Spectroscopic Methods for the Determination of Protein Interactions

Among the spectroscopic methods available in a standard biochemistry laboratory (e.g., absorption spectroscopy, fluorescence spectroscopy, circular dichroism), fluorescence spectroscopy is often the most suitable for studying the formation of protein complexes. This unit focuses on the use of fluorescence spectroscopy for the quantification of protein-protein interactions. The methods presented are restricted to steady-state fluorescence, since time-resolved fluorescence experiments (which are used to determine the lifetimes of various species) require relatively expensive equipment and rather complicated data analysis techniques.

Although studies of protein-protein interactions have been conducted using absorption spectroscopy or circular dichroism to monitor conformational changes upon interaction, fluorescence measurements offer a much broader range of possibilities and can usually be performed with only small amounts of protein.

The fluorescence of a protein is characterized by its excitation and emission spectra and corresponding emission maximum ($\lambda_{\text{max}}$), as well as by its quantum yield (i.e., the percentage of absorbed photons that result in emitted photons) and anisotropy (i.e., the extent to which fluorescence intensity differs when measured in different directions). These parameters depend on the local environment of the fluorophore and therefore can change upon interaction of the protein with another protein or ligand. As a result, these characteristics can often be used to measure the extent of complex formation involving a given protein (Fig. 20.8.1).

![Figure 20.8.1](image)

**Figure 20.8.1** Changes in fluorescence intensity and emission maximum wavelength ($\lambda_{\text{max}}$) upon complex formation. Compound S (gray line) does not contain tryptophan and thus does not contribute any fluorescence signal between 330 and 360 nm. Addition of compound S to protein A (dashed line), which contains five tryptophan residues, leads to an approximately twofold increase in fluorescence intensity and a shift in the fluorescence maximum from 335 nm to 328 nm upon complex formation (black solid line). AU, arbitrary units.
The source of fluorescence can be an intrinsic fluorophore, such as tryptophan or tyrosine; a covalently or noncovalently attached fluorescent dye (i.e., a label); or a fluorescent binding partner (e.g., a cofactor, such as NADH, or a nucleotide) that binds specifically to the complex under investigation.

This unit provides an overview of fluorescent signals suitable for monitoring protein interactions. The protocols presented describe the determination of the optimal wavelength for a titration experiment (Support Protocol 1), the preparation of necessary controls (Support Protocol 2), and the execution of the actual titration (Basic Protocol 1). In addition, a section on the fluorescent labeling of proteins (Basic Protocol 2) and an introduction to appropriate data analysis are included (Basic Protocol 3). An example of a fluorescence titration experiment and the data analysis that follows is also presented. Many of the topics addressed in this unit can also be applied to other spectroscopic methods.

**STRATEGIC PLANNING**

In the quantification of protein-protein interactions via spectroscopic methods, the aims are to obtain a significant signal change upon binding and to determine conditions under which the system is sufficiently stable to allow the performance of a titration experiment to evaluate binding affinity and stoichiometry. As a guideline, for best results, the signal should change by at least 30% upon binding. If the signal is unstable and decreases with time due to photobleaching or protein aggregation, it is imperative that these decreases not be misinterpreted as being indicative of binding events.

The first option to examine is the use of intrinsic tryptophan (or tyrosine) fluorescence as a monitor for the interaction. If the associated change in signal is sufficiently large, then this option is preferable to external labeling, which requires additional time and may influence the binding properties of the protein. However, if the protein does not contain a suitable intrinsic fluorophore, an extrinsic label must be attached at an appropriate position within the protein. In such cases, it is advantageous to have structural information about the protein in order to determine a suitable position for the label. Extrinsic labels usually have a much higher quantum yield and therefore allow the use of much lower protein concentrations. Nonetheless, the labeling procedure can cause unwanted modification of the protein of interest, thereby affecting its activity or stability. Hence, it is crucial to verify that the activity (e.g., enzymatic activity or ligand-binding behavior) of the labeled protein is similar to that of the wild-type version.

**Quantification of Protein Interactions**

Using spectroscopic techniques, it is possible to quantify protein-protein interactions to obtain two key parameters: the equilibrium binding constant ($K$; dissociation constant $K_d = 1/K$) and the stoichiometry value ($p$) for the binding partners under investigation. In this unit, the following assumption is made about the protein-protein interaction under study: Protein A can bind $p$ copies of compound/protein S with equal affinity. For analysis of more complex binding mechanisms, see APPENDIX 5A.

The following complexes can be formed upon interaction between A and S: AS, AS$_2$, ... AS$_p$. Spectroscopic methods rely on differences between the specific spectroscopic signal associated with one of the reaction partners (A or S) and the signals associated with the expected complexes (AS, AS$_2$, ... AS$_p$). We assume further that the fluorescence of the complex is proportional to the number of molecules of S bound (as would be the case if, for example, protein A was a symmetrical dimer). Thus, the complex AS$_2$ gives rise to twice the signal yielded by AS, and so forth. In order to obtain well-defined binding curves, the aim should be to maximize the fluorescence amplitude ($\Delta F$), which is the difference between the fluorescence of the complex ($F_{as}$) and the sum...
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Figure 20.8.2  Binding curve as inferred from changes in the fluorescence maximum. A simulated binding curve constructed using saturation values (solid circles) is compared with a “binding curve” generated using the observed change in $\lambda_{\text{max}}$ (open circles). Full saturation is normalized to 1. The simulated spectra on which the determination of $\lambda_{\text{max}}$ is based are shown in Figure 20.8.9. The change in $\lambda_{\text{max}}$ due to complex formation is not linear and thus cannot be used to represent the true binding curve.

of the individual fluorescence signals of protein A and compound S ($F_a$ and $F_s$); thus, $\Delta F = F_{\text{as}} - F_a - F_s$.

To determine binding parameters, the change in fluorescence that results from the interaction is monitored while the concentration of one of the reaction partners is varied. The signal employed could be fluorescence intensity, anisotropy, fluorescence resonance energy transfer (FRET), or change in fluorescence intensity as a function of accessibility to a quencher molecule for the complex as compared with the unbound protein. Each type of signal is described below.) It may not be suitable to simply measure the shift in the emission $\lambda_{\text{max}}$ (open circles), since this shift is not necessarily linearly correlated with the amount of complex formed (see Fig. 20.8.2) and thus does not represent the true binding curve (solid circles).

Intrinsic Fluorescence

Tryptophan is the dominant amino acid contributor to the fluorescence spectrum of a protein. The quantum yield and emission maximum wavelength ($\lambda_{\text{max}}$) associated with intrinsic fluorescence are very sensitive to the polarity of the environment (Hutnik et al., 1991; Xia et al., 2003). If tryptophan fluorescence is used as a signal for monitoring an interaction, an excitation wavelength that does not induce significant tyrosine fluorescence (e.g., 295 nm) should be chosen (Fig. 20.8.3). Tyrosine fluorescence is often less sensitive to changes in environment and thus produces a constant background signal upon excitation. Monitoring of emission at a wavelength close to the Raman band (see UNIT 7.7) should be avoided, especially when working with low protein concentrations.

A common problem in the study of protein-protein interactions is that although both proteins contain several tryptophan residues, the fluorescence of only a few residues is affected by binding (Fig. 20.8.4). If only one of the proteins being studied contains
**Figure 20.8.3** Fluorescence emission spectra of 10 μM tryptophan and 10 μM tyrosine at excitation wavelengths ($\lambda_{ex}$) of 280 and 295 nm. AU, arbitrary units.

