically upon excitation, and excited state ionization can occur. Emission from phenylalanine is observed only when the sample protein lacks both tyrosine and tryptophan residues, which is a rare occurrence (Chapter 16).

The emission of tryptophan is highly sensitive to its local environment, and is thus often used as a reporter group for protein conformational changes. Spectral shifts of protein emission have been observed as a result of several phenomena, including binding of ligands, protein–protein association, and protein unfolding. The emission maxima of proteins reflect the average exposure of their tryptophan residues to the aqueous phase. Fluorescence lifetimes of tryptophan residues range from 1 to 6 ns. Tryptophan fluorescence is subject to quenching by iodide, acrylamide, and nearby disulfide groups. Tryptophan residues can be quenched by nearby electron-deficient groups like $–NH_3^+$, $–CO_2H$, and protonated histidine residues. The presence of multiple tryptophan residues in proteins, each in a different environment, is one reason for the multi-exponential intensity decays of proteins.

3.1.1. Fluorescence Enzyme Cofactors

Enzyme cofactors are frequently fluorescent (Figure 3.1). NADH is highly fluorescent, with absorption and emission maxima at 340 and 460 nm, respectively (Figure 3.3). The oxidized form, NAD^+, is nonfluorescent. The fluorescent group is the reduced nicotinamide ring. The lifetime of NADH in aqueous buffer is near 0.4 ns. In solution its fluorescence is partially quenched by collisions or stacking with the adenine moiety. Upon binding of NADH to proteins, the quantum yield of the NADH generally increases fourfold,[4] and the lifetime increases to about 1.2 ns. How-

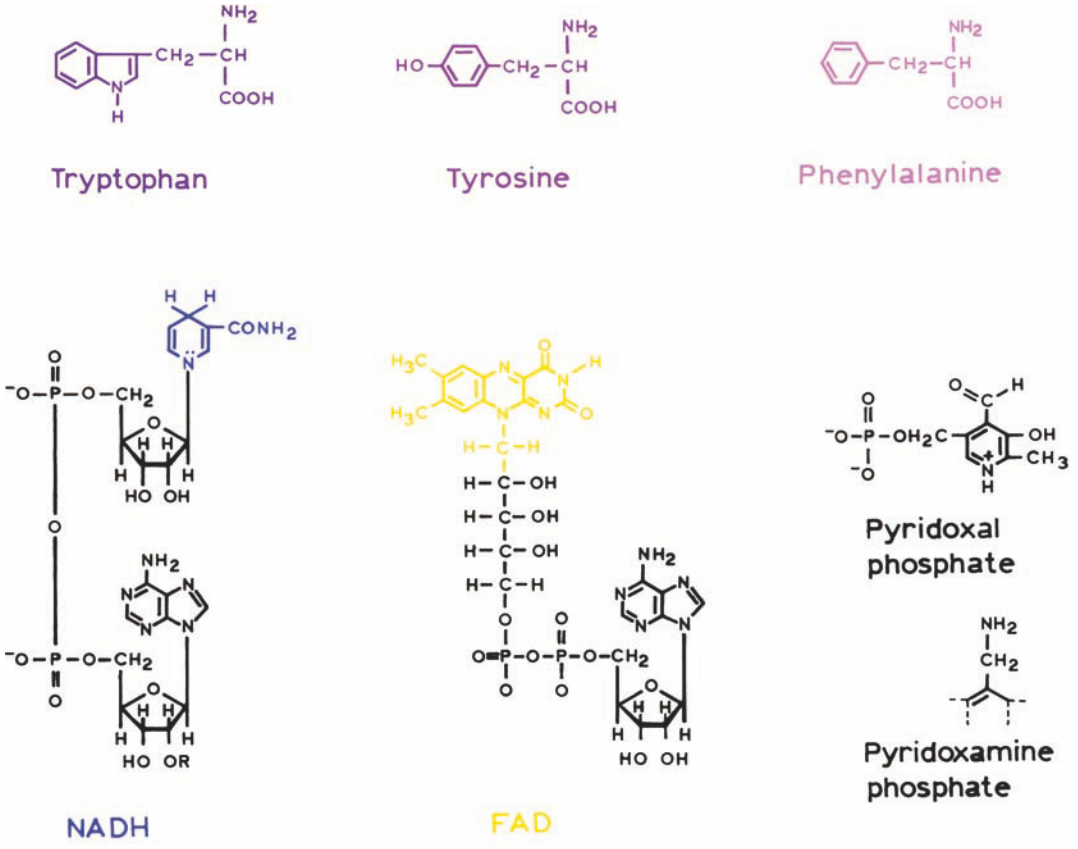

Figure 3.1. Intrinsic biochemical fluorophores. R is a hydrogen in NADH, and a phosphate group in NADPH.

ever, depending on the protein, NADH fluorescence can increase or decrease upon protein binding. The increased yield is generally interpreted as binding of the NADH in an elongated fashion, which prevents contact between adenine and the fluorescent-reduced nicotinamide group. Lifetimes as long as 5 ns have been reported for NADH bound to horse liver alcohol dehydrogenase[5] and octopine dehydrogenase.[6] The lifetimes of protein-bound NADH are typically different in the presence and absence of bound enzyme substrate.

The cofactor pyridoxyl phosphate is also fluorescent (Figure 3.4).[7–14] Its absorption and emission spectra are dependent upon its chemical structure in the protein, where pyridoxyl groups are often coupled to lysine residues by the aldehyde groups. The emission spectrum of pyridoxamine is at shorter wavelengths than that of pyridoxyl phosphate. The emission spectrum of pyridoxamine is dependent on pH (not shown), and the emission spectrum of the pyridoxyl group depends on its interaction with proteins. The spectroscopy of pyridoxyl groups is complex, and it seems that this cofactor can exist in a variety of forms.

Riboflavin, FMN (Flavin mononucleotide), and FAD (Flavin adenine dinucleotide) absorb light in the visible range (≃450 nm) and emit around 525 nm (Figure 3.3). In contrast to NADH, the oxidized forms of flavins are fluorescent, not the reduced forms. Typical lifetimes for FMN and FAD are 4.7 and 2.3 ns, respectively. As for NADH, the flavin fluorescence is quenched by the adenine. This

Table 3.1. Fluorescence Parameters of Aromatic Amino Acids in Water at Neutral pH

Species[a]	λ_{ex} (nm)	λ_{ex} (nm)	Bandwidth (nm)	Quantum yield	Lifetime (ns)
Phenylalanine	260	282	–	0.02	6.8
Tyrosine	275	304	34	0.14	3.6
Tryptophan	295	353	60	0.13	3.1 (mean)

[a]From [1].

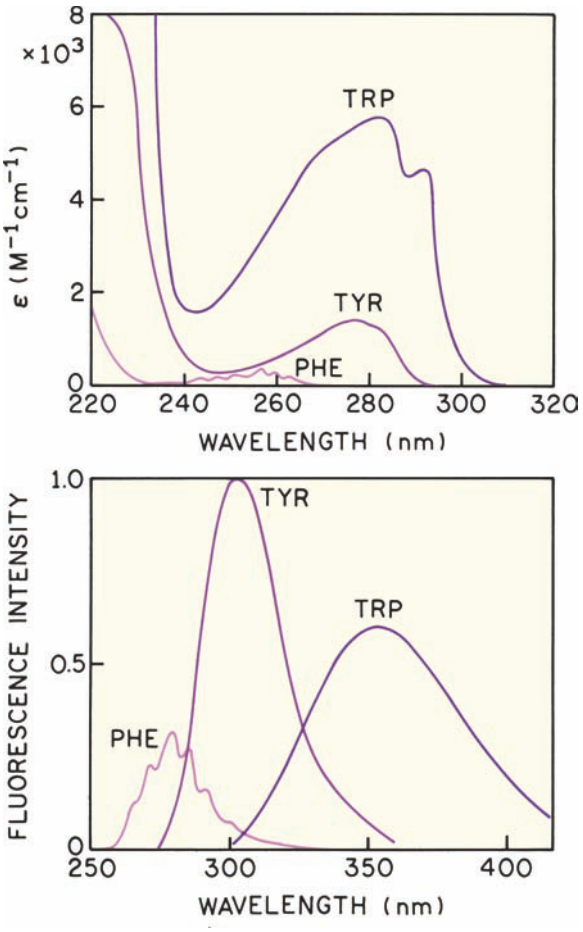

Figure 3.2. Absorption and emission spectra of the fluorescent amino acids in water of pH 7.0.

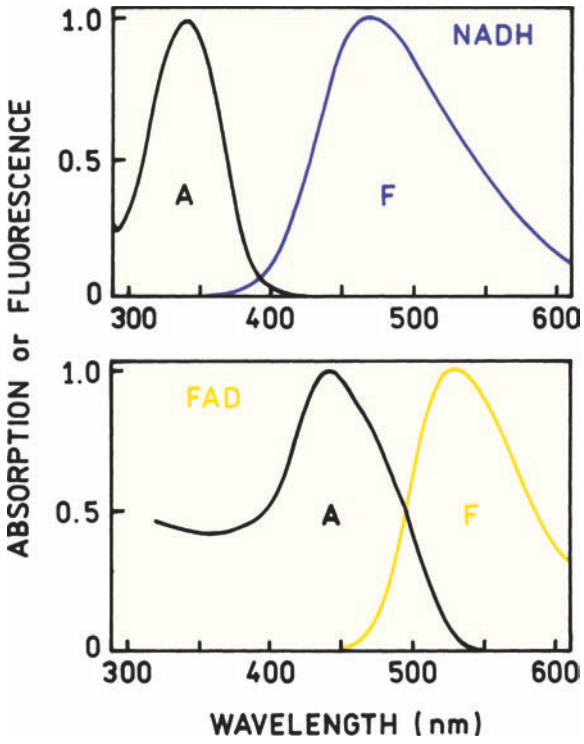

Figure 3.3. Absorption and emission spectra of the enzyme cofactors NADH and FAD.

quenching is due to complex formation between the flavin and the adenosine.[15] The latter process is referred to as static quenching. There may also be a dynamic component to the quenching due to collisions between adenine and the reduced nicotinamide moiety. In contrast to NADH, which is highly fluorescent when bound to proteins, flavoproteins are generally weakly fluorescent[16–17] or nonfluorescent, but exceptions exist. Intensity decays of protein-bound flavins are typically complex, with multi-exponential decay times ranging from 0.1 to 5 ns, and mean decay times from 0.3 to 1 ns.[18]

Nucleotides and nucleic acids are generally nonfluorescent. However, some exceptions exist. Yeast $tRNA^{PHE}$ contains a highly fluorescent base, known as the Y-base, which has an emission maximum near 470 nm and a lifetime near 6 ns. The molecules described above represent the dominant fluorophores in animal tissues. Many additional naturally occurring fluorescence substances are known and have been summarized.[19]

There is presently interest in the emission from intrinsic fluorophores from tissues, from fluorophores that are not enzyme cofactors.[20–25] Much of the fluorescence from cells is due to NADH and flavins.[26–27] Other fluorophores are seen in intact tissues, such as collagen, elastin lipo-pigments and porphyrins (Figure 3.4). In these cases the emission is not due to a single molecular species, but represents all the emitting structures present in a particular tissue. The emitting species are thought to be due to crosslinks between oxidized lysine residues that ultimately result in hydroxypyridinium groups. Different emission spectra are observed with different excitation wavelengths. Much of the work is intrinsic tissue fluorescence, to identify spectral features that can be used to identify normal versus cancerous tissues, and other disease states.

3.1.2. Binding of NADH to a Protein

Fluorescence from NADH and FAD has been widely used to study their binding to proteins. When bound to protein NADH is usually in the extended conformation, as shown in

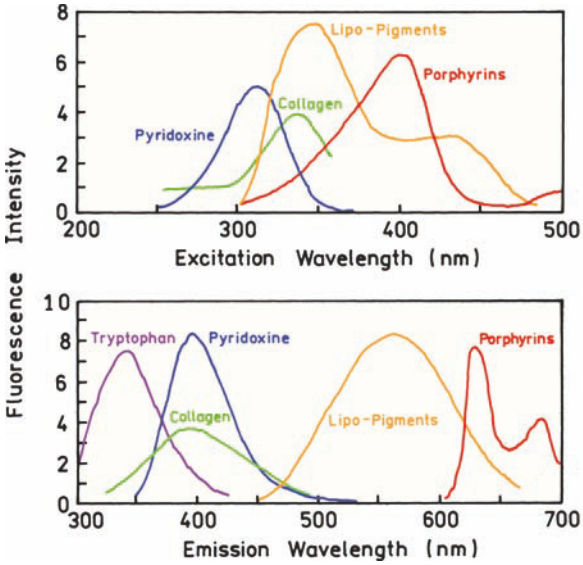

Figure 3.4. Emission spectra from intrinsic tissue fluorophores. Revised from [25].

Figure 3.5. This is shown for the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD), which catalyzes the last step in the biosynthesis of estradiol from estrogen.[28] The protein consists of two identical subunits, each containing a single tryptophan residue. 17β-HSD binds NADPH as a cofactor. Binding prevents quenching of the reduced nicotinamide by the adenine group. As a result the emission intensity of NADPH is usually higher when bound to protein than when free in solution.

Emission spectra of 17β-HSD and of NADPH are shown in Figure 3.6. NADPH is identical to NADH (Figure

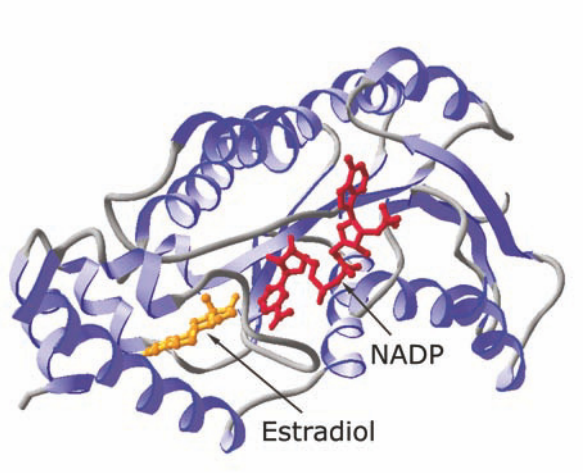

Figure 3.5. Structure of 17β-hydroxysteroid hydrogenase (β-HSD) with bound NADPH. From [28].

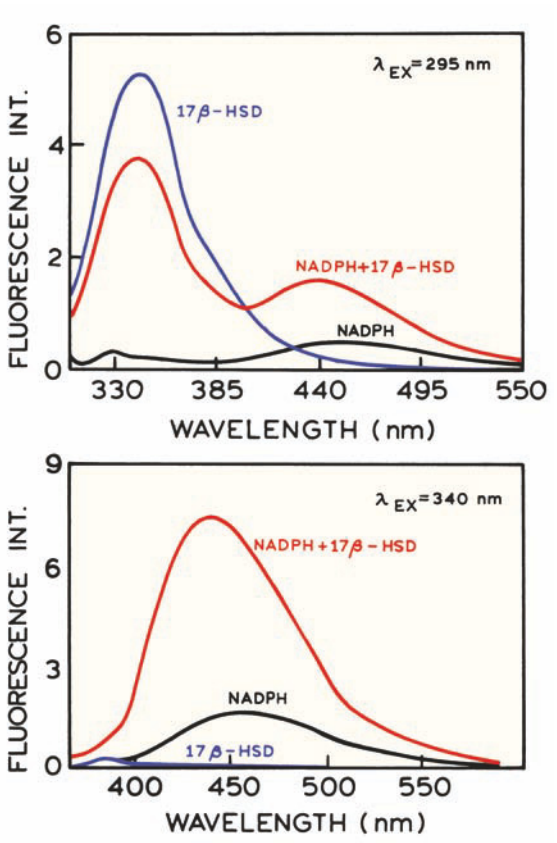

Figure 3.6. Emission spectra 17β-hydroxysteroid dehydrogenase (β-HSD) in the presence and absence of NADPH. Revised from [29].

