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-

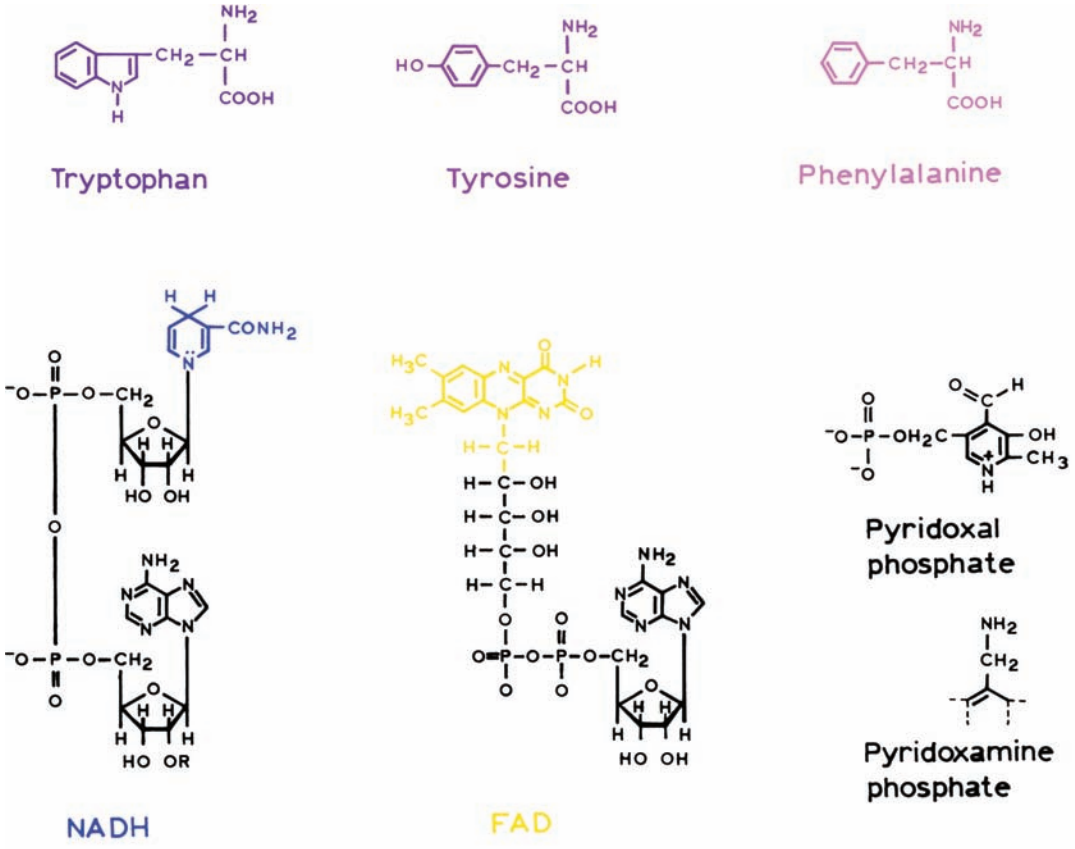

Figure 3.1. Intrinsic biochemical fluorophores. R is a hydrogen in NADH, and a phosphate group in NADPH.

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].