**Figure 20.8.4** Typical fluorescence titration data for a protein-protein interaction. Protein S was added in stepwise fashion to a solution of protein A (0.9 μM). The relative fluorescence signal ($F_{[S]}/F_{[S]=0}$) is plotted against the concentration of protein S. Binding of protein S quenches the fluorescence of protein A, which results in a decrease in signal. However, the relative change in fluorescence is only 17%, due to the presence of tryptophan residues not affected by the interaction. AU, arbitrary units.
Figure 20.8.5 Effect of the fluorescence of compound S on binding data. Simulated data for the addition of S to a solution originally containing 15 µM protein A ($K_d = 5$ µM, $p = 1$) are shown. Fluorescence is plotted against the concentration of compound S. In the simplest case, the fluorescence signal arises only from protein A (squares). If compound S also contributes to the fluorescence signal (solid circles), a nonhyperbolic total fluorescence curve is observed (open circles). In order to correct for the contribution of S, linear regression of the data obtained in the absence of protein A is performed, and the results of the regression are subtracted from the raw binding data. Abbreviations: AU, arbitrary units; $C_s$, total concentration of compound S.

tryptophan residues, then the signal change may nonetheless be strong enough to allow determination of a binding curve. However, if both proteins contain tryptophan side chains, titration of the second component may lead to a linearly changing background (see Basic Protocol 3 and Fig. 20.8.5). If so, this background must be subtracted correctly to obtain meaningful results.

Recently, the biosynthetic incorporation of irregular or unnatural amino acids, such as azatryptophan, has been reported. (Proteins can contain multiple tryptophan residues, making it difficult to assign changes in tryptophan fluorescence to a particular site. The advantage of using unnatural amino acids is that their fluorescence differs from the intrinsic fluorescence of the components used.) However, this is not a standard procedure and may not be feasible in a standard protein laboratory (Becker et al., 2003; Li et al., 2003).

Extrinsic Fluorescence

Extrinsic labels (fluorescent dyes) can be attached to reactive cysteine residues or amino groups (e.g., those found in lysine residues or at the N termini of proteins). Cysteine residues are preferred for such reactions, because they are generally not very abundant in proteins (as compared with lysine), and labeling can therefore be quite specific. In some cases, excess cysteine residues may have to be removed or specific cysteine residues
introduced using site-directed mutagenesis. It is very important to determine whether these mutations affect the stability, folding, or activity of the protein in question.

If cysteine residues have to be introduced for labeling, there are two possibilities for their positioning. In one scenario, the label can be positioned at the putative interaction interface. This would maximize the probability of environmental change upon complex formation, but at the same time, the dye could also influence binding affinity or even inhibit complex formation. Alternatively, the label could be attached at a position far from the interface. This would be recommended if a conformational change was expected at the labeled site upon complex formation, as such a change would lead to a difference in fluorescence intensity. Another reason for choosing a remote position would be the expectation of a significant change in the rotational diffusion coefficient (see Background Information, “Fluorescence anisotropy”) upon complex formation, as remote labeling would allow the change in anisotropy to be monitored in such a case. A third strategy involves labeling both reaction partners and monitoring energy transfer (FRET; see below and Background Information) as an indicator of complex formation.

It is important to ensure that the labeling procedure yields a homogenous population of labeled protein molecules. If, for example, some protein A molecules contain two labels, the associated \( F_a \) and \( F_{as} \) values might differ from the corresponding values for the singly labeled species, leading to more complicated binding curves.

Since most fluorescent dyes have absorption and emission spectra in the visible region (500 to 600 nm), an unwanted contribution to the signal from the unlabeled protein component (i.e., tryptophan or tyrosine fluorescence) is unlikely, unless prosthetic groups are present. These groups, although often not fluorescent, can lead to signal changes due to their absorption properties (inner filter effect; see UNIT 7.7).

Molecular Probes (http://www.probes.com) offers a broad selection of amino- and cysteine-specific labels and also provides detailed information about the labeling procedure, as well as background information on the dyes offered. Other companies, including Amersham Biosciences (CyDye fluorors, convenient for labeling antibodies, proteins, and oligonucleotides), Sigma-Aldrich (Fluka fluorescent probes), Pierce (fluorescein and rhodamine labeling kits, designed for antibody labeling, but applicable to all proteins), Roche Applied Science, and PerkinElmer, to name a few, also provide fluorescent probes and easy-to-use labeling kits.

**Fluorescence Resonance Energy Transfer (FRET)**

If the fluorescence intensity of (labeled) protein A does not change significantly upon binding to compound S, it may be possible to monitor binding using resonance energy transfer instead. This method does not depend on environmental changes around the fluorophore, but rather on the proximity of a particular pair of fluorophores (or a fluorophore and a chromophore), each attached to one of the two molecules under investigation.

The spectroscopic requirement for FRET is a significant overlap between the emission spectrum of one fluorophore (the donor) and the absorption spectrum of the other (the acceptor; see Background Information). The structural requirement is that the distance between the donor and acceptor be minimal during the interaction. The efficiency of FRET depends inversely on the sixth power of the distance between donor and acceptor, providing a sensitive molecular ruler. The distance at which the energy transfer efficiency is 50% is defined as \( R_0 \) (the Förster radius), the value of which depends on the spectroscopic properties of the pair of fluorophores used (see Background Information). A change in the extent of FRET can be measured either as a decrease in donor fluorescence or as an increase in acceptor fluorescence.
**Intensity or Anisotropy?**

When complex formation leads to a significant shift in the spectrum of the fluorophore or to a large change in the quantum yield, the intensity at a specific emission wavelength (associated with a particular excitation wavelength) serves as a suitable monitor for the quantification of complex formation. The excitation and emission wavelengths should be chosen to maximize the change in signal (see Support Protocol 1). However, if the spectral properties do not change significantly upon complex formation, it may be possible to use anisotropy to monitor the interaction (Bai et al., 1997; LeTilly and Royer, 1993). In this case, the excitation and emission wavelengths should be chosen to minimize the change in signal intensity upon complex formation, because the results have to be corrected for any such changes (see Background Information). One disadvantage associated with the use of anisotropy is that the signal-to-noise ratio is usually rather small.

**Fluorescence Quenching**

Upon binding of a fluorescent protein to another protein or ligand, a decrease in fluorescence intensity due to direct quenching of the fluorophore by the bound protein/ligand can occur (Kelly and von Hippel, 1976). In a standard titration experiment, this quenching would be indistinguishable from a decrease caused by a change in the polarity of the environment. The accessibility of the binding site to external quenchers (e.g., acrylamide) or dyes [e.g., anilinonaphthalenesulfonate (ANS)] can be used as an alternative signal in cases in which fluorescence intensity and anisotropy do not change significantly upon complex formation ([UNIT 7.7](#)). Specifically, titration of protein A with compound S can be performed in the presence of such a quencher or dye. One example of this approach involves the binding of ε-ADP to DNA B helicase (Jezewska and Bujalowski, 1997).

**FLUORESCENCE TITRATION TO MONITOR COMPLEX FORMATION AND DETERMINE BINDING CONSTANTS**

This protocol describes how to perform a titration experiment using any of the signals mentioned in Strategic Planning—namely, fluorescence intensity of a single label, fluorescence intensity due to FRET (also see Background Information), or fluorescence intensity as influenced by the accessibility of the fluorophore to a quencher molecule. In this third case, the starting solution consists of protein A in the presence of a suitable amount of quencher.

The same protocol applies if a change in anisotropy is employed to monitor complex formation (in which case a polarization device has to be set accordingly; see Basic Protocol 1) or if the fluorescence of a third ligand or cofactor that binds specifically to the complex is chosen as the signal to be measured (also see Background Information).

**Choice of concentrations**

It is essential that the concentrations of the proteins in both solutions be known and that no inactive protein fractions be present.

In general, with regard to the protein solution in the reaction cuvette, use of the lowest concentration possible is recommended. Doing so minimizes possible inner filter effects, the amount of material used, and possible problems due to reduced solubility. In addition, if the concentration of the protein in the cuvette is significantly lower than the $K_d$ for binding and the stoichiometry of the interaction is already known, data analysis becomes much simpler (see Basic Protocol 3). However, if the concentration is too low, protein content may be lost through binding to the cuvette walls (see Support Protocol 2). If stoichiometry and affinity values are to be determined from a single titration experiment, the concentration of protein A should be 1 to 3 times the expected $K_d$. 

