[16] Green Fluorescent Protein Chimeras to Probe Protein–Protein Interactions

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Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is autofluorescent. The fluorophore of GFP forms on translation of the protein, without the action of any additional factors. GFP can be expressed in a wide variety of nonnative cell types. To date, GFP has been used largely *in vivo* as a marker for gene expression and as a fusion tag to monitor protein localization in living cells.[1–6]

GFP has exceptional physical and chemical properties besides spontaneous fluorescence. These properties include high thermal stability and resistance to detergents, organic solvents, and proteases. These properties endow GFP with enormous potential for biotechnical applications.[7–9] Since the cDNA of GFP was cloned,[10] a variety of GFP variants have been generated that broaden the spectrum of its application.[11–16] Among those

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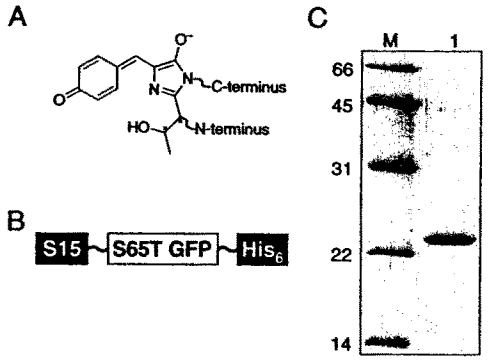

FIG. 1. Green fluorescent protein chimera. (A) Chemical structure of the fluorophore in the core of the S65T variant of green fluorescent protein.[17] This fluorophore forms spontaneously from residues Thr-65, Gly-66, and Tyr-67. The quinone methide resonance form shown here is likely to be responsible for the characteristic fluorescence of S65T GFP. (B) Structure of S15–GFP(S65T)–His_6, in which S15 is fused to the N terminus and six histidine residues are fused to the C terminus of S65T GFP. Two amino acid residues (Pro-Asp and Leu-Glu, respectively) link the tags to S65T GFP. (C) SDS–PAGE analysis of purified GFP chimera. Lane M, Molecular mass markers (in kDa); lane 1, S15–GFP(S65T)–His_6.

variants, S65T GFP (Fig. 1A) is unique in having increased fluorescence intensity, faster fluorophore formation, and altered excitation and emission spectra than that of the wild-type protein.[14,17] The wavelengths of the excitation and emission maxima of S65T GFP (490 and 510 nm, respectively) resemble closely those of fluorescein. The fluorescein-like spectral characteristics of S65T GFP enable its use with instrumentation that has been designed specifically for use with fluorescein.

Here, we describe the use of S65T GFP to probe protein–protein interactions *in vitro*.[18] This method requires fusing GFP to one of the target proteins to create a GFP chimera. The interaction of this fusion protein with another protein can be analyzed by two distinct methods.

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The first method is a fluorescence gel retardation assay. The gel retardation assay has been used widely to study protein–DNA interactions.[19] This assay is based on the electrophoretic mobility of a protein–DNA complex being less than that of either molecule alone. In a fluorescence gel retardation assay, electrophoretic mobility is detected by the fluorescent properties of S65T GFP. The fluorescence gel retardation assay is a rapid method to demonstrate the existence of a protein–protein interaction and to estimate the equilibrium dissociation constant (K_d) of the resulting complex.

The second method is a fluorescence polarization assay. The fluorescence polarization assay is an accurate method to evaluate the K_d in a specified homogeneous solution. Fluorescence polarization assays usually rely on fluorescein as an exogenous fluorophore. S65T GFP can likewise serve in this role. Further, the fluorescence polarization assay can be adapted for the high-throughput screening of protein or peptide libraries.