3.1) except for a phosphate group on the 2'-position of the ribose. For excitation at 295 nm both the protein and NADPH are excited (top). Addition of NADPH to the protein results in 30% quenching of protein fluorescence, and an enhancement of the NADPH fluorescence.[29] The Förster distance for trp→NADPH energy transfer in this system is 23.4 Å. Using eq. 1.12 one can readily calculate a distance of 26.9 Å from the single tryptophan residue to the NADPH.

For illumination at 340 nm only the NADPH absorbs, and not the protein. For excitation at 340 nm the emission spectrum of NADPH is more intense in the presence of protein (Figure 3.6, lower panel). An increase in intensity at 450 nm is also seen for excitation at 295 nm (top panel), but in this case it is not clear if the increased intensity is due to a higher quantum yield for NADPH or to energy transfer from the tryptophan residues. For excitation at 340 nm the emission intensity increases about fourfold. This increase is due to less quenching by the adenine group when NADPH is bound to the protein. The increased quantum yield can be

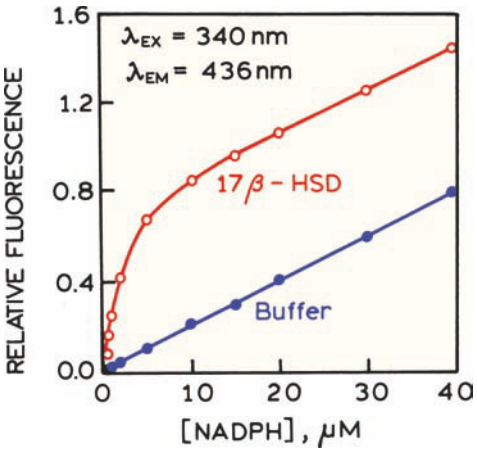

Figure 3.7. Fluorescence intensity of NADPH titrated into buffer (●) or a solution of 17β-HSD (○). Revised from [29].

used to study binding of NADPH to proteins (Figure 3.7). In the absence of protein the emission increases linearly with NADPH concentration. In the presence of protein the intensity initially increases more rapidly, and then increases as in the absence of protein. The initial increase in the intensity of NADPH is due to binding of NADPH to 17β-HSD, which occurs with a fourfold increase in the quantum yield of NADPH. Once the binding sites on 17β-HSD are saturated, the intensity increases in proportion to the concentration of unbound NADPH. In contrast to NADH, emission from FAD and flavins is usually quenched upon binding to proteins.

3.2. EXTRINSIC FLUOROPHORES

Frequently the molecules of interest are nonfluorescent, or the intrinsic fluorescence is not adequate for the desired experiment. For instance, DNA and lipids are essentially devoid of intrinsic fluorescence (Figure 1.18). In these cases useful fluorescence is obtained by labeling the molecule with extrinsic probes. For proteins it is frequently desirable to label them with chromophores with longer excitation and emission wavelengths than the aromatic amino acids. Then the labeled protein can be studied in the presence of other unlabeled proteins. The number of fluorophores has increased dramatically during the past decade. Useful information on a wide range of fluorophores can be found in the Molecular Probes catalogue.[30]

3.2.1. Protein-Labeling Reagents

Numerous fluorophores are available for covalent and noncovalent labeling of proteins. The covalent probes can have a variety of reactive groups, for coupling with amines and sulfhydryl or histidine side chains in proteins. Some of the more widely used probes are shown in Figure 3.8. Dansyl chloride (DNS-Cl) was originally described by Weber,[31]

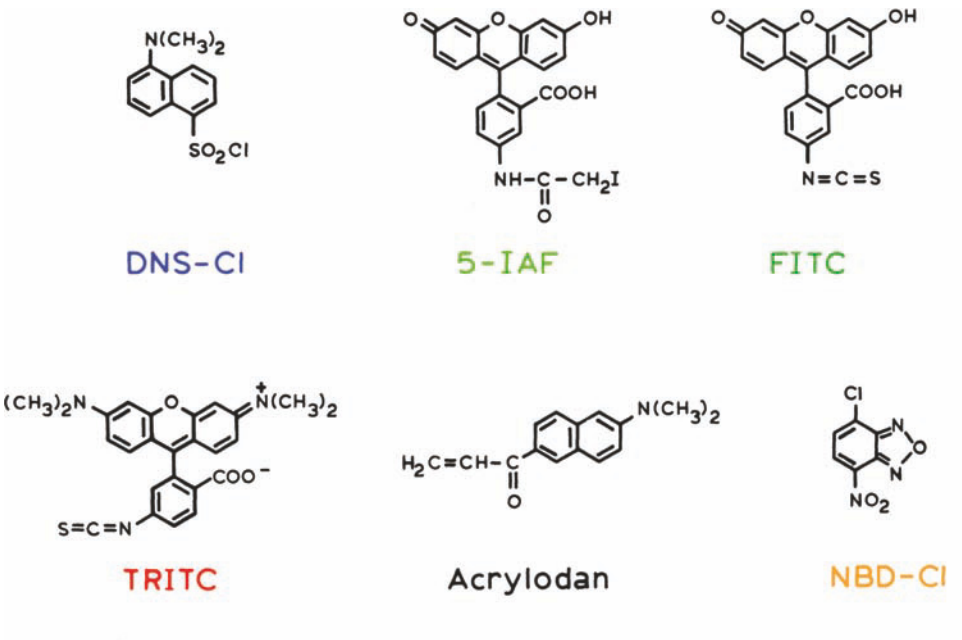

Figure 3.8. Reactive probes for conjugation with macromolecules.

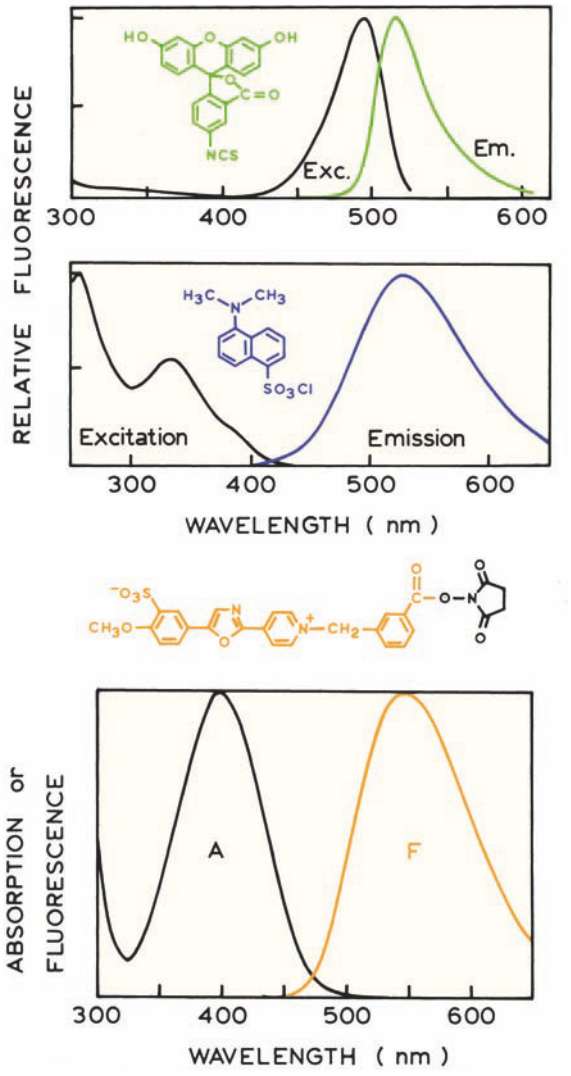

Figure 3.9. Excitation and emission spectra of FITC (top) and DNS-Cl (middle) labeled antibodies. Also shown in the excitation and emission spectra of Cascade Yellow in methanol (bottom).

and this early report described the advantages of extrinsic probes in biochemical research. Dansyl chloride is widely used to label proteins, especially where polarization measurements are anticipated. This wide use is a result of its early introduction in the literature and its favorable lifetime (≃10 ns). Dansyl groups can be excited at 350 nm, where proteins do not absorb. Since dansyl groups absorb near 350 nm they can serve as acceptors of protein fluorescence. The emission spectrum of the dansyl moiety is also highly sensitive to solvent polarity, and the emission maxima are typically near 520 nm (Figure 3.9).

Brief History of Gregorio Weber 1916–1997

The Professor, as he is referred to by those who knew him, was born in Buenos Aires, Argentina in 1916. He received an M.D. degree from the University of Buenos Aires in 1942 and went on to graduate studies at Cambridge University. Dr. Weber's talents were recognized by Sir Hans Krebs, who recruited him to the University of Sheffield in 1953. During his years at Sheffield, Professor Weber developed the foundations of modern fluorescence spectroscopy. While at Sheffield, the Professor developed the use of fluorescence polarization for studying macromolecular dynamics. In 1962 Professor Weber joined the University of Illinois at Urbana-Champaign, remaining active until his death in 1997. Dr. Weber's laboratory at the University of Illinois was responsible for the first widely used phase modulation fluorometer, a design that went on to successful commercialization. Professor Weber stressed that fluorescence spectroscopy depends on the probes first and instrumentation second.

While dansyl chloride today seems like a common fluorophore, its introduction by Professor Weber represented a fundamental change in the paradigm of fluorescence spectroscopy. Professor Weber introduced molecular considerations into fluorescence spectroscopy. The dansyl group is solvent sensitive, and one is thus forced to consider its interactions with its local environment. Professor Weber (Figure 3.10) recognized that proteins could be labeled with fluorophores, which in turn reveal information about the proteins and their interactions with other molecules. The probes that the Professor developed are still in widespread use, including dansyl chloride, 1-anilinonaphthalene-6-sulfonic acid (ANS), 2-(p-toluidinyl)naphthalene-6-sulfonic acid (TNS), and Prodan derivatives.

Fluoresceins and rhodamines are also widely used as extrinsic labels (Figure 3.11). These dyes have favorably long absorption maxima near 480 and 600 nm and emission wavelengths from 510 to 615 nm, respectively. In contrast to the dansyl group, rhodamines and fluoresceins are not sensitive to solvent polarity. An additional reason for their widespread use is the high molar extinction coefficients near 80,000 M^{-1} cm^{-1}. A wide variety of reactive derivatives are available, including iodoacetamides, isothiocyanates, and maleimides. Iodoacetamides and maleimides are typically used for labeling sulfhydryl groups, whereas isothiocyanates, N-hydroxysuccinimide, and sulfonyl chlorides are used for labeling amines.[32] Frequently, commercial labeling reagents are a mixture of isomers.

Figure 3.10. Professor Gregorio Weber with the author, circa 1992.

One common use of fluorescein and rhodamine is for labeling of antibodies. A wide variety of fluorescein- and rhodamine-labeled immunoglobulins are commercially available, and these proteins are frequently used in fluorescence microscopy and in immunoassays. The reasons for selecting these probes include high quantum yields and the long wavelengths of absorption and emission, which minimize the problems of background fluorescence from biological samples and eliminate the need for quartz optics. The lifetimes of these dyes are near 4 ns and their emission spectra are not significantly sensitive to solvent polarity. These dyes are suitable for quantifying the associations of small labeled molecules with proteins via changes in fluorescence polarization.

The BODIPY dyes have been introduced as replacements for fluorescein and rhodamines. These dyes are based on an unusual boron-containing fluorophore (Figure 3.12). Depending on the precise structure, a wide range of emission wavelengths can be obtained, from 510 to 675 nm. The BODIPY dyes have the additional advantage of displaying high quantum yields approaching unity, extinction coefficients near 80,000 M^{-1} cm^{-1}, and insensitivity to solvent polarity and pH. The emission spectra are narrower than those of fluorescein and rhodamines, so that more of the light is emitted at the peak wavelength, possibly allowing more individual dyes to be resolved. A disadvantage of the BODIPY dyes is a very small Stokes shift.[33] As a result the dyes transfer to each other with a Förster distance near 57 Å.

3.2.2. Role of the Stokes Shift in Protein Labeling

One problem with fluoresceins and rhodamines is their tendency to self-quench. It is well known that the brightness of fluorescein-labeled proteins does not increase linearly with the extent of labeling. In fact, the intensity can decrease as the extent of labeling increases. This effect can be understood by examination of the excitation and emission spectra (Figure 3.9). Fluorescein displays a small Stokes shift. When more than a single fluorescein group is bound to a protein there can be energy transfer between these groups. This can be understood by realizing that two fluorescein groups attached to the same protein are likely to be within 40 Å of each other, which is within the Förster distance for fluorescein-to-fluorescein transfer. Stated differently, multiple fluorescein groups attached to a protein result in a high local fluorescein concentration.

Examples of self-quenching are shown in Figure 3.13 for labeled antibodies.[30,34] Fluorescein and Texas-Red both show substantial self-quenching. The two Alexa Fluor dyes show much less self-quenching, which allows the individually labeled antibodies to be more highly fluorescent. It is not clear why the Alexa Fluor dyes showed less self-quenching since their Stokes shift is similar to that of fluo-

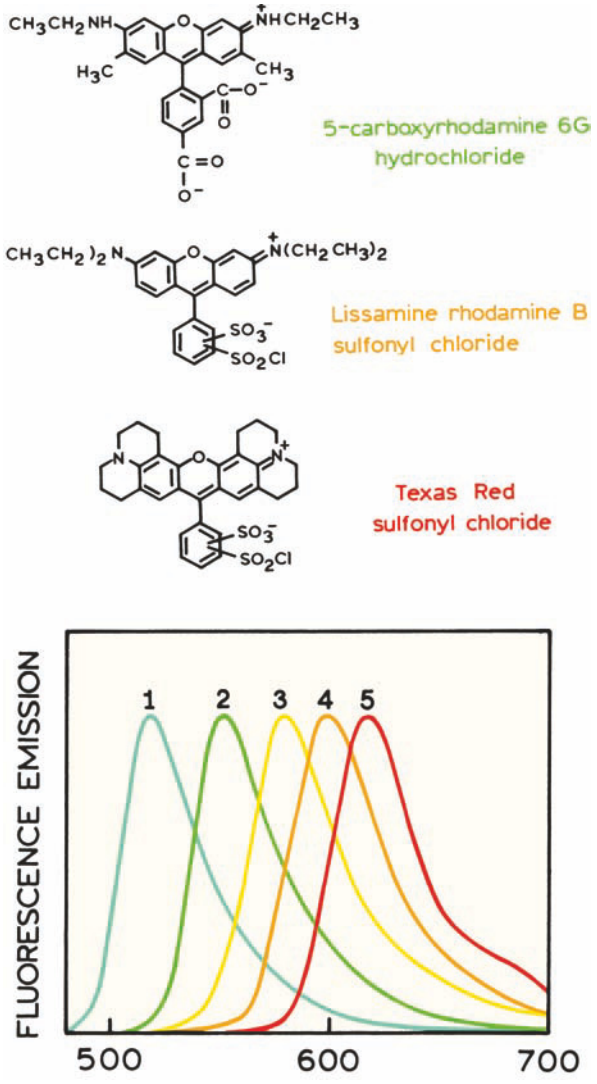

Figure 3.11. Structures and normalized fluorescence emission spectra of goat anti-mouse IgG conjugates of (1) fluorescein, (2) rhodamine 6G, (3) tetramethylrhodamine, (4) Lissamine rhodamine B, and (5) Texas Red dyes. Revised from [30].