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**BASIC PROTOCOL 1**

**FLUORESCENCE TITRATION TO MONITOR COMPLEX FORMATION AND DETERMINE BINDING CONSTANTS**

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**Quantitation of Protein Interactions**

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Assuming that no information is known about the affinity of the interacting molecules, start with the following: Use a concentration of \(~0.5\) to \(1\) \(\mu\)M in a volume of \(1\) ml for protein A, and add small volumes (1 to 5 \(\mu\)l) of a stock solution (\(~100\) \(\mu\)M) of compound S until the signal reaches saturation. An example of such a titration and a description of the subsequent data analysis are given in Basic Protocol 4. For the determination of the equilibrium constant, it is essential to obtain sufficient data points in the region between 10% and 90% complex formation. It is advisable to reach nearly complete saturation so that any deviations from the simplest (and therefore anticipated) binding behavior can be detected and the final fluorescence amplitude can be accurately determined. Thus, the titration increments should initially be relatively small and become larger toward the end of the titration.

When preparing the stock solution of component S, two criteria should be taken into consideration. The volume added should be minimized (to minimize dilution of protein component A), but it should be large enough to allow reproducible results. Reproducibility should be confirmed by titrating a corresponding volume of a simple fluorescent compound (e.g., tryptophan). It should also be ensured that the buffer solution has the same viscosity (e.g., the same concentration of glycerol or saccharose, if present) as the titrant. Another option for keeping the concentration of protein A constant is to prepare solution S with the inclusion of protein A at the same concentration as is present in the cuvette. (Since solution S is usually quite concentrated, the fraction of S that is bound to A in this solution can be neglected.) This is a useful (although not very widely used) approach. As an example, in a titration of 1 \(\mu\)M protein A with 5-\(\mu\)l aliquots of a 100 \(\mu\)M solution of compound S, 1 \(\mu\)M protein A would be added to the stock solution of compound S. Then, regardless of the volume of stock solution added to the cuvette, the concentration of protein A in the cuvette would remain constant at 1 \(\mu\)M.

**Materials**

- Filtered and degassed buffer solution for diluting protein samples (see UNIT 7.7)
- Protein “A” and compound “S” solutions, purified and of known concentration
- Calibrated fluorescence spectrometer (see UNIT 7.7)
- Cleaned cuvettes (see UNIT 7.7)
- Stirring devices

**NOTE:** Always make sure that the solution has reached the required temperature before starting the measurement. Extra time must be allowed for temperature equilibration if the protein solution has been on ice before the experiment, as fluorescence is strongly temperature dependent. Samples in plastic cuvettes tend to require more time to reach their final temperature than do samples in glass or quartz cuvettes.

**NOTE:** All of the experiments mentioned in this unit are equilibrium experiments. Thus, it is very important to ensure that samples are at equilibrium (i.e., that the fluorescence reaches a constant value) after every titration step. Depending on the speed of complex formation at the concentrations chosen, it may be necessary to wait for some time (i.e., several minutes) before recording data.

**Prepare spectrometer**

1. Switch on the fluorescence spectrometer and allow it to warm up (see UNIT 7.7).
2. Set excitation and emission wavelengths (Support Protocol 1).

  *If photobleaching occurs, the shutters should be closed during equilibration (see Support Protocol 2).*
Perform titration

3. Fill cuvette with buffer solution and wait for solution to equilibrate at the desired experimental temperature. Add protein A, stir the sample if possible (see Support Protocol 2), and monitor the fluorescence signal (see UNIT 7.7).

   In some fluorescence spectrometers, a magnetic stirring device is already mounted below the cuvette holder. Alternatively, samples can be stirred manually using a small plastic stirrer. If the protein is sensitive to stirring, mixing can be achieved by gently pipetting the solution up and down or by applying a lid to the cuvette and inverting once or twice.

4. Once the fluorescence signal has stabilized, add stepwise aliquots of the stock solution of compound S. Stir solution continuously if possible (see Support Protocol 2). Measure an emission spectrum or acquire a time trace at a single wavelength (preferably for at least ~50 sec, so the stability of the signal can be monitored).

   If a spectrum is obtained after each addition, select a wavelength and read the fluorescence value at this wavelength each time; plot the resulting fluorescence values against the concentration of compound S. This technique permits the investigator to judge whether the titration contains a sufficient number of data points. Saturation can also be estimated from an overlay of the spectra obtained.

   If fluorescence is recorded at a single wavelength, the titration may be monitored continuously (time trace) to measure the signal change throughout the experiment. In this case, the display would resemble the final binding curve. This kind of experiment requires a fluorimeter, in which the shutters close automatically upon opening of the lid. If a continuous time trace is not obtained, a spectrum must be recorded after each titration step, and the fluorescence intensities recorded at the emission wavelength must be plotted against the concentration of compound S.

5. Plot the observed equilibrium fluorescence against the concentration of compound S. Check the plot visually for unusual behavior in the final part of the binding curve (at levels near saturation). If there are any indications of processes such as photobleaching or damage to the protein, perform the control experiments described in Support Protocol 2.

   If photobleaching and other artifacts can be ruled out, the presence of multiple distinctive slopes in the plot may be an indication of nonspecific binding.

6. Analyze data using an appropriate model (see Basic Protocol 3).

INTRODUCTION OF A FLUORESCENT LABEL INTO A PROTEIN AND DETERMINATION OF THE DEGREE OF LABELING

Commonly used fluorescent labels include fluorescein, dansyl, rhodamine, and NBD [N,N'-dimethyl-N-(acetyl)-N'-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]. Molecular Probes offers a selection of new chromophores, including the Alexa dyes (coumarin derivatives), BODIPY, and others, that have very high extinction coefficients and quantum yields. Compared with “older” labels such as fluorescein, these newer compounds are quite photostable and much less sensitive to pH (with activity over a pH range of 4 to 10), and they are available for a large range of excitation and emission wavelengths. A disadvantage, however, is that these novel fluorescent probes contain very complex and bulky aromatic groups, which could disturb the interaction of the labeled protein with its ligand or potentially lead to nonspecific binding due to hydrophobic interactions. It should also be borne in mind that some of these newer compounds are less sensitive to changes in their environment compared with more traditional labeling compounds (e.g., fluorescein).
All of the fluorescent labels mentioned can be attached to cysteine residues (as is typically done with maleimides or iodoacetamides) or amino groups (as is typically done with succinimidyl esters) on the protein of interest.

One easy way to label a protein is to use a commercially available labeling kit. These kits usually contain a premeasured amount of dye, so that only a certain amount of pure protein has to be added to the dye vial. They also include the buffer solutions and columns needed to purify the modified protein (typically gravity-feed size-exclusion columns or prepacked desalting columns).

The following protocol explains how to label a protein with a fluorescent dye without using such a kit. The protocol can also be modified according to the instructions of the label’s supplier (e.g., to incorporate optimal pH and buffer conditions and optimal temperature and time for the labeling reaction).

Materials

- Protein, purified and of known concentration (5 to 20 mg/ml), to be labeled
- Reaction buffer (as recommended by supplier of labeling compound)
- 10 mg/ml fluorescent dye in DMSO or DMF: Use succinimidyl esters (or sulfonyl chlorides) for the modification of amine groups, and use maleimides (or iodoacetamide) for the modification of thiol groups (in cysteine residues)
- Filtered and degassed buffer solution (see UNIT 7.7)
- Dialysis membranes or Slide-A-Lyzer dialysis kits (Pierce)
- PD-10 or NAP-5 columns (Amersham Biosciences)
- Cuvettes (see UNIT 7.7)
- UV-vis spectrometer
- Additional reagents and equipment for dialysis (APPENDIX 3B) or buffer exchange using a desalting column (UNIT 8.3)

CAUTION: Dimethylsulfoxide (DMSO) and dimethylformamide (DMF) should be handled with caution, as they are known to facilitate the entry of organic molecules into tissues. It is strongly recommended that double gloves be used when handling these solvents.

NOTE: The amount of protein used for labeling depends mainly on the availability of the protein. Typically, 5 to 10 mg is used.

NOTE: Some fluorescent dyes are very photosensitive; thus, the labeling procedure should be allowed to take place under minimal light. Ideally, samples should always be kept in the dark or wrapped in aluminum foil and, if possible, kept on ice during and between experiments.

