Chapter 19

Fluorescence Polarization Assay to Quantify Protein-Protein Interactions: An Update

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Abstract

A fluorescence polarization assay can be used to evaluate the strength of a protein-protein interaction. A green fluorescent protein variant is fused to one of the protein partners. The formation of a complex is then deduced from an increase in fluorescence polarization, and the equilibrium dissociation constant of the complex is determined in a homogeneous aqueous environment. The assay is demonstrated by using the interaction of the S-protein and S-peptide fragments of ribonuclease A as a case study.

Key words Fluorescence anisotropy, Fluorescence polarization, Fusion protein, Green fluorescent protein, Protein-protein interaction

1 Introduction

Fluorescence polarization can be used to analyze macromolecular interactions in which one of the reactants is labeled with a fluorophore (*see* **Note 1**). In this assay, the formation of a complex is deduced from an increase in fluorescence polarization, and the equilibrium dissociation constant (K_d) of the complex is determined in a homogeneous aqueous environment (*see* **Note 2**). Most fluorescence polarization assays have used a small molecule such as fluorescein as a fluorophore [1–4].

Here, a variant (S65T) of green fluorescent protein (GFP) is used as the fluorophore in a polarization assay [5–7]. The advantages of using S65T GFP as the fluorophore are the ease with which a protein can be fused to GFP by using recombinant DNA techniques, the high integrity of the resulting chimera, and the broad chemical and physical stability of GFP compared to small-molecule fluorophores. To quantify the formation of a complex of two proteins (X and Y), a GFP fusion protein (GFP–X) is produced by using recombinant DNA technology (*see* **Note 3**).

Protein GFP–X is then titrated with protein Y, and the equilibrium dissociation constant is obtained from the increase in

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fluorescence polarization that accompanies complex formation. Like a free fluorescein-labeled ligand, free GFP–X is likely to rotate more rapidly and therefore to have a lower rotational correlation time than does the GFP–X·Y complex. An increase in rotational correlation time upon binding results in an increase in fluorescence polarization, which can be used to assess complex formation [8].

In a fluorescence polarization assay, the interaction between the two proteins is quantified in a homogeneous solution. The fluorescence polarization assay thereby allows for the determination of accurate values of K_d in a wide range of solution conditions. GFP is particularly well suited to this application because its fluorophore is held rigidly within the protein, as revealed by the three-dimensional structure of wild-type GFP and the S65T variant [9, 10]. Such a rigid fluorophore minimizes local rotational motion, thereby ensuring that changes in polarization report on changes to the *global* rotational motion of GFP, as affected by a protein-protein interaction.

2 Materials

1. 20 mM Tris–HCl buffer (varying pH).
2. Solution of aqueous NaCl (varying concentration).
3. Purified GFP–X and purified protein Y.
4. Fluorometer equipped with polarization measurement capability.
5. Graphics software capable of nonlinear regression analysis (e.g., DeltaGraph or SigmaPlot).

3 Methods

3.1 Fluorescence Polarization Assay

1. Mix protein GFP–X (0.50–1.0 nM) with various concentrations of protein Y in 1.0 mL of 20 mM Tris–HCl buffer, pH 8.0, with or without NaCl at 20 °C (*see* **Note 4**). Conditions such as buffer, pH, temperature, and salt can be varied as desired.
2. After mixing, take five to seven polarization measurements at each concentration of protein Y (*see* **Notes 5** and **6**). For a blank measurement, use a mixture that contains the same components except for protein GFP–X.

3.2 Data Analysis

1. Fluorescence polarization (P) is defined as

$$P = \frac{I_{||} - I_{\perp}}{I_{||} - I_{\perp}} \qquad (1)$$

where $I_{||}$ is the intensity of the emission light parallel to the excitation light plane and $I_{\perp}$ is the intensity of the emission

light perpendicular to the excitation light plane. *P*, the ratio of light intensities, is a dimensionless number with a maximum value of 0.5. Calculate values of K_d by fitting the data to the equation:

$$P = \frac{\Delta P \cdot F}{K_d + F} + P_{min} \tag{2}$$

In Eq. 2, P is the measured polarization, ΔP ($=P_{max} - P_{min}$) is the total change in polarization, and F is the concentration of free protein Y (*see* **Note 7**).