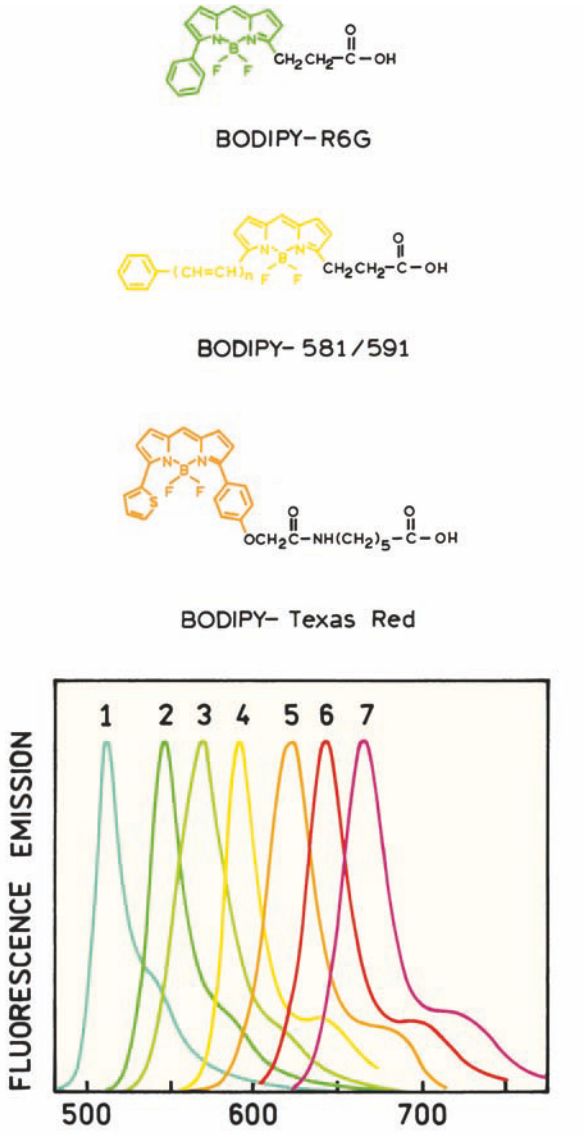

Figure 3.12. Normalized fluorescence emission spectra of BODIPY fluorophores in methanol. Revised from [30].

rescein and rhodamine. The BODIPY dyes have a small Stokes shift and usually display self-quenching. New dyes are being developed that show both a large Stokes shift and good water solubility. One such dye is Cascade Yellow, which displays excitation and emission maximum near 409 and 558 nm, respectively (Figure 3.9). The large Stokes shift minimizes the tendency for homotransfer, and the charges on the aromatic rings aid solubility.

In contrast to fluorescein, rhodamines, and BODIPYs, there are fluorophores that display high sensitivity to the polarity of the local environment. One example is Prodan[35] (Figure 3.8), which is available in the reactive form—called acrylodan.[36] In the excited state there is a charge separation from the amino to the carbonyl groups. When bound to membranes, Prodan and its derivatives display large spectral shifts at the membrane phase-transition temperature.

3.2.3. Photostability of Fluorophores

One of the most important properties of a probe is its photostability. Almost all fluorophores are photobleached upon continuous illumination, especially in fluorescence microscopy where the light intensities are high. Fluorescein is one of the least photostable dyes (Figure 3.14). The Alexa

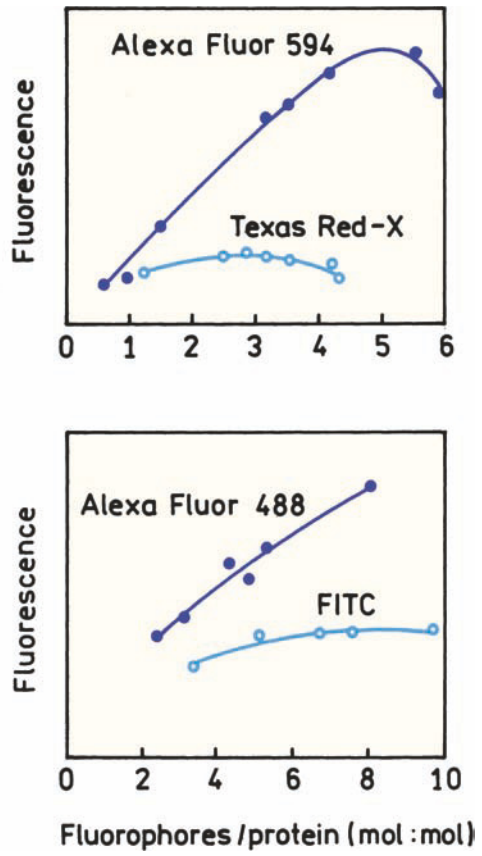

Figure 3.13. Effect of the fluorophore-to-protein ratio on the intensity of covalently labeled antibodies. Revised from [30].

Fluor dyes are more photostable and appear to have been developed for this reason. The chemical structures of Alexa Fluor dyes are not available. The emission maximum of Alexa Fluor dyes ranges from 442 to 775 nm. The photostability of a dye can be affected by its local environment. In some cases photostability is increased by removal of oxygen, and in other cases oxygen has no effect. There appears to be no general principles that can be used to predict photostability.

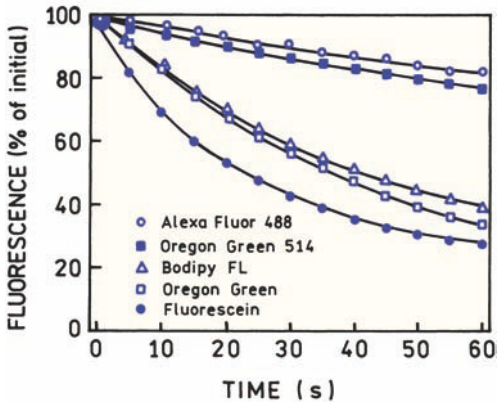

Figure 3.14. Comparison of the photostability of labeled antibodies in cells on fixed slides. The intensities were measured using a fluorescence microscope. Revised from [30].

3.2.4. Non-Covalent Protein-Labeling Probes

There are a number of dyes that can be used to non-covalently label proteins. These are typically naphthylamine sulfonic acids, of which 1-anilinonaphthalene-6-sulfonic acid (ANS) and 2-(p-toluidinyl)naphthalene-6-sulfonic acid (TNS) are most commonly used.[37] Dyes of this class are frequently weakly or nonfluorescent in water, but fluoresce strongly when bound to proteins[38] or membranes. Figure 3.15 shows the emission spectra of BSA excited at 280 nm as the sample is titrated with ANS. In the absence of BSA

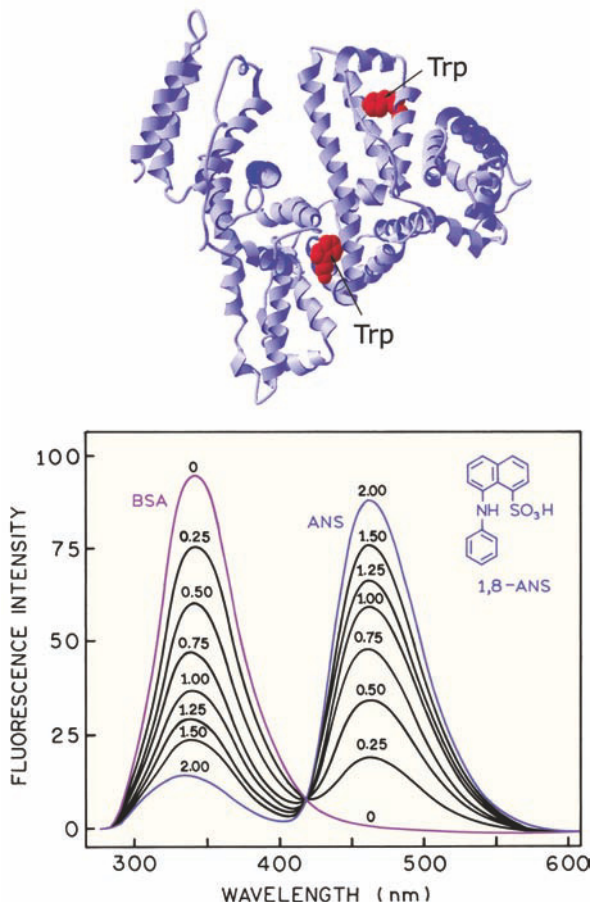

Figure 3.15. Fluorescence emission spectra of bovine serum albumin (BSA) in the presence of increasing ANS concentration. The numbers indicate the average number of ANS molecules bound per BSA molecule. Excitation at 280 nm. The structure shows the crystal structure of HSA modified to contain two tryptophanes. Revised from [38].

Figure 3.16. Color photograph of solutions of HSA, ANS and a mixture when illuminated with a UV hand lamp. From [39].

the emission from the ANS dissolved in buffer would be insignificant (not shown). Tryptophan emission from BSA is quenched upon addition of ANS, and the ANS emission increases as the BSA emission decreases. There is no observable emission from ANS alone, which shows an emission maximum above 500 nm in water. ANS-type dyes are amphiphatic, so that the nonpolar region prefers to adsorb onto nonpolar regions of macromolecules. Since the water-phase dye does not contribute to the emission, the observed signal is due to the area of interest, the probe binding site on the macromolecule.

Binding of ANS to BSA or human serum albumin (HSA) can be used as a visible demonstration. Take an aqueous solution of ANS (about 10^{-5} M) and BSA (about 10 mg/ml) and observe them under a UV hand lamp. Little emission will be seen from either sample. Any emission seen from the ANS solution will be weak and greenish. Then mix the two solutions while illuminating with the UV hand lamp. There will be an immediate increase in fluorescence intensity and a shift of the ANS emission to the blue (Figure 3.16). We frequently use this demonstration to illustrate fluorescence to students.

3.2.5. Membrane Probes

Labeling of membranes is often accomplished by simple partitioning of water-insoluble probes into the nonpolar regions of membranes. DPH, 1,6-diphenyl-1,3,5-hexatriene, is one of the most commonly used membrane probes. Addition of DPH to a membrane suspension results in complete binding, with no significant emission from DPH in the aqueous phase. All the emission from DPH is then due to DPH in the membrane environment. The tasks of labeling membranes have been made easier by the availability of a wide variety of lipid probes. A few examples are shown in Figure 3.17. Lipid probes can be attached to the fatty acid chains or to the phospholipids themselves. The depth of this probe in the bilayer can be adjusted by the length of the various chains, as shown for the anthroyl fatty acid. DPH, often used as a partitioning probe, can be localized near the membrane–water interface by attachment of a trimethylammonium group to one of the phenyl rings (TMA-DPH).[40] Unsaturated fatty acids can also be fluorescent if the double bonds are conjugated as in parinaric acid.[41]

Membranes can also be labeled by covalent attachment of probes to the lipids. This is useful with more water-soluble probes like fluorescein or rhodamine. The probes can be forced to localize in the membrane by attachment to long acyl chains or to the phospholipids themselves (Figure 3.17). Depending on chemical structure, the fluorescent group can be positioned either on the fatty acid side chains (Fluorenyl-PC) or at the membrane–water interface (Texas Red-PE). The fluorophore Texas Red is often used for long-wavelength absorption and high photostability. Pyrene has been attached to lipids (pyrenyl lipid) to estimate diffusive processes in membranes by the extent of excimer formation. The pyrenyl PC probe displays unusual spectral properties. The emission spectra of pyrenyl-PC liposomes are highly dependent on temperature (Figure 3.18). The unstructured emission at higher temperatures is due to excimer formation between the pyrene groups.[42] If the pyrenyl-PC is present at a lower mole fraction the amount of excimer emission decreases. The relative amounts of monomers and excimer emission can be used to estimate the rate of lateral diffusion of lipids in the membranes.

3.2.6. Membrane Potential Probes

There are membrane probes that are sensitive to the electrical potential across the membrane. Typical membrane potential probes are shown in Figure 3.19. A number of mechanisms are thought to be responsible, including partitioning of the dye from the water to the membrane phase, reorientation of the dyes in the membrane, aggregation of dyes in the membrane, and the inherent sensitivity of the dyes to the electric field.[43–48] The carbocyanine dyes typically respond to potential by partitioning and/or aggregation in the membranes,[49–50] whereas the stryryl dyes seem to respond directly to the electric field.[51] The merocyanine

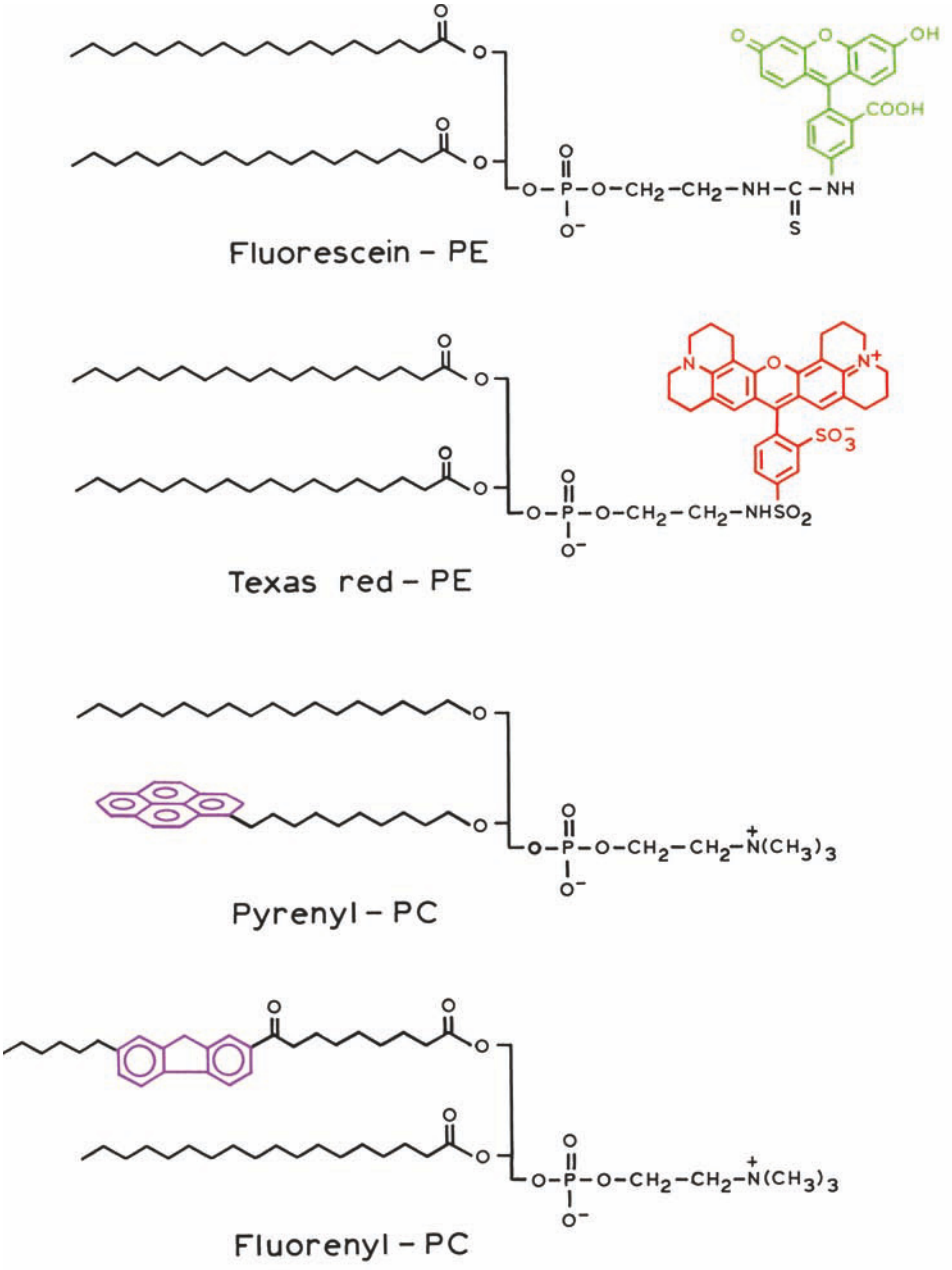

Figure 3.17. Fluorescent phospholipid analogues. PC = phosphatidylcholine; PE = phosphatidylethanolamine.

dyes probably respond to membrane potential by both mechanisms.[51–53] There are continuing efforts to develop improved dyes.[54–55] With all these probes the effect of potential is small, typically a few percent, so that intensity ratios are often used to provide more stable signals.[56–57]

Because of the small size of fluorophores it is difficult to obtain a significant change in voltage across the fluorophore. The sensitivity to voltage can be improved by using RET and a dye that translocates across the membrane in response to voltage.[58–59] This is accomplished by positioning a fluorophore (coumarin-lipid) on one side of the membrane and allowing a second dye (oxonal) to partition into the membrane (Figure 3.20). The oxonal is an acceptor for coumarin. There was minimal absorption by oxonal at the coumarin excitation wavelength so that RET was the dominant origin of the oxonal emission. Changes in voltage

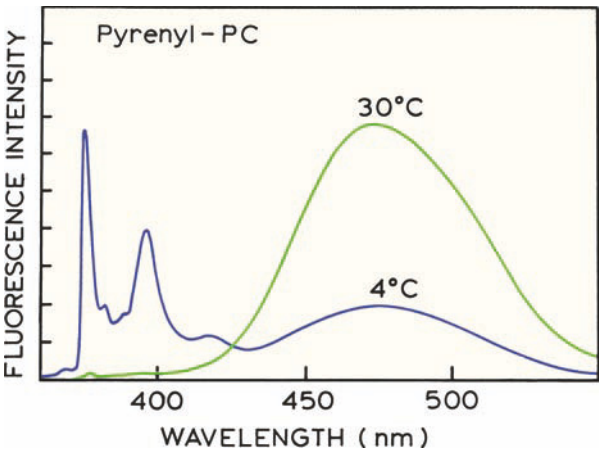

Figure 3.18. Fluorescence emission spectra for pyrenyl-PC measured at 4 and 30°C. The lipid probe was dispersed in water. From [42] and reprinted with permission from Springer-Verlag Inc.

result in changes in oxonal concentration near the coumarin and hence change in intensity of the oxonal emission. This system is about fivefold more sensitive to voltage than the ASP class of dyes.