Label ε-amino or thiol groups on target protein

1. Dialyze the protein to be labeled (APPENDIX 3B) using the recommended reaction buffer (e.g., 0.1 M sodium bicarbonate, pH 8.0), or else change buffer using an NAP desalting column (UNIT 8.3).

   Primary amine buffer compounds, such as Tris, should be avoided, especially when using amine-reactive dyes, as amine-containing buffer molecules will compete with the amino groups on the protein.

   The pH of the labeling reaction should be chosen with care. For amine-reactive dyes, a basic pH (8 to 9) should be used if the ε-amino groups of lysine residues are to be labeled, whereas a lower pH (7 to 7.5) should be chosen if the protein is to be specifically labeled at the N terminus. Furthermore, the pH of the buffer solution should not be too close to (i.e., should differ by > 0.5 pH units from) the isoelectric point (pI) of the target protein.
If thiol groups (on cysteine residues) are to be labeled, they must first be reduced. This is usually achieved by adding reducing reagents such as DTT or mercaptoethanol. Unfortunately, these reducing agents tend to react with the dye and thus compete with the labeling reaction; therefore, they should be avoided if possible. A suitable alternative is TCEP [Tris-(2-carboxyethyl)phosphine], which is much more stable than DTT and does not contain thiol groups. Nevertheless, TCEP may also react with certain labeling reagents, such as maleimides; thus, it is useful to check the supplier’s protocol for reaction buffer recommendations prior to labeling. In many cases, the protein can be reduced with DTT or TCEP and then rapidly desalted (e.g., using a small PD10 column) immediately before labeling.

2. Add 100 µl dye solution (1 mg) to 1 ml protein solution in a small vessel (e.g., a 2- to 5-ml plastic tube). Stir continuously for 1 hr using a magnetic stirrer or by gently rocking the tube.

    Depending on the amount of protein used, the incubation time may have to be adjusted. If the protein is not stable at room temperature, the incubation can be performed at 4°C and allowed to continue for a longer period of time.

3. Remove free dye by applying the reaction solution to an equilibrated PD-10 or NAP-5 column and eluting with the appropriate buffer. Collect eluate.

    Ideally, a buffer similar to the one used in fluorescence titration will be used for elution.

    Free dye molecules can be separated from the conjugated protein using these desalting columns. (Typically, 0.5- to 1-ml columns are used.) Note, however, that this procedure does not separate labeled protein from unlabeled protein.

    Depending on the specific protein, samples can usually be stored in a refrigerator for 1 to 2 days between experiments. Samples should be frozen in liquid nitrogen and kept at −20°C for medium-length storage (up to 2 weeks) or at −70°C for long-term storage. Light-sensitive samples should always be wrapped in aluminum foil.

**Determine yield of labeled protein**

4. Using a UV-vis absorption spectrophotometer (UNIT 7.2), measure the absorbance of the solution at 280 nm and at the wavelength at which the dye exhibits dominant absorbance (denoted as λ).

    The concentrations of labeled and unlabeled protein can be determined as long as the label and the unlabeled protein exhibit distinct absorption spectra.

    Typically, the protein will have an aromatic absorbance around 280 nm. The total absorbance should be between 0.1 and 1.0 to ensure accurate readings; dilute protein solution with storage or desalting buffer if necessary to achieve this condition.

5. Calculate the concentrations of dye and protein in the solution.

    Concentrations of dye and protein are calculated differently depending on the spectral characteristics of these compounds. In the following equations, \( \varepsilon_{280}^{dye} \) and \( \varepsilon_{\lambda}^{dye} \) are the extinction coefficients of the dye at 280 nm and \( \lambda \) (the wavelength at which the dye exhibits dominant absorbance), respectively, and \( \varepsilon_{280}^{p} \) and \( \varepsilon_{\lambda}^{p} \) are the extinction coefficients of the protein at 280 nm and \( \lambda \), respectively.

    Case 1: No spectral overlap:

    \[
    [\text{dye}] = \frac{\text{OD}_{\lambda}}{\varepsilon_{\lambda}^{dye}}
    \]

    Equation 20.8.1

    \[
    [\text{protein}] = \frac{\text{OD}_{280}}{\varepsilon_{280}^{p}}
    \]

    Equation 20.8.2
Case 2: Dye absorbs at 280 nm, but protein does not absorb at $\lambda_{\text{dye}}$:

$$[\text{dye}] = \frac{\text{OD}_{\lambda}}{\varepsilon_{\lambda}}$$

Equation 20.8.1

$$[\text{protein}] = \frac{\text{OD}_{280} - c_{\text{dye}}\varepsilon_{280}}{\varepsilon_{280}^p}$$

Equation 20.8.3

Case 3: Dye absorbs at 280 nm, and protein absorbs at $\lambda_{\text{dye}}$:

$$[\text{dye}] = \frac{\text{OD}_{280}}{\varepsilon_{280}^p} - \frac{\text{OD}_{\lambda}}{\varepsilon_{\lambda}^p} = \frac{\varepsilon_{280}^p - c_{\text{dye}}\varepsilon_{280}^p}{\varepsilon_{280}^p - \varepsilon_{\lambda}^p}$$

Equation 20.8.4

$$[\text{protein}] = \frac{\text{OD}_{280} - c_{\text{dye}}\varepsilon_{280}}{\varepsilon_{280}^p}$$

Equation 20.8.3

All calculations assume an optical path length of 1 cm. The ratio of dye concentration to protein concentration equals the yield of labeled protein.

Example: The following absorbances were obtained from a protein labeling reaction mixture (path length, 1 cm): $\text{OD}_{280} = 2.0$ and $\text{OD}_{400} = 2.9$. The extinction coefficients for the protein were 0.039 $\mu$M$^{-1}$cm$^{-1}$ (280 nm) and 0.005 $\mu$M$^{-1}$cm$^{-1}$ (400 nm), and the corresponding extinction coefficients for the dye were 0.010 $\mu$M$^{-1}$cm$^{-1}$ and 0.090 $\mu$M$^{-1}$cm$^{-1}$, respectively. Calculation according to Case 3 above yields the following:

$$C_{\text{dye}} = \frac{2.0 - 2.9}{0.039 - 0.005} = \frac{51 - 580}{0.01 - 0.09} = \frac{30}{3} = 30 \mu$M$$

Equation 20.8.5

$$C_{\text{p}} = \frac{2.0 - 30 \times 0.01}{0.039} = 44 \mu$M$$

Thus, the yield of labeled protein is $30 \mu$M/44 $\mu$M = 68%.

Typical yields for the labeling of proteins vary considerably, from 30% to 100%, depending on the protein and labeling procedure used. Hence, samples can still contain unconjugated protein after the labeling reaction is run. Although this results in a decrease in the total change in fluorescence intensity upon binding, this decrease should not affect the standard titration experiments described here.
ANALYSIS OF TITRATION EXPERIMENTS: DETERMINATION OF BINDING AFFINITY AND STOICHIOMETRY

This protocol describes corrections that may be necessary after a binding curve has been obtained and explains how to determine the binding affinity (K) and stoichiometry value (p) using the data yielded by a titration experiment.

Correct data

The raw data (denoted $F_{obs}$) obtained in the titration experiment (Basic Protocol 1) may deviate from an ideal binding curve for several reasons, including the following: (1) the contribution of compound S to the fluorescence signal (denoted $F_{add}$); (2) the dilution of protein A upon titration with compound S; and (3) bleaching or stirring artifacts. (Support Protocol 1 describes how to test for such artifacts.) Dilution effects can be avoided as described in Basic Protocol 1 or corrected for as described in this protocol. If corrections for both dilution effects and the contribution of compound S to the total fluorescence signal have to be made, then the latter correction must be made first.

If compound S contributes to the fluorescence signal:

If the added compound S contributes to the fluorescence signal, a fraction of the observed fluorescence will change linearly with the concentration of S. An example is shown in Figure 20.8.5.

There are two possible ways to account for this: Either subtract the contribution prior to data analysis or include the contribution of S in the analysis (see Analyze Data below for a description of the latter option). Correction of data requires that the contribution of compound S be measured accurately. If the contribution of compound S is included in the data analysis, the additional measurements described below are not necessary; this may be advantageous if the amount of compound S available is limited.