2. Calculate the fraction of bound protein (f_B) by using the equation

$$f_B = \frac{P - P_{min}}{\Delta P} = \frac{F}{K_d + F} \tag{3}$$

Plot f_B versus F to show the binding isotherms.

3.3 Case Study

Fluorescence polarization was used to determine the effect of salt concentration on the formation of a complex between the S15 and S-protein fragments of ribonuclease A [11–15]. A GFP chimera of S-peptide [S15–GFP(S65T)–His_6] was produced from bacteria and titrated with free S-protein. The value of K_d increased fourfold when NaCl was added to a final concentration of 0.10 M (Fig. 1). A similar salt dependence for the dissociation of RNase S had been observed previously [16]. The added salt is likely to disturb the

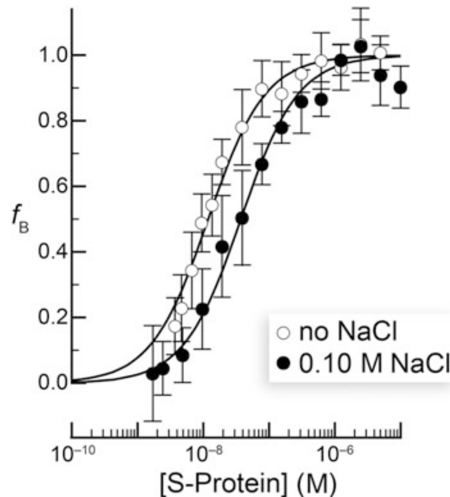

Fig. 1 Fluorescence polarization assay of a protein-protein interaction: S15–GFP (S65T)–His_6 with S-protein. S-Protein is added to 20 mM Tris–HCl buffer, pH 8.0, in a volume of 1.0 mL. Each data point is an average of 5–7 measurements. Curves are obtained by fitting the data to Eq. 3. The values of K_d in the presence of 0 and 0.10 M NaCl are 1.1×10^{-8} M and 4.2×10^{-8} M, respectively

water molecules hydrating the hydrophobic patch in the complex between S15 and S-protein, resulting in a decrease in the binding affinity [17]. Finally, the value of $K_d = 4.2 \text{ Å} \times 10^{-8}$ M observed in 20 mM Tris–HCl buffer (pH 8.0) containing NaCl (0.10 M) is similar (i.e., threefold lower) to that obtained by titration calorimetry in 50 mM sodium acetate buffer (pH 6.0) containing NaCl (0.10 mM) [18].

4 Notes

1. We used the term "polarization" instead of "anisotropy" herein. Fluorescence polarization (P) and fluorescence anisotropy (A) are related [$A = 2P/(3 - P)$] and contain equivalent physical information with respect to monitoring macromolecular complex formation. Many instruments report on both polarization and anisotropy and either parameter can be used to evaluate K_d.
2. Polarization is proportional to the rotational correlation time, which is defined as

$$P \propto \tau = \frac{3\eta V}{RT} \tag{4}$$

   In Eq. 4, rotational correlation time (τ) is the time taken for a molecule to rotate 68.5° and is related to the solution viscosity (η), molecular volume (V), gas constant (R), and absolute temperature (T). Thus, under conditions of constant viscosity and temperature, polarization is directly proportional to the molecular volume, which increases upon complex formation.
3. Using the "superfolder" variant of GFP could facilitate the creation of a GFP–X fusion protein [19].
4. In the assay solution, [GFP–X] should be significantly lower than the value of K_d ([GFP–X] $< < K_d$) but still be high enough to generate detectable fluorescence in the spectrometer. In the case study, [GFP–X] = 1 nM and $K_d > 10$ nM.
5. Data collection must be done at equilibrium. To estimate the time to reach equilibrium, a pilot experiment can be performed in which Y is added at [Y] = K_d, and the polarization is monitored until it reaches a stationary value.
6. At each [Y], the sample should be blanked with an identical mixture that lacks GFP–X.
7. The change in polarization (ΔP) upon complex formation must be detectable. For example, if the value of τ for GFP–X does not change significantly upon formation of the GFP–X·Y complex, then the value of ΔP is small and the data analysis is difficult.

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