3.3. RED AND NEAR-INFRARED (NIR) DYES

The cyanine dyes were initially used as membrane potential probes and evolved into some of the more commonly used long-wavelength dyes. Long-wavelength probes are of current interest for several reasons. The sensitivity of fluorescence detection is often limited by the autofluorescence of biological samples. As the excitation wavelength becomes longer, the autofluorescence decreases, and hence detectability over background increases.[60] Long-wavelength dyes can be excited with laser diodes. The most familiar long-wavelength dyes are the cyanine dyes, such as the Cy-3, Cy-5 and Cy-7 in Figure 3.21. Such dyes have absorption and emission wavelengths above 550 nm.[61–63] The cyanine dyes typically display small Stokes shift, with the absorption maxima about 30 nm blue shifted from the emission maxima, as shown for Cy3. A wide variety of conjugatable cyanine dyes are available. Charged side chains are used for improved water solubility or to prevent self-association, which is a common cause of self-quenching in these dyes. Lipid side-chains are used to bind these dyes to membranes.

Additional long-wavelength dyes are shown in Figure 3.22. Some rhodamine derivatives display long absorption and emission spectra, as seen for Rhodamine 800.[64] The oxazine dyes display surprising long absorption and emission maxima given their small size.[65] Extended conjugated systems result in long absorption and emission wavelengths, as shown for IR-125 and thiazole orange. Dyes of this class have been extensively characterized for use as long-wavelength probes and in DNA sequencing.[66] The dye

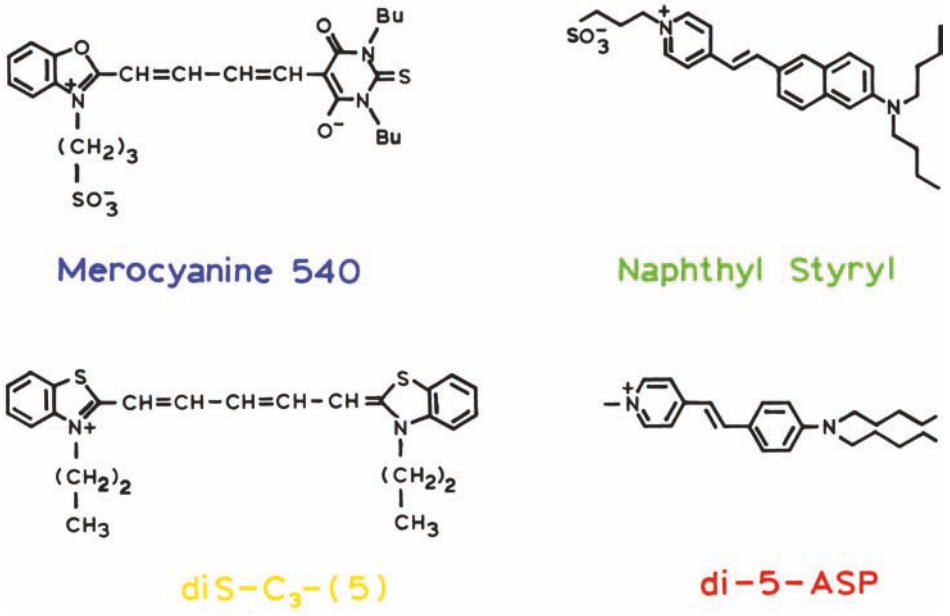

Figure 3.19. Membrane potential probes.

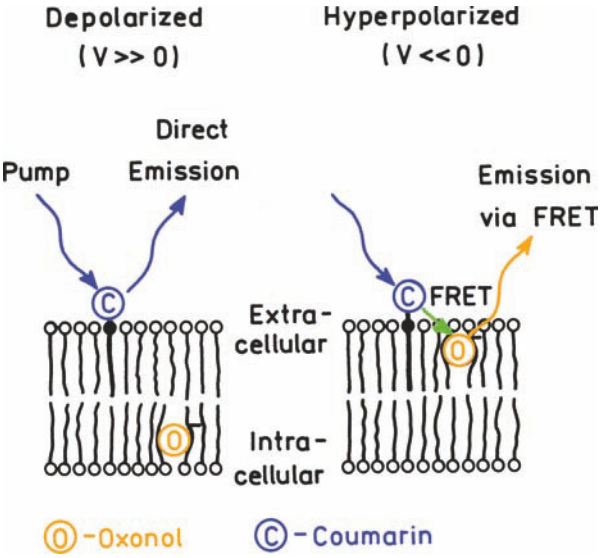

Figure 3.20. Measurement of membrane potential using RET and a mobile dye. The donor is a coumarin lipid and the acceptor is oxonal. Revised from [59].

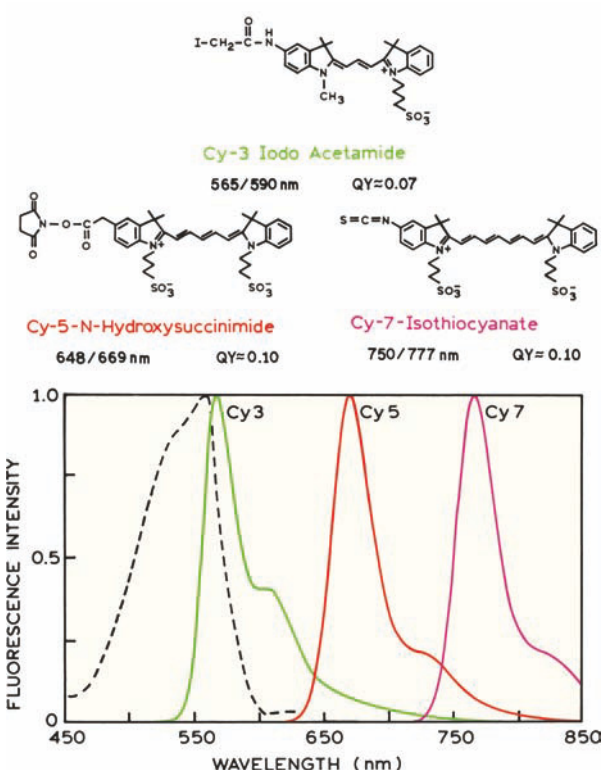

Figure 3.21. Chemically reactive cyanine dyes. The dashed line shows the absorption spectrum of Cy3.

thiazole orange can be excited at 735 nm and binds strongly to DNA. Dyes of this type are also used for staining DNA restriction fragments during capillary electrophoresis. Another class of long-wavelength dyes are the phthalocyanines and naphthalocyanines (Figure 3.22). At present these dyes are used less in biochemistry due to a lack of water solubility and a tendency to aggregate.[67] There is ongoing work to improve the phahalacyanines[68] and to develop other red-NIR dyes.[69–72]

3.4. DNA PROBES

While very weak intrinsic emission has been observed from unlabeled DNA, this emission is too weak and too far in the UV for practical applications. Fortunately, there are numerous probes that spontaneously bind to DNA and display enhanced emission.[73–75] Several representative DNA probes are shown in Figure 3.23. One of the most widely used dyes is ethidium bromide (EB). EB is weakly fluorescent in water, and its intensity increases about 30-fold upon binding to DNA. The lifetime of ethidium bromide is about 1.7 ns in water, and increases to about 20 ns upon binding to double-helical DNA. The mode of binding appears to be due to intercalation of the planar aromatic ring between the base pairs of double helical DNA. Many DNA probes such as acridine orange also bind by intercalation. Other types of probes bind into the minor groove of DNA, such as DAPI and Hoechst 33342. The fluorescence of DAPI appears to be most enhanced when adjacent to AT-rich regions of DNA.[76] Hoechst 33358 binds with some specificity to certain base-pair sequences.[77–79] In recent years improved DNA dyes have been developed that bind to DNA with high affinity. Typical high-affinity dyes are dimers of known DNA probes, such as the ethidium homodimer[80] and elongated positively charged dyes like TOTO-1.[81] Such dyes remain bound to DNA during gel electrophoresis and allow DNA detection with high sensitivity.

3.4.1. DNA Base Analogues

The native bases of DNA are not useful as fluorescent probes, and thus the use of extrinsic DNA probes is necessary. However, DNA can be made fluorescent by the use of DNA base analogues. 2-amino purine (2-AP) is an analogue of adenine, and isoxanthopterin (IXP) is an analogue of guanine (Figure 3.24). In solution, 2-amino purine has a high quantum yield and a single exponential decay time near 10 ns. Upon incorporation into double-stranded DNA

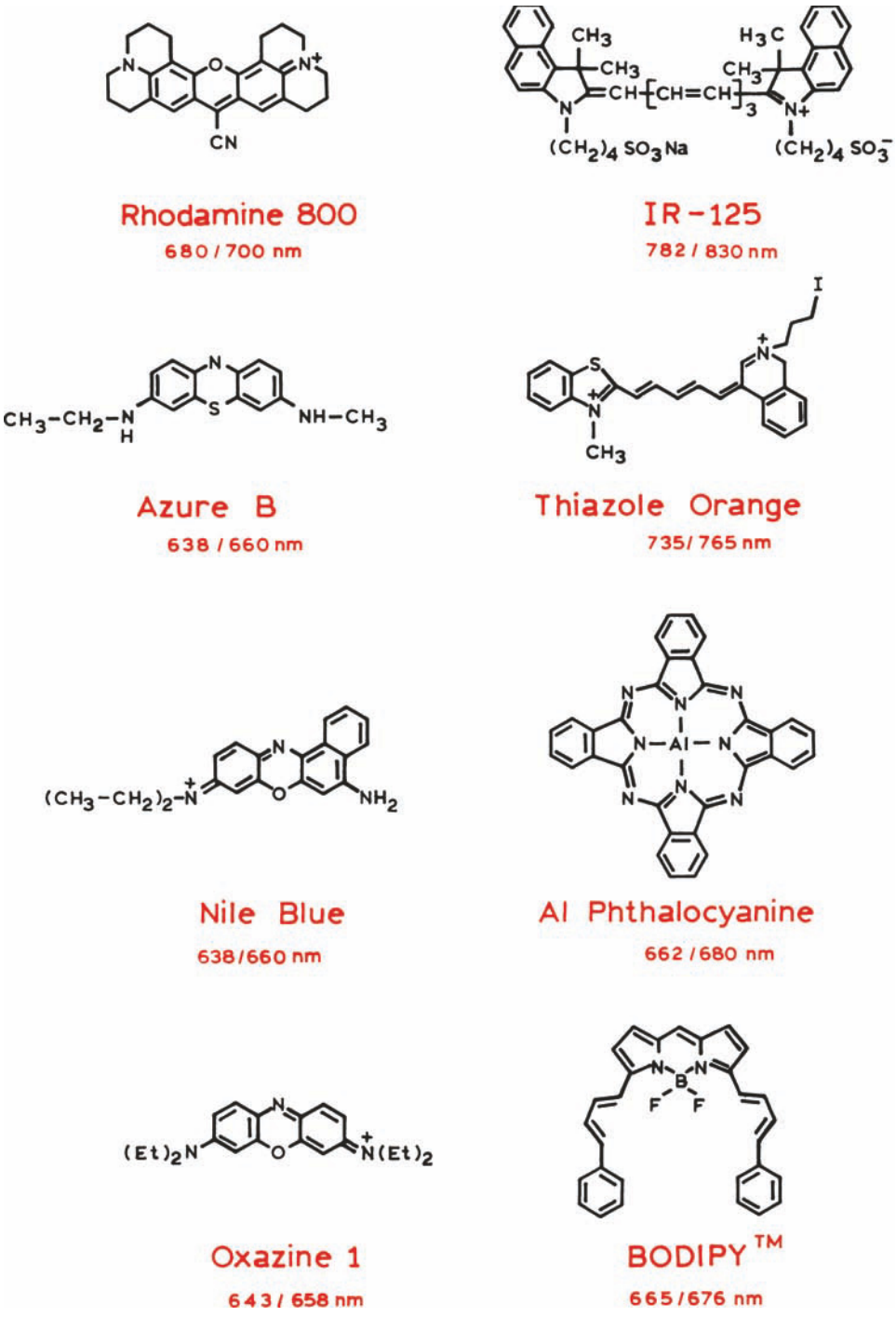

Figure 3.22. Representative NIR dyes.

oligomers, its fluorescence is partially quenched and its decay becomes complex.[82] The sensitivity of 2-AP to its environment makes it a useful probe for studies of DNA conformation and dynamics.[83–86]

Figure 3.24 shows emission spectra of isoxanthopterin (IXP). Like 2-AP, IXP is partially quenched when in double-helical DNA (Figure 3.25), but more fluorescent when present in a dinucleotide.[87–88] The dependence of the IXP

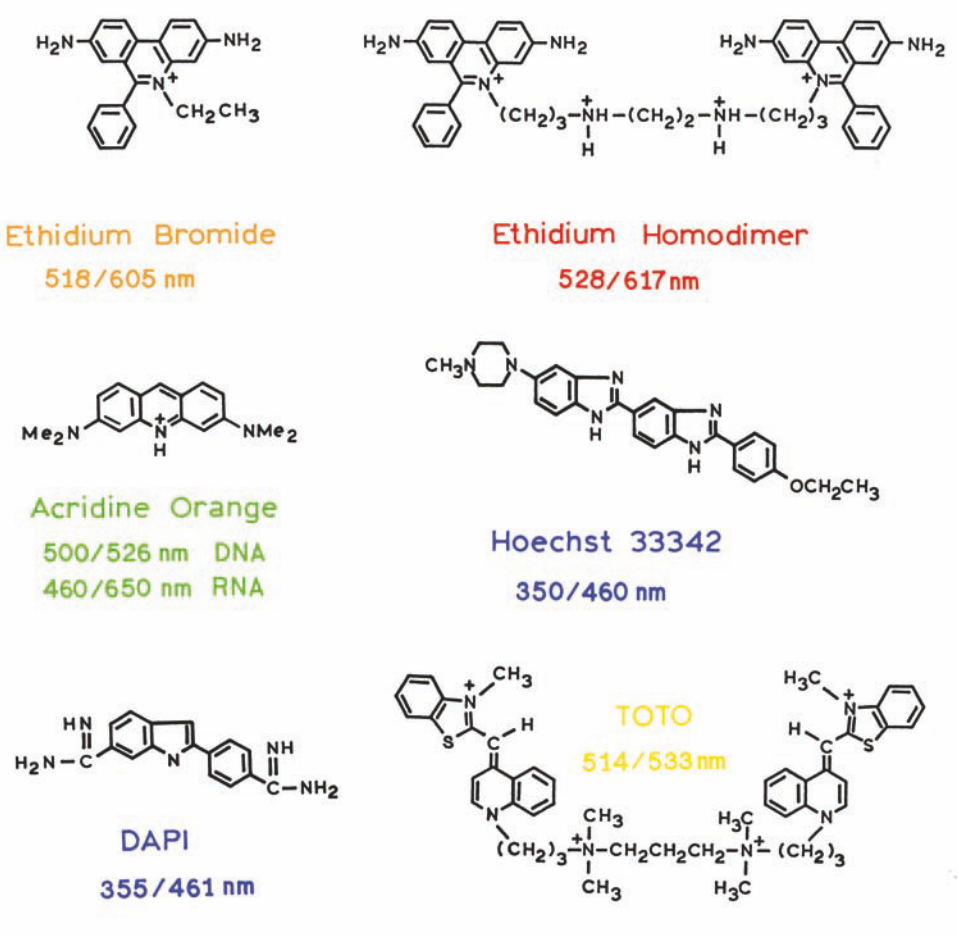

Figure 3.23. Representative DNA probes. Excitation and emission wavelengths refer to DNA-bound dye.