The first step in the correction process is to perform a titration as in Basic Protocol 1, but with protein A absent from the sample buffer in the cuvette. The recorded fluorescence signal should change linearly with the total concentration of S ($C_s$; see Fig. 20.8.5, black circles). Next, using the fluorescence data obtained, perform a linear regression to determine the fractional fluorescence intensity of compound S ($F_s$)

$$F_{add} = F_s C_s$$

Once $F_s$ has been determined from this equation, a corrected fluorescence reading ($F_{corr}$) for each data point in the titration curve from Basic Protocol 1 can be obtained using the following equation:

$$F_{corr} = F_{obs} - F_s C_s$$

In Figure 20.8.5, linear regression analysis of the fluorescence data obtained by titrating with compound S in the absence of protein A yields a slope of $F_s = 2$ (black circles). Using this slope, the contribution of compound S to the signal observed in Basic Protocol 1 can be calculated (second column, Table 20.8.1). This contribution (denoted $F_{add}$) is then subtracted from $F_{obs}$ (Fig. 20.8.5, open circles) to yield the corrected binding curve (Fig. 20.8.5, open squares).
Table 20.8.1  Corrections for the Contribution of Compound S to the Total Fluorescence Signal$^{a,b}$

<table>
<thead>
<tr>
<th>$\bar{C}_s$</th>
<th>$F_{\text{add}} = 2\bar{C}_s$</th>
<th>$F_{\text{obs}}$</th>
<th>$F_{\text{corr}} = F_{\text{obs}} - F_{\text{add}}$</th>
</tr>
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<tbody>
<tr>
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<td>128</td>
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</table>

$^{a}\bar{C}_s$, total concentration of compound S; $F_{\text{add}}$, contribution of compound S to observed fluorescence signal; $F_{\text{obs}}$, total observed fluorescence signal; $F_{\text{corr}}$, corrected fluorescence signal.

$^{b}$ All fluorescence values in arbitrary units.

If dilution effects need to be corrected for:

As a rule, if the solution containing protein A is diluted by more than 5% during titration, the following correction should be performed:

$$F_{\text{corr}} = F_{\text{obs}} \frac{V_0 + dV}{V_0}$$

Equation 20.8.6

Here, $F_{\text{obs}}$ is the fluorescence observed prior to correction for dilution effects (and after correction for the contribution of compound S, if necessary), $V_0$ is the total volume of protein A solution at the beginning of the experiment, and $dV$ is the total volume of compound S solution added during titration. Examples of data prior to and after correction for dilution effects are shown in Figure 20.8.6.

Analyze data

The data analyses presented in APPENDIX 5A are based on the transformation of data into a form in which the concentration of the complex is plotted against the free ligand concentration. This requires knowledge of the stoichiometry value ($p$, which can be determined via stoichiometric titration, for example; see Fig. A.5A.2) prior to data analysis. For the most simple binding mechanism ($p$ identical binding sites), such a transformation yields a hyperbolic binding curve.
Figure 20.8.6 Quadratic binding curve and dilution effects. Typical experimental data were simulated for a case in which analysis based on a quadratic function was warranted. Black circles represent raw data, and triangles represent the data after correction for dilution. Curves correspond to the fits as discussed in the text. AU, arbitrary units.

If the stoichiometry cannot be determined separately, it is still possible to obtain at least the binding constant \( K \) using the experimental data, and under specific conditions, \( p \) can be determined as well. Here, two cases must be distinguished from one another:

**Hyperbolic binding curve**

At concentrations of protein A that are much smaller than \( K_d \), the concentration of complex present after each addition of compound S is always much smaller than the total concentration of S (see example in Basic Protocol 4). If \([A] < 0.1 K_d\), then at half-saturation, the total and free concentrations of compound S differ by \(<5\%\). Thus, the concentration of free compound S \( (\overline{C_s}) \) can safely be approximated as the total concentration of S \( (\overline{C_s}) \). In this case, no information about stoichiometry can be obtained, but the binding constant can be determined. The corresponding binding curve is hyperbolic and can be described by the following equation:

\[
F = \overline{C_a} F_a + \Delta F \overline{C_s} = \overline{C_a} F_a + \Delta F \frac{\overline{C_s}}{K_d + \overline{C_s}} p\overline{C_a}
\]

\[
\approx \overline{C_a} F_a + \Delta F \frac{\overline{C_s}}{K_d + \overline{C_s}} p\overline{C_a}
\]

**Equation 20.8.7**

where \( F_a \) is the fluorescence of the free protein A, \( F_{as} \) is the fluorescence of the complex AS, \( \overline{C_a} \) is the total concentration of protein A, and \( \Delta F = F_{as} - F_a \).

The data in these curves can either be linearized (see APPENDIX 5A) or, preferably, analyzed with nonlinear fitting routines to determine \( K_d \) and \( p\Delta F\overline{C_a} \).
**Quadratic (or square root) binding curve**

If the concentration of protein A is \( \geq 0.2 K_d \), the concentration of free compound S present at each titration step is reduced significantly upon complex formation. In this case, mass balance must be used to obtain the relevant function for the concentration of complex formed:

\[
\bar{C}_{as} = \frac{pC_a C_s}{K_d} = \frac{(p\bar{C}_a - C_{as})(\bar{C}_s - C_{as})}{K_d}
\]

Equation 20.8.8

This yields a quadratic equation for \( C_{as} \), which denotes the concentration of occupied binding sites on protein A:

\[
C_{as}^2 - (\bar{C}_s + K_d + p\bar{C}_a)C_{as} + p\bar{C}_a\bar{C}_s = 0
\]

The value of \( C_{as} \) that satisfies this quadratic equation is a square root function involving \( \bar{C}_s \) and \( \bar{C}_a \) (the total concentrations of compound S and protein A, respectively):

\[
C_{as} = \frac{1}{2} \left[ g - \sqrt{g^2 - h} \right]
\]

Equation 20.8.9

where \( g = \bar{C}_s + K_d + p\bar{C}_a \) and \( h = 4p\bar{C}_a\bar{C}_s \)

Thus, the total fluorescence signal observed is given by:

\[
F = F_a \times \bar{C}_a + \Delta F \times C_{as} = F_a \times \bar{C}_a + \Delta F \times \frac{1}{2} \left[ g - \sqrt{g^2 - h} \right]
\]

Equation 20.8.10

where \( \Delta F = F_{as} - F_a \).

The determination of \( p \) via this method, although less exact than direct measurement in a stoichiometric experiment, might sometimes be the only possibility (e.g., when the reactants have limited solubility). In order to obtain a reliable estimate of the stoichiometry value \( p \), the concentration of protein A should be 1 to 3 times \( K_d \) (estimated value).

**Inclusion of signal arising from compound S**

If compound S, which is used to titrate the fluorescent protein A, also contributes to the fluorescence signal, this contribution has to be taken into account. This can be done either by performing a separate titration of compound S in the absence of protein A (see above) or by explicitly including the contribution of compound S in the data analysis. In the latter case, the observed fluorescence is given by:

\[
F = \bar{C}_a F_a + \Delta F C_{as} + \bar{C}_s F_s
\]

where \( \Delta F = F_{as} - F_a - F_s \).

Then, the parameters in the equation (either quadratic or hyperbolic) describing \( C_{as} \), as well as the slope \( F_s \), are to be optimized with regard to fitting of the experimental data.
Note that when a nonfluorescent compound S binds nonspecifically to protein A and alters the fluorescence of the latter, the binding curve that results can resemble the curve that arises when fluorescent compound S contributes directly to the observed signal. However, if compound S is not fluorescent, then a titration involving compound S in the absence of protein A will yield no significant fluorescence signal. For the analysis of data in the event of nonspecific binding, refer to APPENDIX 5A.

EXAMPLE: STANDARD PROTEIN-PROTEIN TITRATION WITH DATA ANALYSIS

As an example, the results of a hypothetical titration of 1 µM protein A with a 100 µM stock solution of compound S are presented in Figure 20.8.6. A 1-ml volume of buffer solution containing 1 µM protein A, which carries the fluorophore, is dispensed into the fluorimeter cell, and the fluorescence signal is recorded at a constant wavelength. Aliquots of the compound S solution (Table 20.8.2) are subsequently added to the protein A solution in the cell, with the fluorescence signal being monitored at this same wavelength after each addition.