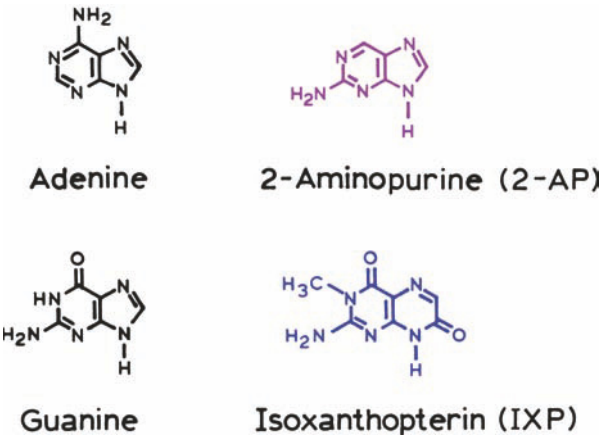

Figure 3.24. DNA purine bases (left) and fluorescent base analogues (right).

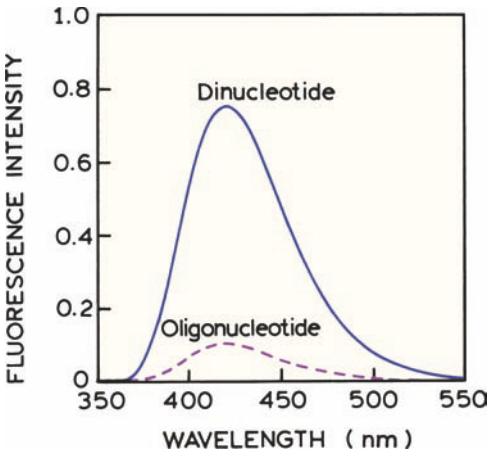

Figure 3.25. Emission spectra of the isoxanthopterin nucleotide in a dinucleotide and an oligonucleotide. From [87].

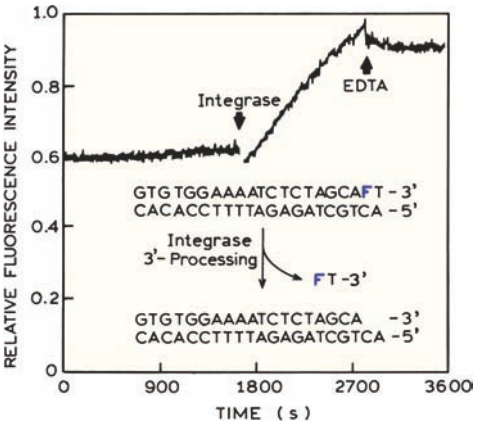

Figure 3.26. HIV integrase assay based on release of the isoxanthopterin-containing nucleotide (F). Revised from [87], and reprinted with permission of Oxford University Press.

on DNA structure was used as an assay for the HIV integrase protein. This protein is responsible for integration of HIV DNA into the host cell's genome.[89–90] The assay was based on a DNA oligonucleotide that has the sequence specific for HIV integrase (Figure 3.26). The enzyme mechanism involves cleavage of a dinucleotide from the 3'-end of HIV DNA, followed by ligation to the 5'-end of the host DNA. The IXP nucleotide was positioned near the 3'-end of the synthetic substrate. Incubation with HIV integrase resulted in release of the dinucleotide, which was detected by an increase in IXP fluorescence.

3.5. CHEMICAL SENSING PROBES

It is often desirable to detect spectroscopically silent substances such as Cl^-, Na^+, or Ca^{2+}. This is possible using

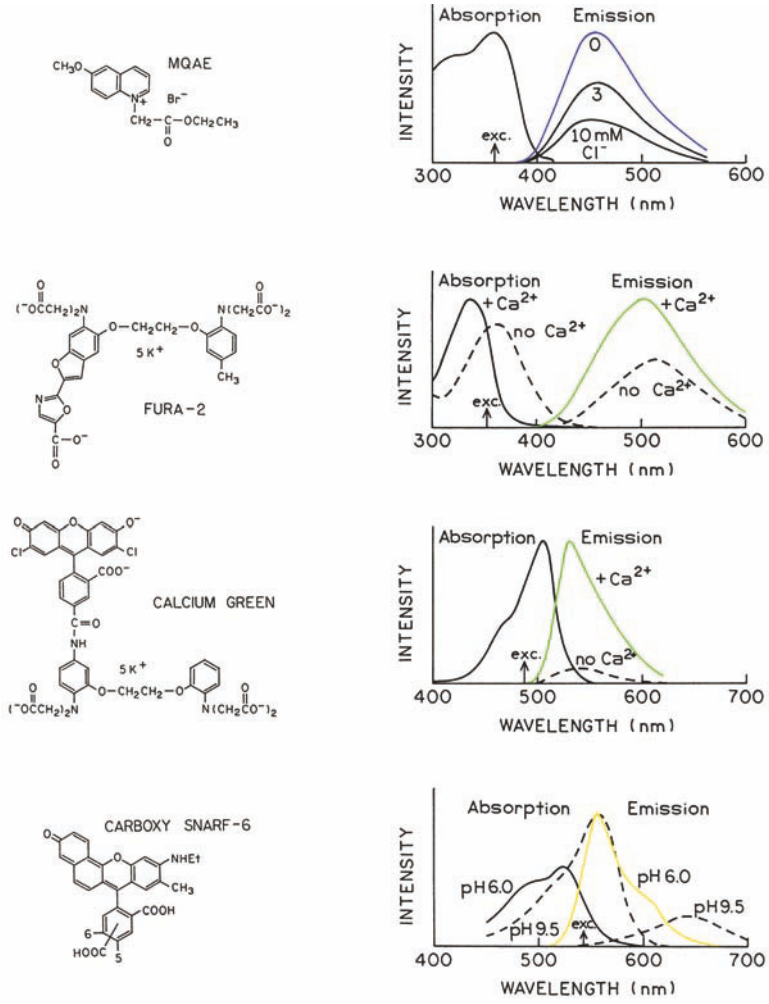

Figure 3.27. Chemical sensing probes (left) and thus spectra (right).

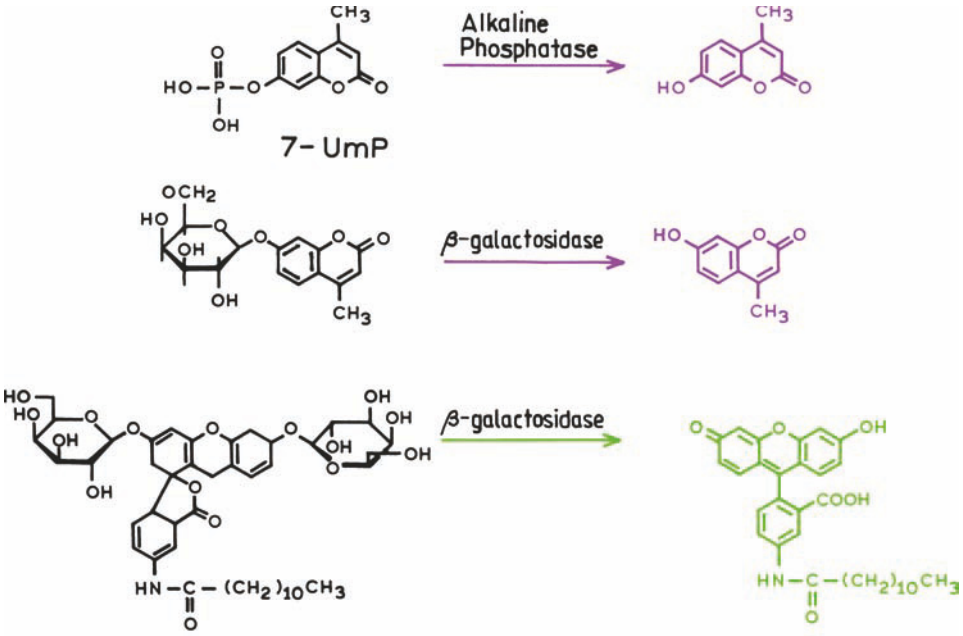

Figure 3.28. Fluorogenic probes.

sensing probes, some of which are shown in Figure 3.27. The probe MQAE is collisionally quenched by chloride according to the Stern-Volmer equation (eq. 1.6), allowing the chloride concentration to be estimated from the extent of quenching.[91] Other probes allow measurement of free Ca^{2+}. Probes such as Fura-2 display Ca^{2+}-dependent spectral shifts. Such probes are called wavelength-ratiometric probes because the analyte (Ca^{2+}) concentration can be determined from a ratio of intensities at different excitation or emission wavelengths. Other probes such as Calcium Green display a Ca^{2+}-dependent increase in intensity but no spectral shift. Wavelength-ratiometric and non-ratiometric probes are known for many species,[92] including H^+, Na^+, K^+, and Mg^{2+}, amines, and phosphate.[93–96] These dyes typically consist of a fluorophore and a region for analyte recognition, such as on azacrown ether for Na^+ or K^+, or a BAPTA group for Ca^{2+}. Such dyes are most often used in fluorescence microscopy and cellular imaging, and are trapped in cells either by hydrolysis of cell-permeable esters or by microinjection.

3.6. SPECIAL PROBES

3.6.1. Fluorogenic Probes

Another class of probes is the fluorogenic probes.[97–99] These are dyes that are non- or weakly fluorescent until some event occurs, such as enzymatic cleavage. Typical fluorogenic probes are shown in Figure 3.28. 7-Umbelliferyl phosphate (7-UmP) is nonfluorescent as the phosphate ester, but becomes highly fluorescent upon hydrolysis. 7-UmP is used to measure the activity of alkaline phosphatase. This enzyme is often used as the basis of enzyme-linked immunoadsorbent (ELISA) assays, and is also used in enzyme-amplified DNA assays.

It is often important to measure β-galactosidase activity in cells. This enzyme is often used as a gene marker in cells. Its activity can be detected by a galactoside of umbelliferone or 7-hydroxy-4-methylcoumarin (Figure 3.28, middle). An improved probe is shown in the lower panel. This fluorescein derivative contains a fatty acid chain that serves to retain the probe at the site of hydrolysis. This allows the cells with β-galactosidase activity to be identified under a microscope.

Another class of fluorogenic reagents are those that are initially nonfluorescent, and become fluorescent upon reacting with amines (Figure 3.29). While they have been used for labeling proteins, they are more commonly used in protein sequencing, determination of protein concentration, or for detection of low molecular weight amines in chromatography.

Fluorogenic probes can also be based on energy transfer.[100–102] One example is shown in Figure 3.30, in which a peptide is labeled with a donor and acceptor. The sequence

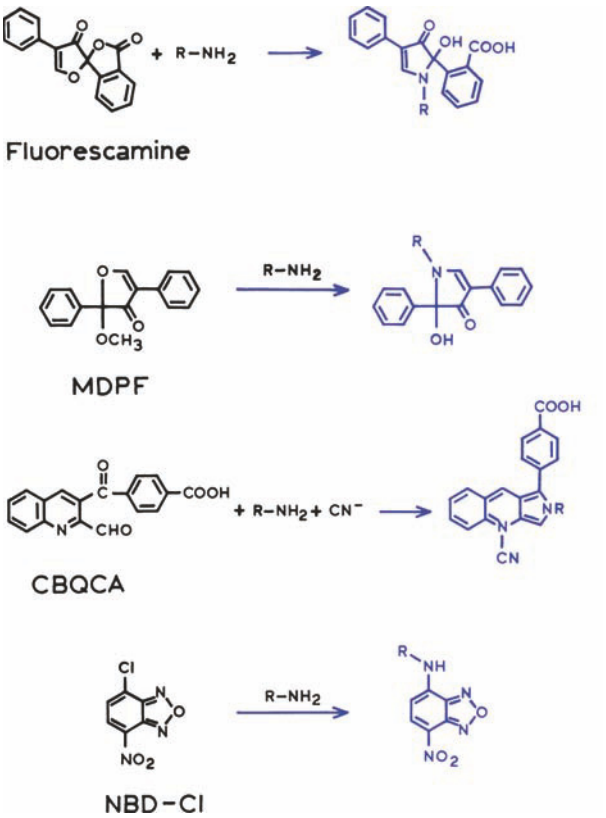

Figure 3.29. Fluorogenic reagents for amines.

of amino acids was selected to be specific for a protease found in HIV. Cleavage of the peptide results in a greater distance between the donor and acceptor and increased donor intensity. This concept of a decrease in energy transfer upon cleavage has also been applied to lipases that hydrolyze phospholipids.

3.6.2. Structural Analogues of Biomolecules

One approach to designing fluorophores is to make the shape similar to the parent biomolecule. This approach was used to make fluorescent analogues of steroids. Two examples are shown in Figure 3.31. Cholesterol is an essential component of cell membranes, and estradiol is important for the expression of female sexual characteristics. Both molecules are nonfluorescent, but structurally similar molecules have been synthesized which display useful fluorescence. Dehydroergosterol is a fluorescent analogue of cholesterol that displays absorption and emission maxima near 325 and 390 nm, respectively. Dehydroergosterol has been used as a probe for the interactions of steroids with membranes.[103–106] Ligand analogues have also been reported for the estrogen receptor.[107–108]

3.6.3. Viscosity Probes

Fluorescent quantum yields are often dependent on viscosity, but there are relatively few fluorophores characterized

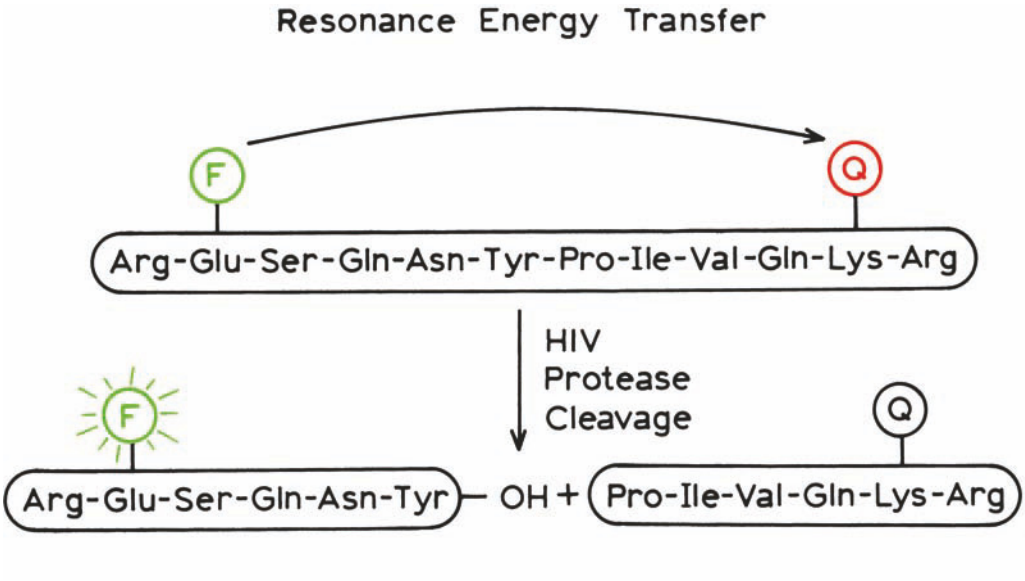

Figure 3.30. Fluorogenic probes for HIV protease. The fluorescence signal is generated when HIV protease releases the fluorophore (F) from the quenching effects of the nearby acceptor chromophore (Q).

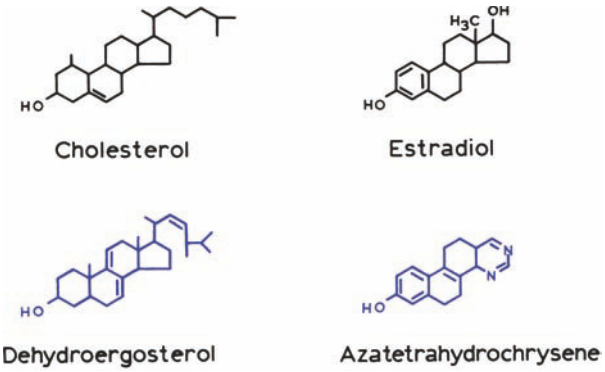

Figure 3.31. Nonfluorescent steroid cholesterol and estradiol, and fluorescent analogues dehydroergosterol and 1,3-diaza-9-hydroxy-5,6,11,12-tetrahydrochrysene. Revised from [103].

as viscosity probes. One such probe is shown in Figure 3.32. These probes display charge transfer in the excited state, presumably from the amine to the vinyl or cyano group. In a highly viscous environment the molecule cannot distort as needed for charge transfer, and the decay is radiative.[109–111] In a less viscous environment the molecule displays internal rotation and charge transfer, which results in radiationless decay. As a result the quantum yield depends on solvent viscosity. These probes have been used to study the viscosities of membranes, and the rigidity of binding sites on proteins. These viscosity probes can be regarded as a subclass of the TICT probes, which are probes that distort in the excited state to form twisted intramolecular charge transfer states.[112] For probes like DPH the quantum yield is only weakly dependent on the viscosity. For DPH the viscosity is determined from the anisotropy.

3.7. GREEN FLUORESCENT PROTEINS

An important addition to the library of probes has been the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria*. The bioluminescence of the primary photoprotein aqueorin is blue. The bioluminescence from the jellyfish is green due to a closely associated green fluorescent protein. GFP contains a highly fluorescent group within a highly constrained and protected region of the protein. The chromophore is contained within a barrel of β-sheet protein[113] (Figure 3.33). The remarkable feature of the GFP is that the chromophore forms spontaneously upon folding of the polypeptide chain[114–115] without the need for enzymatic synthesis (Figure 3.34). As a result, it is possible to express the gene for GFP into cells, and to obtain proteins which are synthesized with attached

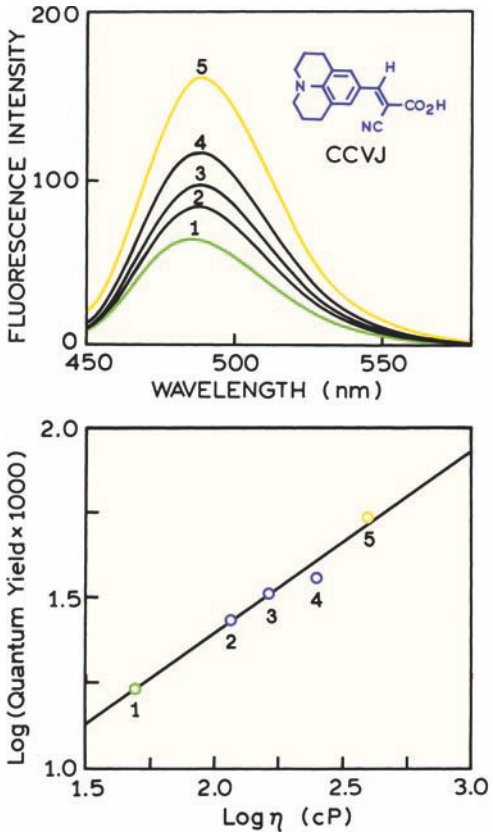

Figure 3.32. Fluorescence emission spectra and relative quantum yields of CCVJ in ethylene glycol/glycol mixtures of varying viscosity. Reprinted with permission from [111]. Copyright © 1993, American Chemical Society.

GFP.[116–118] It is even possible to express GFP in entire organisms.[119–121]

GFPs with different spectral properties have been created by introducing mutations into the amino-acid sequence. Mutants are known that display longer absorption and emission wavelengths (Figure 3.35) and have higher photostability.[122–124] In general GFPs have good photostability and display high quantum yields, which is probably because the β-barrel structure shields the chromophore from the local environment.

For a time it was thought that GFP was only present in *Aequorea victoria*. It is known that similar naturally fluorescent proteins are present in a number of Anthrozoa species, in corals.[125–129] As a result, a large number of fluorescent proteins are now available with emission maxima ranging from 448 to 600 nm (Figure 3.35). Because of the variety of fluorescent proteins the terminology has become confusing. The fluorescent proteins from coral are often referred to as yellow (YFPs) or red (RFPs) fluorescent pro-

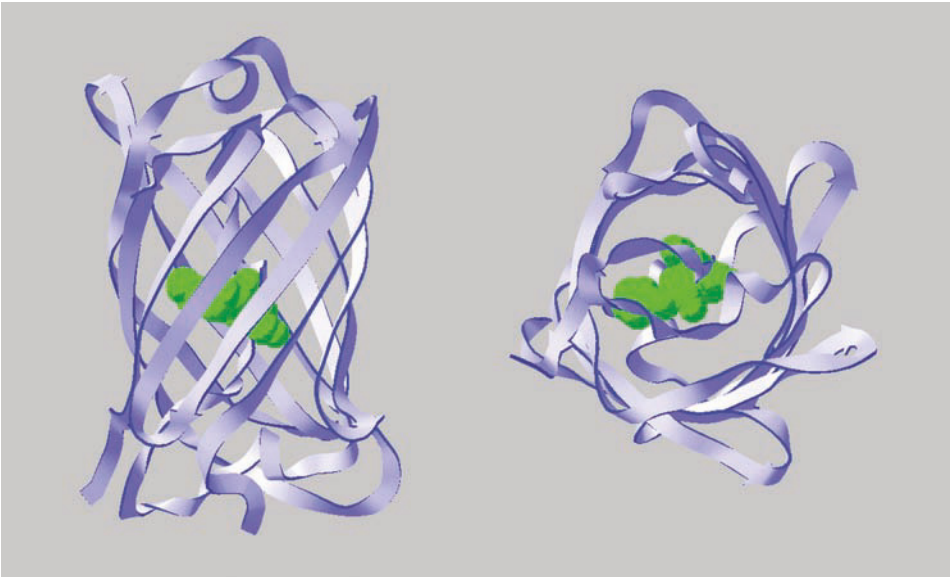

Figure 3.33. β-Barrel structure of GFP. Side and top view. The chromophore is linked covalently to the protein.

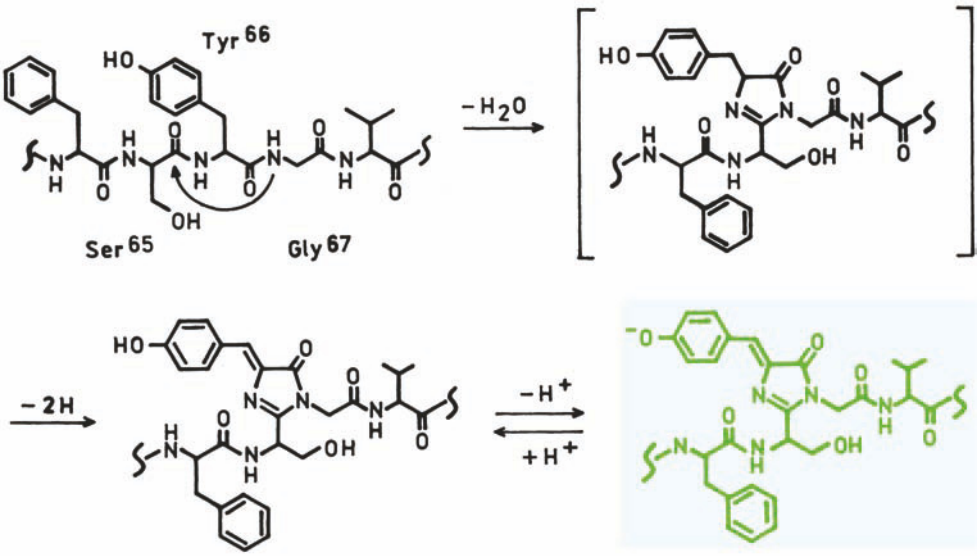

Figure 3.34. Spontaneous formation of the fluorophore in GFP by the serine–tyrosine–glycine residues. From [115].

teins. However, this is a misnomer because green fluorescent proteins can also come from coral.[127] It has been suggested that proteins derived from *Aequores victoria* be called AFPs to indicate their origin with this jellyfish.[130] For simplicity we will refer to all these proteins as GFPs.

An unfavorable property of all these fluorescent proteins is their tendency to self-associate. AFPs tend to form weakly bound dimers. The proteins from coral form strongly bound tetramers.[130–132] This self-association makes them less useful as intracellular probes, particularly with fusion proteins where the nonfluorescent proteins will be artificially brought into close proximity to each other. Another difficulty with the most widely used red fluorescent proteins, DsRed, is that the fluorescence develops too slowly and can take 30 hours to become fluorescent. These problems have been mostly solved by mutating the sequence to disrupt the self-association and to obtain proteins that mature more rapidly.[132–136]

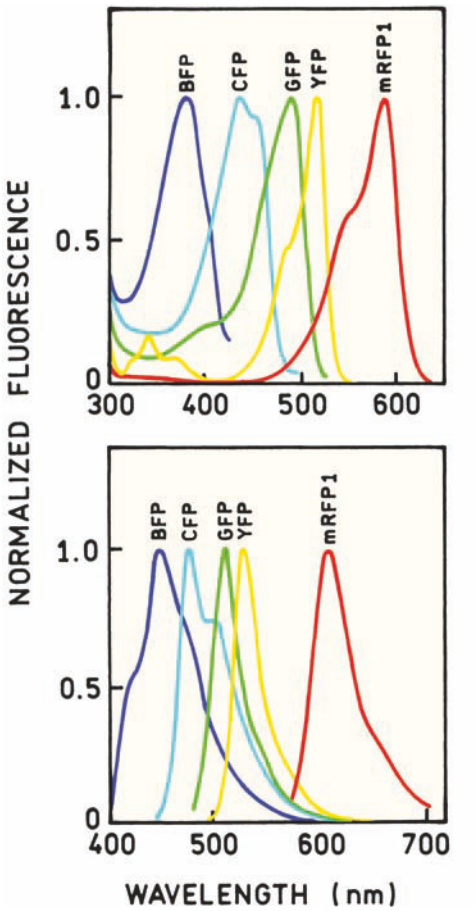

Figure 3.35. Excitation (top) and emission spectra (bottom) of GFP mutants. Revised from [129].

The fluorophores in GFPs are formed autocatalytically, and it is not necessary to add a fluorophore or enzymes to synthesize the fluorophore. All that is needed is the gene or mRNA that codes for the amino-acid sequence. As a result organisms that contain the gene or mRNA can express the fluorescent protein. This possibility is shown in Figure 3.36 for a *Xenopus* embryo. While in the blastomer stage the right and left blastomers were injected with mRNA for a green or red fluorescent coral protein, respectively. One week later the descendants of the injected blastomer showed fluorescence from the fluorescent proteins coded by the mRNAs.

3.8. OTHER FLUORESCENT PROTEINS

3.8.1. Phytofluors: A New Class of Fluorescent Probes

A new type of fluorescent probe is the so-called "phytofluors." These fluorescent probes are derived from the phytochromes, which are light-sensitive proteins present in photosynthetic organisms. These proteins allow the organisms to adjust to external light conditions, and are important in seed germination, flowering, and regulation of plant growth. Phytochromes typically contain a nonfluorescent chromophore that interconverts between two stable forms. Some phytochromes spontaneously form covalent adducts[137–139] with phycoerythrobilin. The absorption and

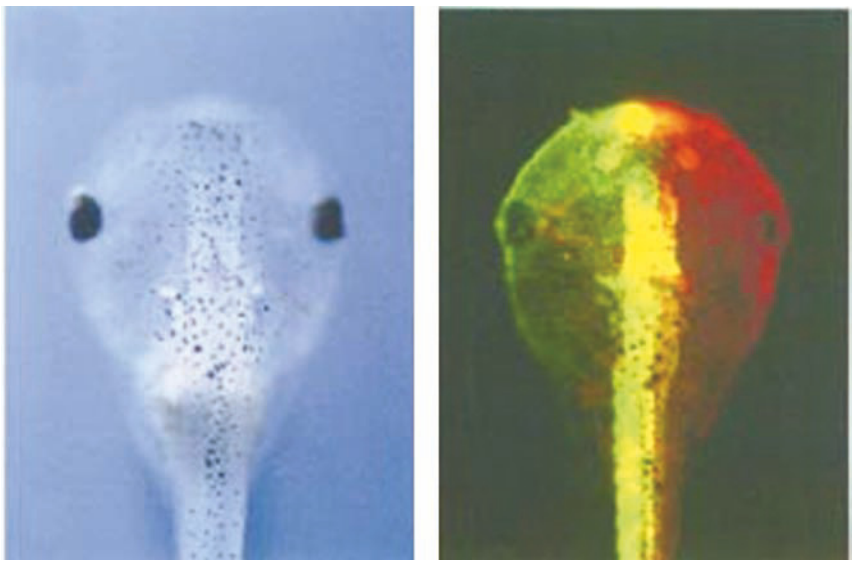

Figure 3.36. Color photograph (right) of a *Xenopus* embryo injected with the mRNA from a green fluorescent coral protein (right side) and a red fluorescent coral protein (left side). The left side shows a white light photograph. Reprinted from [125].

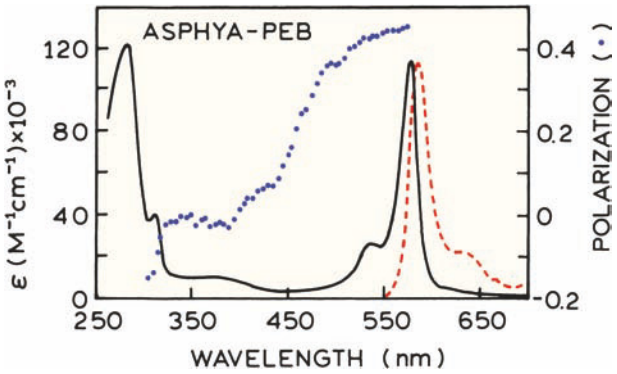

Figure 3.37. Absorption, emission, and excitation polarization spectra of a phytochrome from the plant *Avena sativa*, which contains bound phycoerythrobilin (PEB). Revised and reprinted with permission from [139]. Copyright © 1997, Current Biology Ltd.

emission spectra of one phytofluor protein are shown in Figure 3.37. The spectra are at favorably long wavelengths, and the quantum yield is near 0.70. A favorable property of these proteins is their high anisotropy, which occurs because, in contrast to the phycobiliproteins, there is only a single chromophore.