The binding curve corresponding to this sample experiment is depicted in Figure 20.8.6. A first estimation of $K_d$ can be obtained as follows: 50% of protein A is complexed with compound S at a total concentration of $\sim 1$ µM compound S. At this point on the curve, the concentration of free S is about 0.5 µM (assuming that $p = 1$), and therefore, $K_d$ is estimated to be approximately 0.5 µM as well. This estimate suggests that the binding data obtained should be analyzed using a quadratic model, since the concentration of free S differs from the total concentration of S. Note that this difference would be even larger if the stoichiometry value $p$ was greater than 1. Analysis of the data shown in Figure 20.8.6 using a quadratic model (Eq. 20.8.10) yields a $K_d$ of 0.51 µM and a stoichiometry value ($p$) of 0.96.

<table>
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<tr>
<th>Point no.</th>
<th>$dV$ (µl)</th>
<th>[S] (µM)</th>
<th>[A] (µM)</th>
<th>$F_{obs}$</th>
<th>$F_{corr} = F_{obs} \times (1000 + dV)/1000$</th>
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</table>

*a$dV$, volume of 100 µM compound S solution added; $F$, fluorescence signal; $F_{obs}$, total observed fluorescence signal; $F_{corr}$, corrected fluorescence signal.*

*bAll fluorescence values in arbitrary units.*
Spectroscopic Methods for the Study of Protein Interactions

Figure 20.8.7 Hyperbolic binding curve and dilution effects. Typical experimental data were simulated for a case in which analysis based on a hyperbolic function was warranted ([A] < 0.1K_d). Black circles represent raw data, and triangles represent the data after correction for dilution. AU, arbitrary units.

Figure 20.8.7 shows data from a simulated experiment in which 0.01 μM protein A is titrated with 100 μM compound S under otherwise identical conditions. Here, when the total concentration of compound S is ~0.5 μM, about 50% of protein A is complexed with S. Thus, the concentration of free S at this point is 0.5 μM - (50% × 0.01 μM) = 0.495 μM, assuming that p = 1. In this case, the concentration of free S can safely be approximated as the total concentration of S, and the binding curve should be analyzed using the hyperbolic model (Eq. 20.8.7).

SUPPORT PROTOCOL 1

DETERMINATION OF OPTIMAL EMISSION AND EXCITATION WAVELENGTHS FOR FLUORESCENCE TITRATION EXPERIMENTS

In order to obtain a suitable binding curve for the quantification of a protein-ligand interaction, a sufficiently large signal change upon complex formation is required. The approximate excitation and emission maxima for the protein of interest are usually known. (If an extrinsic label is used, this information should be available from the supplier of the label.) The strategy presented here applies to the use of changes in fluorescence intensity as a monitor for complex formation.

Materials

Filtered and degassed buffer solution for diluting protein samples (see UNIT 7.7)
Protein “A” and compound “S” solutions, purified and of known concentration
Calibrated fluorescence spectrometer (see UNIT 7.7)
Cleaned cuvettes (see UNIT 7.7)
1. Switch on the fluorescence spectrometer and allow it to warm up (UNIT 7.7).

2. Set the excitation wavelength as suggested by the dye manufacturer, or use 280 or 295 nm if tryptophan fluorescence is to be measured. Fill cuvette with buffer solution, add protein A, stir the sample if possible, and obtain an emission spectrum.

   Make certain that the buffer solution has equilibrated to the required temperature before performing this step.

3. Identify the wavelength corresponding to maximum emission in the emission spectrum obtained in step 2. Set the emission wavelength of the fluorescence spectrometer to this value, and acquire an excitation spectrum for protein A alone.

   The excitation spectrum must be corrected for the lamp spectrum (as the lamp monochromator intensity varies as a function of wavelength). Lamp spectra for such corrections are generally obtained using rhodamine B. Usually, lamp spectrum corrections are made by the supplier of the instrument prior to shipping, and the supplier also typically provides rhodamine B as a calibration standard for the investigator.

4. Repeat steps 2 and 3 using a solution of protein A saturated with compound S.

   The concentration of compound S necessary for saturation must be determined experimentally.

5. Repeat steps 2 and 3 for a solution containing only compound S at the saturating concentration, and also for buffer alone.

6. Choose the pair of excitation and emission wavelengths that yields the maximum difference between fluorescence signal background (i.e., signal attributable to buffer plus signal attributable to protein A and compound S individually) and signal attributable to the complex AS.

---

**DETERMINATION OF THE STABILITY AND PROPENSITY FOR PHOTOBLEACHING OF A FLUORESCENT PROTEIN**

In a typical titration experiment, compound S is titrated into a solution of protein A. Thus, because the same protein A sample is used throughout the course of the experiment, it is important to make sure that extended exposure to light and/or mechanical stress due to stirring do not induce alterations in the protein or protein-ligand complex.

**Materials**

- Filtered and degassed buffer solution for diluting protein samples (see UNIT 7.7)
- Protein “A” and compound “S” solutions, purified and of known concentration
- Calibrated fluorescence spectrometer (see UNIT 7.7)
- Cleaned cuvettes (see UNIT 7.7)

**NOTE:** In the protocol that follows, it is assumed that protein A contains the fluorescent label.

**Prepare spectrometer**

1. Switch on the fluorescence spectrometer and allow it to warm up (UNIT 7.7).

2. Set the excitation and emission wavelengths as determined using Support Protocol 1.
Perform control assessments of protein A

3. Check the stability of the protein A solution by filling the cuvette with this solution under the same conditions as in the titration experiment (see Basic Protocol 1, step 3) and observing the fluorescence signal continuously for a period of time corresponding to the duration of the titration experiment (at least 30 min).

   Make certain that the solution has equilibrated to the required temperature before making fluorescence readings.

   A decrease in the fluorescence signal over time could indicate photobleaching, loss of protein content due to attachment of protein molecules to the cuvette walls, or mechanical damage to the protein as a result of stirring.

4. Check for photobleaching by filling the cuvette with a fresh aliquot of protein A solution and observing the fluorescence signal at intermittent time points (by briefly opening and then closing the shutter on the fluorimeter) over a period of time corresponding to the duration of the titration experiment.

   In total, ~10 to 20 fluorescence signal readings should be obtained in this step.

   If the fluorescence signal decreases to the same extent as in step 3, then photobleaching can be ruled out.

   Photobleaching can be reduced by decreasing the excitation bandwidth or changing the excitation wavelength to one at which the intensity of the lamp is lower. The absolute signal will be reduced by these procedures, but such reductions can be partially compensated for by increasing the emission slit width (particularly when using a monochromator).

5. Check for mechanical damage and loss of protein content due to protein–cuvette wall interactions by repeating step 3 using a new aliquot of protein A solution. If the fluorescence signal decreases over time, compare the rate of decrease with and without stirring the solution.

   Photobleaching can sometimes be distinguished from mechanical damage/loss of protein content by comparing the fluorescence decreases observed with and without stirring. Switching off the stirrer should make the decrease in fluorescence less severe in the case of mechanical damage/loss of protein content due to protein–cuvette wall interactions, whereas it should make the decrease in fluorescence more severe in the case of photobleaching.

6. Repeat steps 3 through 5 using different concentrations of protein A solution.

   Mechanical damage usually results in a concentration-independent change in fluorescence, whereas the fraction of protein content lost to protein–cuvette wall interactions generally decreases with increasing protein concentration.

   If it is found that protein binding to cuvette walls is causing appreciable signal instability, it might be necessary to change buffer conditions, coat the cuvette walls with detergent (e.g., 0.05% Tween 20), or use higher concentrations of protein A (so that the relative loss of protein content becomes smaller). Another option is to add 5% to 10% (v/v) glycerol to the protein dilution buffer (step 3) to stabilize the protein. If detergents are used, their fluorescence profiles should be checked beforehand.