The phytochrome apoproteins can be expressed as recombinant proteins. Phycoerythrobilin binds spontaneously to these phytochromes and becomes fluorescent. These proteins may become useful as probes for gene expression. In contrast to GFP, it is necessary to add the phycoerythrobilin pigment, which needs to be transported into the cells. A recent report has shown that the genes for the apophytochrome, and the two genes needed to synthesize a nonfluorescent pigment from hemes, can be inserted into *E. coli*.[140] It has been shown recently that the phytochromes themselves can be made highly fluorescent.[141] This was accomplished by a tyrosine-to-histidine mutation. This mutation interferes with the light-sensory function of the phytochrome, but causes the protein with its native chromophore to become fluorescent in solution and when expressed in *E. coli* (Figure 3.38). The fluorescent phytochromes may provide another approach to the synthesis of fluorescent proteins within cells or animals of interest.

3.8.2. Phycobiliproteins

The phycobiliproteins are intensely fluorescent proteins from blue-green and red algae.[142–146] These proteins are contained in phycobilisomes, which harvest light and transfer the energy to chlorophyll. The phycobilisomes absorb strongly from 470 to 650 nm (Figure 3.39), in the gap

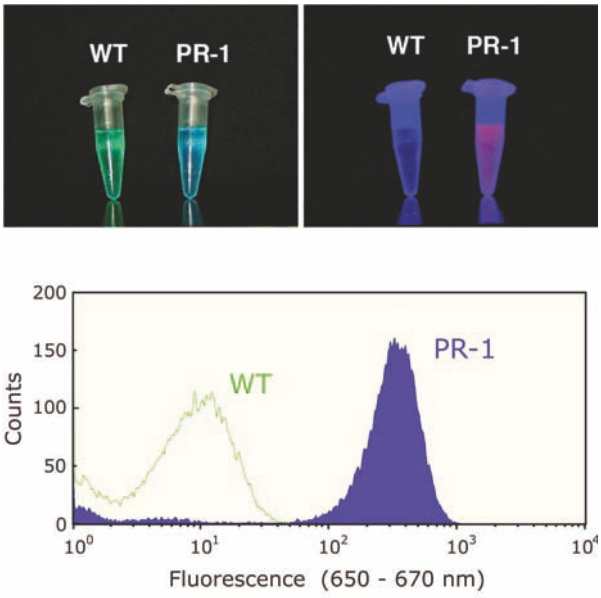

Figure 3.38. White light- and UV-illuminated images of wild type (WT) phytochrome and the fluorescent mutant (PR-1). The lower panel shows flow cytometry data of *E. coli* cells expressing the WT or PR-1 protein. From [141].

between the blue and far-red absorption of chlorophyll. In algae the phycobiliproteins exist as large assemblies that contain phycoerythrin (PE), phycocyanine (PC), and allophycocyanin (APC) (Figure 3.39). Light absorbed by PE is transferred to PC and then to APC, and finally to the photosynthetic reaction center. In intact phycobilisomes the phycobiliproteins are very weakly fluorescent due to efficient energy transfer to photosynthetic reaction centers. However, upon removal from the phycobilisomes the phycobiliproteins become highly fluorescent.

The chromophores in the phycobiliprotein are open-chain tetrapyrol groups called bilins. These chromophores are covalently bound to the phycobiliprotein subunit. Each phycobiliprotein displays different spectral properties, depending on the type of bound bilins. Representative emission spectra are shown in Figure 3.39. Each phycobiliprotein is made up of a number of subunits, with molecular weights ranging from 100,000 to 240,000 daltons (Table 3.2). A remarkable feature of the phycobiliproteins is the high density of chromophores. The 34 bilins in phycoerythrin correspond to a bilin concentration near 80 mM. Also, the large number of chromophores results in high extinction coefficients of 2.4 x 10^6 M^{-1} cm^{-1}, or about 30 times that of fluorescein.

These spectral properties of the bilin groups result in the favorable fluorescence properties of the phycobilipro-

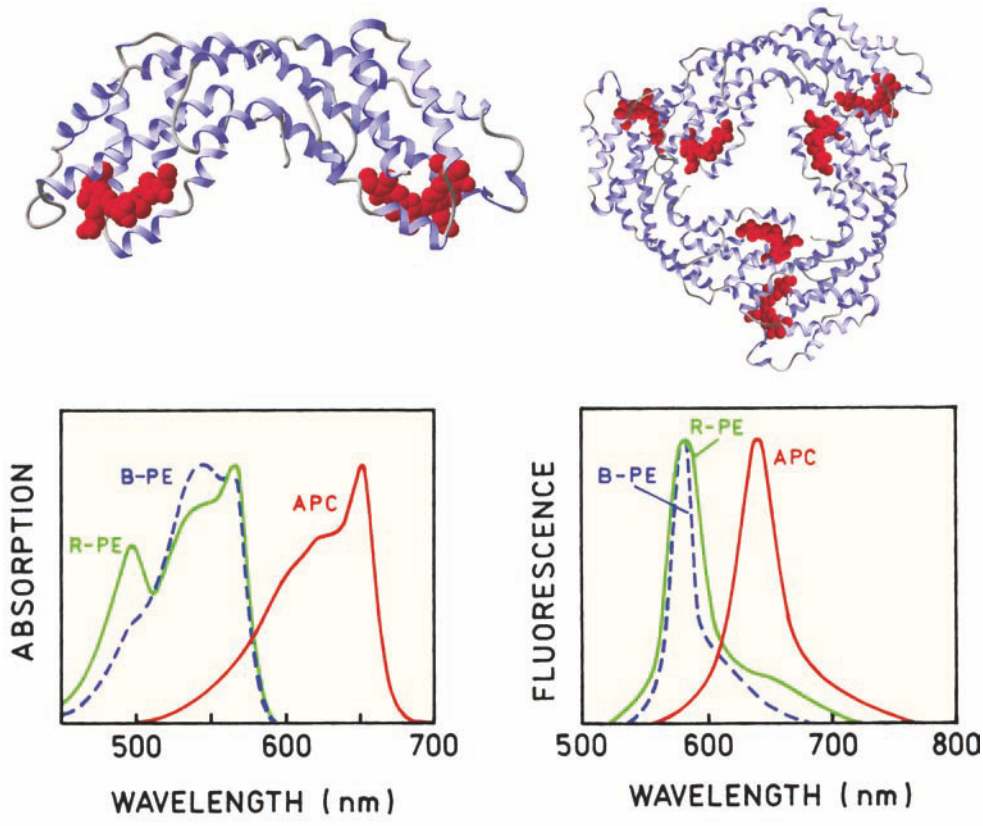

Figure 3.39. Absorption (middle) and fluorescence emission spectra (bottom) of three representative phycobiliproteins. The allophycocyanin is from the filamentous cyanobacterium *Anabaena variabilis*, B-phycoerythrin (B-PE) is from the unicellular red alga *Prophyridium cruentum*, and R-phycoerythrin (R-PE) from the higher red alga *Gastroclonium coulteri*. The structures shown on the top are the αβ monomer of APC and the αβ trimer $(\alpha\beta)_3$. Revised from [142].

teins. They display high quantum yields and are up to 20-fold brighter than fluorescein. They are highly water soluble and stable proteins, which can be stored for long periods of time. They are about 10-fold more photostable than fluorescein.[148] They contain a large number of surface lysine groups and are readily conjugatable to other proteins.[147–149] The long-wavelength absorption and emission make them useful where autofluorescence is a problem. And finally, they display good a Stokes shift. This is not evident from Figure 3.39 unless one realizes that they can be excited at wavelengths below the excitation maxima. Phycobiliproteins have been successfully used for immunoassays,[150–151] for marking of cell-surface antigens in flow cytometry,[152-153] and in single-particle detection.[154]

A minor drawback of the phycobiliproteins is their sensitivity to illumination. This is not due to photobleaching, but to the possibility of exciting more than one chromophore per protein. This results in annihilation of the

Table 3.2. Properties of Some Major Phycobiliproteinsa

Protein	Subunit composition	Approx. mol. wt.	ε(M^{-1} cm^{-})	Total bilins per protein	λ_{ab}^{max} (nm)	λ_{ab}^{max} (nm)	Quantum yield
Allophycocyanin	$(\alpha\beta)_3$	100,000	700,000	6	650	660	0.68
B-Phycoerythrin	$(\alpha\beta)_6\gamma$	240,000	2,400,000	34	543,562	576	0.98
R-Phycoerythrin	$(\alpha\beta)_6\gamma$	240,000	2,200,000	34	495,536,565	576	0.84

[a]From [143] and [144]. C-phycocyanine (620/642 nm) and C-phycoerythrin (562/576 nm) have a subunit structure $(\alpha\beta)_n$, n = 1–6, with molecular weights from 36,500 to 240,000.

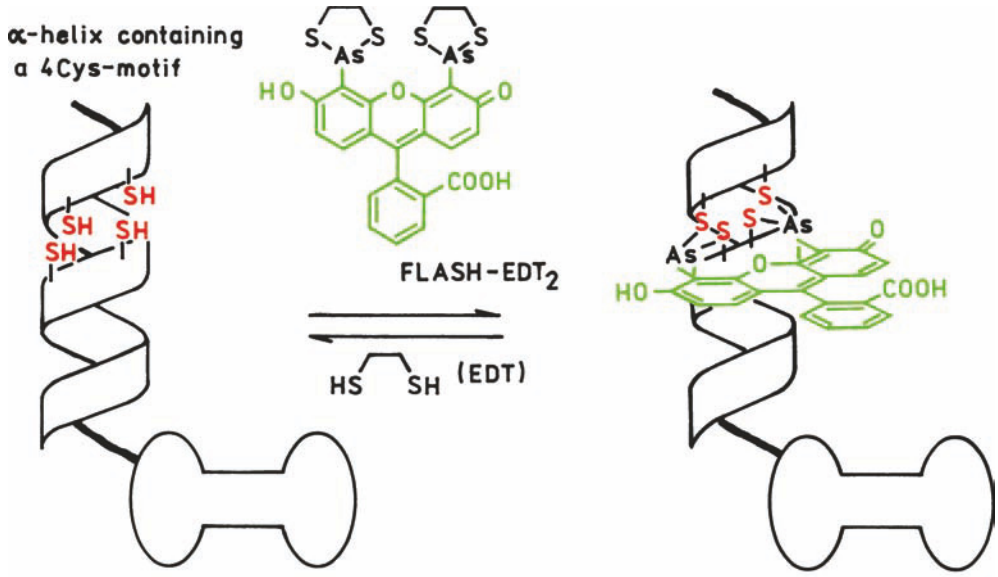

Figure 3.40. Schematic of the reaction of Flash-EDT$_2$ with a recombinant protein containing four cysteine residues at positions i, $i + 1$, $i + 4$, and $i + 5$.

excited state, and a decreased quantum yield and lifetime. The intensity decays are complex, with up to four exponential components ranging from 10 ps to 1.8 ns.[155]

3.8.3. Specific Labeling of Intracellular Proteins

Fusion proteins containing GFP provide a way to specifically label intracellular proteins. GFP is a relatively larger probe and can interfere with the function of the attached protein. A new method for specific labeling of intracellular proteins has recently been reported (Figure 3.40). This method relies on expressing recombinant proteins that contain four cysteine residues in an α-helix.[156–157] These residues are located at positions i, $i + 1$, $i + 4$ and $i + 5$, which positions the SH groups on the same side of the α-helix. This grouping of SH groups does not appear to occur naturally. Proteins containing this motif react specifically with fluorescein analogous containing two trivalent arsenic atoms. Fortunately, the arsenic-containing compounds are not fluorescent, but the reaction product has a quantum yield near 0.49. The reaction can be reversed by addition of excess 1,2-ethanedithiol (EDT). Specific labeling of the intracellular recombinant proteins can be accomplished by exposure of the cells to the bis-arsenic fluorophore, which can passively diffuse into cells.

The fluorophore used for labeling the four-cysteine motif is called Flash-EDT$_2$, meaning fluorescein arsenical helix binder, bis-EDT adduct. This approach is being used in several laboratories[158–159] and is used with other fluorophores to create labeled proteins that are sensitive to their environment.[160–161]

3.9. LONG-LIFETIME PROBES

The probes described above were organic fluorophores with a wide variety of spectral properties, reactivities, and environmental sensitivities. While there are numerous organic fluorophores, almost all display lifetimes from 1 to 10 ns, which limit the dynamic information content of fluorescence. There are several exceptions to the short lifetimes of organic fluorophores. Pyrene displays a lifetime near 400 ns in degassed organic solvents. Pyrene has been derivatized by adding fatty acid chains, which typically results in decay times near 100 ns. In labeled macromolecules the intensity decays of pyrene and its derivatives are usually multi-exponential. Pyrene seems to display photochemical changes.

Another long-lived organic fluorophore is coronene, which displays a lifetime near 200 ns. In membranes the intensity decay of coronene is multi-exponential.[162] Coronene has also been conjugated to lipids.[163] Both pyrene and coronene display low initial anisotropies and are only moderately useful for anisotropy experiments. However, there are two types of organometallic fluorophores which display long lifetimes and other unique features which allow new types of experiments.

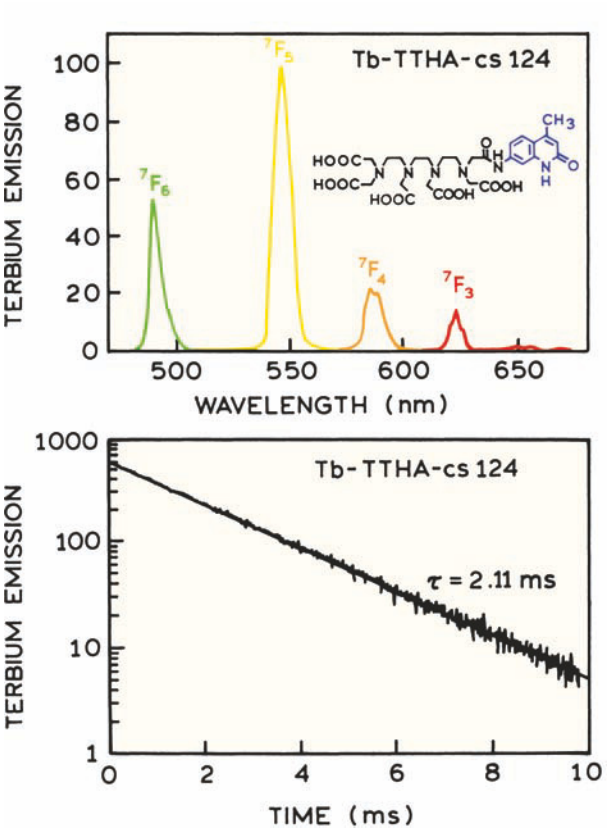

Figure 3.41. Emission spectrum and intensity decay of the lanthanide terbium. Revised and reprinted from [167]. Copyright © 1995, American Chemical Society.

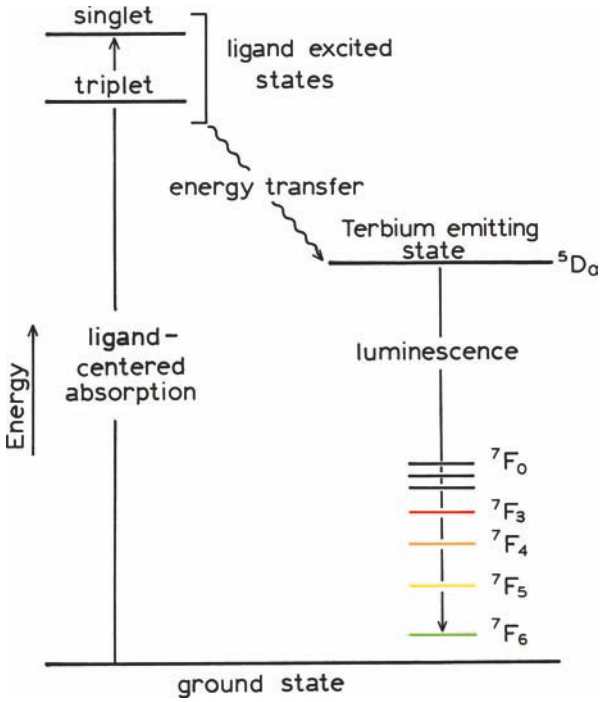

Figure 3.42. Jablonski diagram for excitation of terbium by energy transfer. Modified and redrawn from [165]. Copyright © 1993, with permission from Elsevier Science.

3.9.1. Lanthanides

The lanthanides are uniquely fluorescent metals that display emission in aqueous solution and decay times of 0.5 to 3 ms.[164–167] Emission results from transitions involving 4*f* orbitals, which are forbidden transitions. As a result, the absorption coefficients are very low, less than 10 M^{-1} cm^{-1}, and the emissive rates are slow, resulting in long lifetimes. The lanthanides behave like atoms and display line spectra[164] (Figure 3.41). Because of the weak absorption, lanthanides are usually not directly excited, but rather excited through chelated organic ligands (Figures 3.41 and 3.42). Hence, the excitation spectrum of the complex shown in Figure 3.41 reflects the absorption spectrum of the ligand and not the lanthanide itself.

Lanthanides possess some favorable properties as biochemical probes. They can substitute chemically for calcium in many calcium-dependent proteins.[168–170] A main route of non-radiative decay is via coupling to vibrations of water. For both Eu^{3+} and Tb^{3+} the lifetime in H_2O and D_2O can be used to calculate the number of bound water molecules (n)

$$n = q\left(\frac{1}{\tau_{H_2O}} - \frac{1}{\tau_{D_2O}}\right) \tag{3.1}$$

where q is a constant different for each metal.[165] Hence, the decay times of the lanthanides when bound to proteins can be used to calculate the number of bound water molecules in a calcium binding site. Lanthanides can also be used with proteins that do not have intrinsic binding sites. Reagents have been developed that can be coupled to proteins and chelate lanthanides.[171–172] Because of their sensitivity to water, lanthanide complexes designed as labels generally have most sites occupied by the ligand.

Lanthanides have found widespread use in high sensitivity detection, particularly for immunoassays.[173–174] The basic idea is shown in Figure 3.43. All biological samples display autofluorescence, which is usually the limiting factor in high sensitivity detection. The autofluorescence usually decays on the nanosecond timescale, as do most fluorophores. Because of their long decay times, the lanthanides continue to emit following disappearance of the autofluorescence. The detector is turned on after the excitation flash

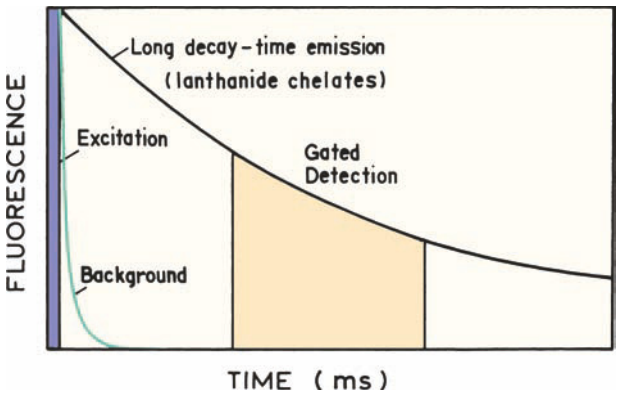

Figure 3.43. Principle of "time-resolved" detection in lanthanide immunoassays. Revised from [174].

to integrate the intensity from the lanthanide. The term "time-resolved" is somewhat of a misnomer, and does not refer to measurement of the decay time. These time-gated immunoassays are essentially steady-state intensity measurements in which the intensity is measured over a period of time following pulsed excitation.

The lanthanides do suffer several limitations. One is the need to chelate the lanthanide in order to obtain significant excitation. The requirement often results in multi-step assays, the last step being addition of the chelator. Another difficulty is the absence of polarized emission, so that the lanthanides cannot be used for anisotropy measurements.

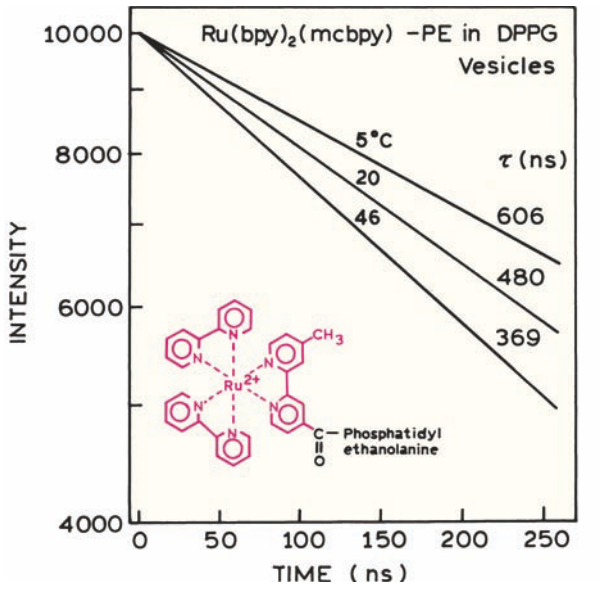

Figure 3.44. Intensity decay of $Ru(bpy)_2(mcbpy)$-PE in DPPG vesicles. Modified From [176].

3.9.2. Transition Metal–Ligand Complexes

Another class of probes with long lifetimes are the transition metal complexes. These are typically complexes of ruthenium (Ru II), rhenium (Re I), or osmium (Os II) with one or more diimine ligands (Figure 3.44). In contrast to the lanthanides, these compounds display molecular fluorescence from a metal-to-ligand charge-transfer state. The transition is partially forbidden, so that the decay times are long. These complexes are highly stable, like covalent bonds, so there is no significant dissociation of the metal and ligands. Transition metal complexes display lifetimes ranging from 10 ns to 10 μs.[175] For example, $Ru(bpy)_2(mcbpy)$ in Figure 3.44 displayed a decay time near 400 ns when conjugated to proteins and lipids.[177] The MLC probes are highly photostable, and display large Stokes shifts, so that probe–probe interactions are not expected. These molecules are known to display polarized emission (Chapter 20) and are thus useful for measurement of dynamic processes on the microsecond timescale.

3.10. PROTEINS AS SENSORS

The use of fluorescence for chemical sensing requires highly specific probes. One approach to obtaining the needed specificity is to rely on proteins that are known to bind the desired analyte. This approach has been used to develop sensing proteins for a variety of analytes.[178–179] One example is a sensor for zinc based on a zinc finger peptide. Zinc fingers are part of transcription factors. These proteins bind zinc with high affinity and specificity, typically to histidine and/or cysteine residues. To make a zinc sensor, the zinc finger amino-acid sequence was modified to contain a covalently linked dansyl group near the middle of the peptide (Figure 3.45).[180] Upon addition of zinc the emission intensity increases, and the emission maxima shift to shorter wavelengths (Figure 3.46). In the absence of zinc the peptide is unfolded, and the dansyl group is exposed to the water. In the presence of zinc the peptide adopts a folded structure that was anticipated to result in shielding the dansyl group from water.

Another example of a protein sensor is shown in Figure 3.47. In this case the protein is calmodulin that was genetically modified to contain a cysteine residue at position 109. This residue was labeled with an environmentally sensitive fluorophore.[181] This labeled protein was sensitive to the antipsychotic drug trifluoperazine. The fluorescence of the coumarin label was almost completely quenched upon addition of this drug.

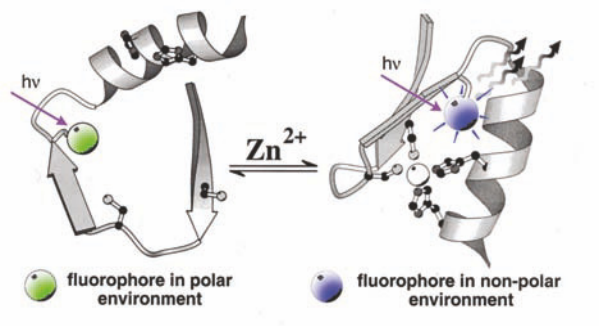

Figure 3.45. A zinc-sensitive peptide based on the dansyl fluorophore. Reprinted with permission from [180]. Copyright © 1996, American Chemical Society.

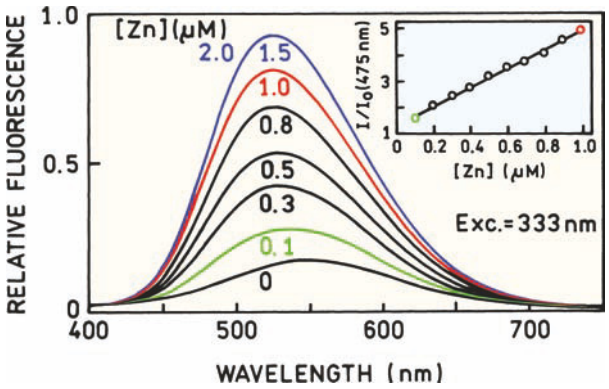

Figure 3.46. Zinc-dependent emission spectra of the dansyl-labeled zinc finger peptide. From [180].

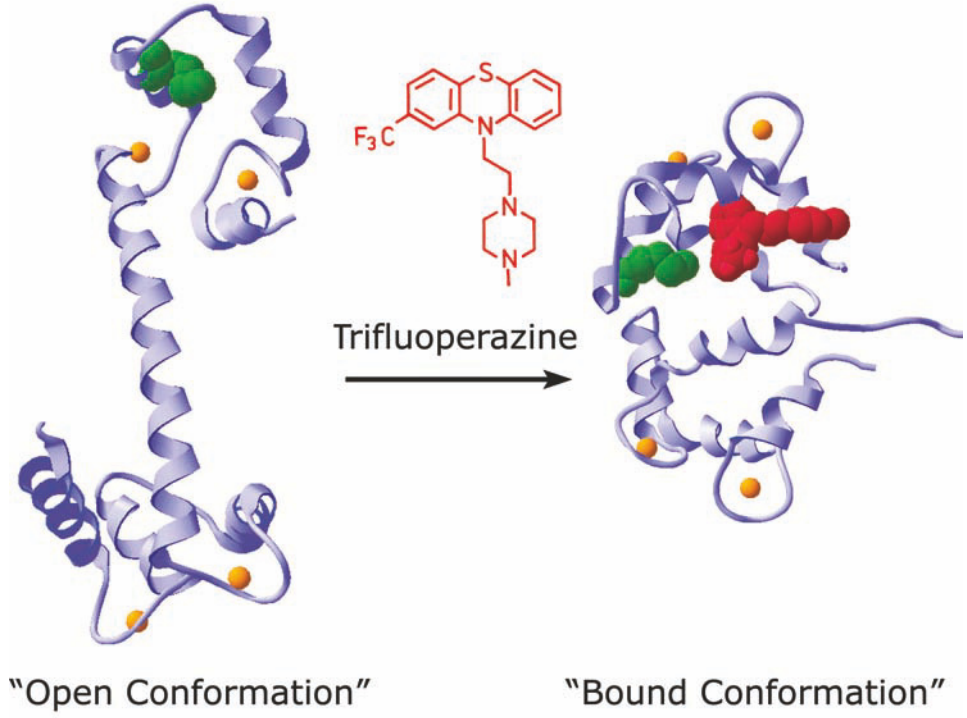

Figure 3.47. Protein sensor for the antipsychotic drug trifluoperazine. The protein is calmodulin labeled with a fluorophore at a genetically inserted cysteine residue at position 109. From [181].

3.11. CONCLUSION

A diversity of molecules display fluorescence, and numerous interactions and processes can alter the spectral properties. Fluorophores can be covalently attached to macromolecules, or designed to interact with specific ions. Emission can occur from the UV to the NIR, and probes are available with short (ns) and long (μs to ms) lifetimes. The technology of probe chemistry is rapidly changing, and new probes are allowing previously impossible experiments to be performed.

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PROBLEMS

P3.1. *Binding of Proteins to Membranes or Nucleic Acids*: Suppose you have a protein that displays tryptophan fluorescence, and you wish to determine if the protein binds to DNA or lipid bilayers. Describe how you could use the tryptophan fluorescence to detect binding. Be specific regarding the spectral observables and expected results, including the use of intrinsic fluorescence, anisotropy and resonance energy transfer.

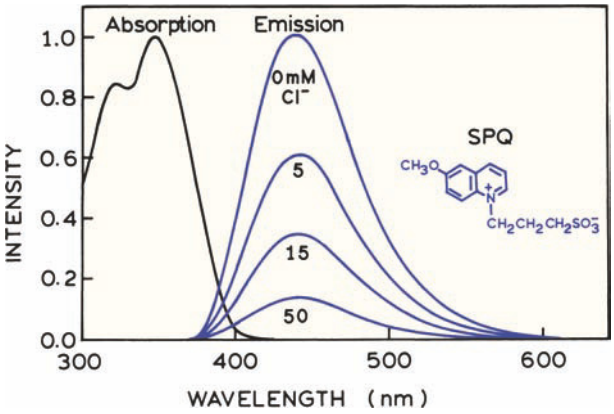

Figure 3.48. Absorption and emission spectra of 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) in water with increasing amounts of chloride. From [182].

P3.2. *Chloride Quenching of SPQ*: Figure 3.48 shows the absorption and emission spectra of the chloride sensitive probe 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) in the presence of increasing amounts of Cl^-. SPQ is collisionally quenched by Cl^-. The unquenched lifetime is 26.3 ns.[182]

A. Use the data in Figure 3.48 to determine the Stern-Volmer quenching constant for chloride.

B. The average concentration of intracellular chloride in blood serum is 103 mM. What is the lifetime and relative intensity of SPQ in blood serum?

C. Suppose the concentration of Cl^- decreases to 75 mM. What is the expected lifetime and relative intensity of SPQ?

D. What factors would complicate interpretation of the SPQ intensities and lifetimes as a measure of Cl^- in blood serum?

P3.3. *Calcium Concentrations Using Calcium Green and Fura-2*: The probes Calcium Green and Fura-2 display spectral changes in the presence of bound Ca^{2+}. Calcium Green shows changes in fluorescence intensity but not in spectra shape. Fura-2 displays a large shift in its absorption spectrum, but little change in the shape of its emission spectrum. Calcium Green and Fura-2 display Ca^{2+} dissociation constants (K_D) near 200 nM.

A. Derive an expression for the fluorescence intensity of Calcium Green relating its intensity to the $[Ca^{2+}]$. For your answer let F_{min} and F_{max} be the fluorescent intensities of Calcium Green in the absence and presence of saturating Ca^{2+}, respectively, and let K_D be the dissociation constant.

B. Derive an expression for the use of Fura-2 as an excitation wavelength-ratiometric probe of $[Ca^{2+}]$. This is a somewhat difficult problem to solve, and the exact form of the answer depends on how one defines the various terms. Let the subscripts 1 and 2 represent the two excitation wavelengths. Let R_{min} and R_{max} be the ratio of intensities of the free (*f*) and calcium-bound (*b*) form of Fura-2.