Perform control assessments of the AS complex

7. Perform steps 3 to 6 using samples that contain the protein complex AS.

   The concentration of complex should ideally be close to half-saturation to produce maximal effects due to changes in fluorescence resulting from adsorption, bleaching or degradation of A, S or AS.
COMMENTARY

Background Information

For a detailed introduction to tryptophan and tyrosine fluorescence spectroscopy, including quenching and fluorescence anisotropy, see UNIT 7.7.

This section provides a discussion on the use of extrinsic probes. Changing the environment of a probe can affect the intensity of the fluorescence signal, the spectral shape, or both. For most labels used in protein interaction assays, a blue shift in the emission maximum occurs when the label is transferred from a hydrophilic to a more hydrophobic environment.

In general, the spectroscopic probe should be located close to the region where the interaction partner is expected to bind (see Fig. 20.8.8). In such cases, the spectral change observed upon addition of compound S could look like the one depicted in Figure 20.8.9. Note the appearance of an isosbestic point, where the same fluorescence intensity is seen in both spectra. When spectra are overlaid...

Figure 20.8.8  Schematic representation of a protein-protein interaction. Protein S binds to protein A, which is fluorescently labeled. Upon binding, the spectral properties and intensities of the emitted fluorescence change.

Figure 20.8.9  Simulated fluorescence spectra as might be observed in a titration experiment (fluorescence in arbitrary units, AU). Note the isosbestic point, where both spectra show the same fluorescence intensity. The existence of an isosbestic point indicates that only two interconverting fluorescent species (protein and complex) are present.
with one another, the appearance of an isosbestic point indicates that only two fluorescent species exist in the system being studied. If binding of the partner S induces a conformational change in protein A, a fluorescent position far from the binding site might also be affected, and the resulting change in signal could be used to monitor complex formation (Hiratsuka, 1998). Another option for avoiding labeling near the protein-ligand interface is to employ FRET (UNIT 19.5).

**Fluorescence resonance energy transfer (FRET)**

Fluorescence resonance energy transfer describes the nonradiative transfer of excited-state energy from a donor (D) to an acceptor (A). This process occurs as the result of a dipole-dipole coupling between the donor and the acceptor. The efficiency of energy transfer ($E$) is given by:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

**Equation 20.8.11**

Here, $R_0$, the so-called Förster radius, is characteristic of the specific donor-acceptor pair, and $r$ is the actual distance between the acceptor fluorophore on protein A and the donor fluorophore on compound S. At $r = R_0$, an energy transfer efficiency of 50% is obtained. The value of $R_0$ depends on the overlap of the emission spectrum of the donor with the excitation spectrum of the acceptor (see Fig. 20.8.10) and on the relative orientation ($\kappa$) of the transition dipole moments of the donor and the acceptor. The latter feature is often not known or not constant during the lifetime of the donor, and therefore, it is usually approximated by $\kappa^2 = 2/3$, which corresponds to a situation in which the orientation is randomized by rotational diffusion prior to energy transfer. Fortunately, variations in the actual value of $\kappa^2$ do not appear to result in major errors in the distances calculated using FRET data (Lakowitz, 1999). Furthermore, when energy transfer is simply used as a signal to quantify the amount of complex formed, knowledge of the exact value of $R_0$ is not necessary.

The pronounced dependence of the efficiency of FRET on the distance between the two interacting species ($1/r^6$) makes this method especially suitable for use as a molecular ruler. For example, FRET can be used to monitor the association of two proteins based on changes in the distance between them, as shown in Figure 20.8.11.
Figure 20.8.11  Monitoring of complex formation using FRET. A schematic representation of the use of FRET to monitor the formation of a complex between protein A and compound S is shown. (A) Assume that compound S is labeled with a donor molecule (grey) and that protein A is labeled with an acceptor molecule (black). (B) In a solution in which both S and A are present but do not interact, irradiation with a wavelength corresponding to the excitation maximum for the donor leads to fluorescence of the donor, but to much less (if any) fluorescence of the acceptor (depending on its excitation spectrum). (C) However, when a complex between S and A is formed, such that the distance between acceptor and donor becomes small enough to allow FRET, the fluorescence of the donor decreases and the fluorescence of the acceptor increases. AU, arbitrary units.
Examples of the use of FRET in the quantification of protein complex formation (either with another protein or with a small ligand) can be found in Cornea and Conn (2002), Johnson et al. (1993), and Wagner et al. (1995). The advantage of this method is that the fluorophores do not have to be located at the interaction interface, thus minimizing the potential for fluorescent labels to interfere with complex formation. The labels just have to be close enough (typically ≤ R₀) in the final complex to allow significant energy transfer. Förster distances (R₀) for typical donor-acceptor pairs range from 20 to 50 Å.

The obvious disadvantages of FRET are that both compounds have to be labeled and that controls for unwanted effects of the labeling procedure have to be performed.

**Fluorescence anisotropy**

A detailed introduction to fluorescence depolarization is given in *UNIT 7.7*. By irradiating the sample with polarized light, molecules whose transition dipole moments are oriented parallel to the plane of polarization are preferentially excited. If the axes of the fluorophores maintain their orientation during this process, the emitted fluorescent light will also be polarized. Relative to this situation, the extent of polarization will decrease if the fluorophores exhibit rotational diffusion during the lifetimes of their excited states. In analogy to the translational diffusion coefficient, the rotational diffusion coefficient is a measure of how rapidly the rotation of a particular protein molecule occurs. This rotation leads to a change in the orientation of the emission transition dipole moment relative to the orientation of the excitation dipole moment. For small fluorescent compounds, the rotational diffusion coefficient is large enough to produce almost complete depolarization. The rotational diffusion coefficient of a fluorescent label will be reduced by attachment to protein A, which increases the mass of the rotating species. Upon binding of compound S to labeled protein A, the rotational diffusion coefficient can be further reduced. In order to characterize the degree to which the emitted fluorescence is polarized, two quantities are commonly used: polarization (P) and anisotropy (r) (see *UNIT 7.7*). In the quantitation of protein complexes, the use of anisotropy is preferred, because it yields simpler equations.

Anisotropy is defined as:

\[
r = \frac{I_{\text{par}} - I_{\text{perp}}}{I_{\text{par}} + 2I_{\text{perp}}}
\]

*Equation 20.8.12*

where \(I_{\text{par}}\) is the intensity of emission parallel to the excitation polarization and \(I_{\text{perp}}\) is the intensity of emission perpendicular to the excitation polarization.

Important with regard to the effect of rotational diffusion on depolarization is the ratio of the lifetime of the fluorophore (\(r\)) to the rotational diffusion time (\(\theta\)). According to the Perrin equation, the anisotropy observed in the presence of rotational diffusion (\(r\)) is related to the anisotropy observed in the absence of rotational diffusion (\(r_0\)) as follows:

\[
\frac{r_0}{r} = \frac{1}{1 + 6D\tau}
\]

*Equation 20.8.13*

where \(D\) is the rotational diffusion coefficient. The anisotropy value can be used to determine the fraction of complexes formed, since it is related linearly to the fractional fluorescence intensity:

\[
r_{\text{obs}} = F_ar_a + F_{\text{as}}r_{\text{as}}
\]

where \(F_a\) and \(F_{\text{as}}\) are the fractional fluorescence values for the labeled protein and the complex, respectively, and \(r_a\) and \(r_{\text{as}}\) represent the anisotropy of the labeled protein and the complex, respectively. Thus, when using anisotropy to quantify complex formation, it is desirable to work at an emission wavelength at which the fluorescence intensity remains unchanged upon complex formation. By doing so, the equation above simplifies to:

\[
r_{\text{obs}} = f_ar_a + f_{\text{as}}r_{\text{as}} = r_a + \Delta rf_{\text{as}}
\]

where \(f_{\text{as}}\) represents the fraction of binding sites that are saturated, \(f_a\) represents the fraction of protein A molecules that are unbound \((f_a = 1 - f_{\text{as}})\), and \(\Delta r = r_{\text{as}} - r_a\). An example of the use of changes in tryptophan fluorescence anisotropy to monitor protein-protein interactions can be found in Lukas et al. (1986).

**Indirect quantification using a labeled ligand**

When the fluorescence of the system of interest does not change upon interaction and labeling of the interacting compounds is not feasible or successful, another approach, which involves the fluorescence of a third compound (usually a cofactor or fluorescent nucleotide), may be appropriate.

This fluorescent ligand (referred to as “L” here) must bind specifically to the complex under investigation and must not interfere with the interaction between protein A and compound S.
When such a ligand is used, the following binding reactions must be considered:

\[ A + S \xrightleftharpoons[K_L]{K_S} AS \]

\[ AS + L \xrightleftharpoons[K_L]{K_S} ASL \]

where L contains a label whose spectroscopic properties change significantly upon binding to the complex AS.

Furthermore, it is assumed that L is present in large excess relative to the complex \([L] > 20K_L\). As a result, \([ASL] = K_L[L][AS] \approx K_L[L]_{tot}[AS] = K_L[L]_{tot}K[A][S] = K_{obs}[A][S]\).

By observing the production of ASL at various concentrations of S (with the concentration of A fixed), the apparent binding constant \(K_{obs}\) can be determined. Then, if \(K_L\) and \([L]_{tot}\) are known, the binding constant \(K_d = 1/K_L\) can be calculated as well.

Discussion of a similar approach, in which the product yielded by enzymatic activity interacts with a fluorescent component, can be found in Solomon and Stansbie (1984).

**Critical Parameters**

- To avoid nonspecific interactions, the protein and ligand should be as pure as possible.

**Temperature**

For fluorescence spectroscopy it is particularly important to maintain the chosen temperature since the fluorescence signal is temperature dependent (even if the interaction is not).

The protein and ligand of interest should be sufficiently stable under the conditions chosen for the experiment, so that measurement of a complete titration curve is possible. Make sure that all solutions are at the required temperature and are in equilibrium before making experimental measurements. This is especially important if some of the solutions are kept on ice.

**Buffers**

The buffer solutions used in titration experiments have to meet two criteria: (1) they should create a stable environment for the protein and ligand under investigation, and (2) they should not interfere with the spectroscopic technique. Absorption and fluorescence spectra of the buffer solution over the relevant spectral range should be obtained so that any interference (inner filter effect or background signal) can be accounted for.

In particular, the following buffer solutions may create problems:

1. Imidazole is not a suitable buffer for absorption or fluorescence spectroscopy involving tryptophan fluorescence since it absorbs at the same wavelength as the fluorophore in this case.

2. Oxidized dithiothreitol absorbs light over a similar range of wavelengths as does tryptophan and should therefore be removed by dialysis; otherwise, reduced DTT should be added immediately before starting the experiment.

In general, when fluorescence signals are low, the buffer fluorescence should always be subtracted. Buffers kept in plastic containers tend to contain small amounts of fluorescing species leached from the container material, with these species usually emitting in the region of tryptophan fluorescence.

For all solutions, great care should be taken to ensure that no light-scattering particles are present. Thus, buffer solutions should be filtered, and it is recommended that all aspects of these experiments be kept as dust-free as possible.

**Troubleshooting**

**Spectral/kinetic data show sharp “spikes”**

This problem is typically due to light scattering from large particles (e.g., dust, aggregated protein). If spikes are observed, clarify the solutions (UNIT 7.7).

**Fluorescence decreases over time**

One possibility is that the solutions are not thermally equilibrated. This can be remedied by incubating solutions for a longer time at the required temperature before use.

It is also possible that one or both of the reagents involved in the interaction are binding to the walls of the cuvette. Try using higher concentrations of the interacting molecules, or add 5% to 10% (v/v) glycerol to stabilize the protein.

A third possibility is that the protein of interest is aggregating. Check to see if the solution is turbid. Stirring of the protein may cause damage, so try different methods of mixing, such as pipetting up and down or using small plastic paddles.

Finally, if photobleaching is suspected, measure the change in fluorescence of the protein A solution over time according to the method described in Support Protocol 2.
**Linear increase in signal throughout titration, with no saturation**

The protein may be aggregating or polymerizing and may therefore exhibit a linear increase in fluorescence. Perform a control titration without protein A; specifically, add compound S in a stepwise fashion to a sample of buffer solution that does not contain protein A.

Another possibility is that the \( K_d \) is very high (e.g., 100× the concentrations being used). Perform the titration using higher concentrations of A and S.

**Possible mixing or stirring artifacts**

Perform a buffer titration (stepwise addition of buffer, instead of compound S, to protein A solution) to determine whether the titration results are real or are an effect of the mixing or stirring procedure.

**Problems with fluorescent labeling of a protein (Basic Protocol 2)**

If the measured ratio of dye to protein is less than 1:1, a possible reason is that the dye concentration used was too low. An increase in dye concentration may lead to a higher yield of the labeled species. However, if the protein to be labeled contains unreactive amino acid residues, increasing the dye concentration will not change this ratio. Note that, in some cases, the extinction coefficient of the dye changes upon interaction with the protein, which means that the amount of conjugated protein appears to be lower than it actually is; in such cases, even an increase in label concentration may not change the calculated ratio. The easiest way to test for a change in the extinction coefficient is to measure the absorption of the label-protein complex and the free dye under native and denaturing conditions (e.g., 6 M guanidine-HCl). If the absorbance of the free dye is not changed by the presence of guanidine-HCl, but the absorbance of the dye in the conjugated protein is changed, then the difference must be due to the interaction of the dye with the protein. In this case, the amount of dye attached to the protein must be calculated using data from the denatured state.

Increasing the dye concentration can lead to multiply labeled (or nonspecifically labeled) protein fractions, particularly when lysine residues are chosen for the attachment of the label. This is undesirable, because it is difficult to separate these differently labeled fractions. Nonetheless, it may be possible to separate such fractions using HPLC (UNIT 8.7).

In cases of multiple labeling (particularly amino-terminal labeling, where several lysine residues may be targeted), it may be important to determine the positions of the labels. This can be achieved by digesting the protein with trypsin (or another protease; UNIT 11.1) and analyzing the fragments using mass spectrometry (Chapter 16).

In general, it is more desirable to retain a certain fraction of unlabeled protein (which simply results in a smaller change in signal) than to risk having multiply labeled proteins. Differently labeled proteins may have different fluorescence signals, which significantly complicate data analysis.

**Anticipated Results**

If the protein does not aggregate or bind to the cuvette walls and does show a significant change in fluorescence upon complex formation, a binding curve such as the one shown in Figure 20.8.2 can be expected (assuming 1:1 stoichiometry and a simple binding mechanism). The equilibrium dissociation constant, as well as the stoichiometry value associated with the interaction, may be obtainable from the experimental data (Basic Protocol 3). Two examples of typical titrations, along with data analysis, are provided in Basic Protocol 3.

**Time Considerations**

Performing a fluorescence titration and the necessary control experiments typically requires ~2 hr. However, the time needed for titration depends largely on the rate of the binding event. If the binding interaction is very slow, the experiment may take considerably longer. In all cases, the reproducibility of the experiment must be verified.

If one of the proteins has to be labeled, the experiment will require a considerably longer period of time. The labeling of the protein and the execution of the necessary control experiments (e.g., measuring the effects of labeling on protein activity and folding) may take several days. If a cysteine residue has to be introduced or removed prior to labeling, 2 to 3 weeks may be required before the fluorescence experiment can be started.

**Literature Cited**


**Key References**


Provides the theory and practical applications of many spectroscopic techniques, including fluorescence and absorption spectroscopy and circular dichroism. Emphasis is placed on the practical aspects of these techniques, and protocols for test experiments as well as lists of background literature are provided. A very good introduction to spectrometry.

Lakowitz, 1999. See above.

Provides basic and in-depth information on fluorescence spectroscopy, including an overview of extrinsic fluorophores. Two chapters discuss time-resolved measurements (advanced).


A basic introduction to the use of spectroscopic methods for detecting conformational changes.

**Internet Resources**

http://www.probes.com

*Molecular Probes Web site. Includes an online version of the Handbook of Fluorescent Probes and Research Products, with up-to-date background and technical information. An e-mail newsletter is also available.*

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