Production, Purification, and Detection of an S65T Green Fluorescent Protein Chimera

To demonstrate the potential of S65T GFP chimeras in exploring protien–protein interactions, we use as a model system the well-characterized interaction of the S-peptide and S-protein fragments of bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5).[20] Subtilisin treatment of RNase A yields two tightly associated polypeptide chains: S-peptide (residues 1–20) and S-protein (residues 21–124).[21] Residues 1–15 of S-peptide (S15) are necessary and sufficient to form a complex with S-protein.[22] A GFP chimera has also been used to explore the interaction of the BALF-1 protein and Bax/Bak, which are Bcl-2 homologs from Epstein–Barr virus.[23]

Protocol

S15–GFP(S65T)–His_6 (Fig. 1B) is produced by standard recombinant DNA techniques.[18] A cDNA fragment encoding S65T GFP is amplified by the polymerase chain reaction (PCR) and inserted into the *Bgl*II/*Xho*I sites of pET-29b (Novagen, Madison, WI), which encodes S15 and hexahistidine (His_6). The resulting expression vector is used to transform *Escherichia coli* strain BL21(DE3). Cells are grown at 37° in 0.5 liter of Luria–Bertani

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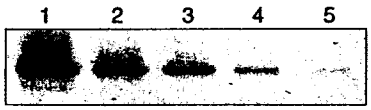

FIG. 2. Fluorimager analysis of purified green fluorescent protein chimera. Lanes 1–5, 10, 3.0, 1.0, 0.3, and 0.1 ng of S15–GFP(S65T)–His_6, respectively.

(LB) medium until the absorbance at 600 nm is 0.5 OD. Isopropylthiogalactoside (IPTG) is then added to a final concentration of 0.5 m*M*, and the cells are grown at 30° for an additional 4 hr. The culture is harvested and resuspended in 25 ml of 50 m*M* HEPES buffer, pH 7.9, containing NaCl (0.3 *M*), dithiothreitol (DTT, 0.5 m*M*), and phenylmethylsulfonyl fluoride (PMSF, 0.2 m*M*), and the cells are lysed by using a French pressure cell. The lysed cells are subjected to centrifugation at 18,000*g*. The supernatant is collected and loaded onto a Ni^{2+}-NTA agarose column (Qiagen, Chatsworth, CA). The column is washed with 50 m*M* HEPES buffer, pH 7.9, containing imidazole (8 m*M*), NaCl (0.3 *M*), and PMSF (0.5 m*M*). S15–GFP(S65T)–His_6 is eluted in the same buffer containing imidazole (0.10 *M*). The green fractions are pooled and purified further by FPLC (fast protein liquid chromatography) on a Superdex 75 gel-filtration column (Pharmacia, Piscataway, NJ) with elution by 50 m*M* HEPES buffer, pH 7.9. Purified S15–GFP(S65T)–His_6 migrates as a single species during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1C).

S15–GFP(S65T)–His_6 remains fluorescent after electrophoresis in a native polyacrylamide gel (Fig. 2). Further, the altered excitation and emission spectra of S65T GFP are well suited for detection by a fluorimager. The sensitivity of S65T GFP detection in a native polyacrylamide gel is $\geq$0.1 ng, which is comparable to that of an immunoblot using an anti-GFP antibody.[24]

The regioisomer of S15–GFP(S65T)–His_6, His_6–GFP(S65T)–S15, can also be produced and analyzed by similar protocols.[18] Surprisingly, His_6–GFP(S65T)–S15 migrates as two distinct species during SDS–PAGE, native PAGE, and zymogram electrophoresis.[25,26] Apparently, two isoforms of His_6–GFP(S65T)–S15 exist that migrate differently during electrophoresis, even in the presence of SDS. We therefore recommend constructing GFP chimeras in which the target protein is fused to the N terminus of GFP, rather than to the C terminus.

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Fluorescence Gel Retardation Assay

Gel mobility retardation is a useful tool for both qualitative and quantitative analyses of protein–nucleic acid interactions.[19] The fluorescence gel retardation assay applies gel retardation of fluorescent species to the study of a protein–protein interaction. In this assay, free and bound S65T GFP chimeras are resolved and visualized in a native polyacrylamide gel.

Example

The fluorescence gel retardation assay is used to quantify the interaction between S-protein and S15–GFP(S65T)–His_6. A fixed quantity of S15–GFP(S65T)–His_6 is incubated with a varying quantity of S-protein prior to electrophoresis in a native polyacrylamide gel. After electrophoresis, the gel is scanned with a fluorimager and the fluorescence intensities of bound and free S15–GFP(S65T)–His_6 are quantified (Fig. 3A). From the relative fluorescence intensities of the bound and free S15–GFP(S65T)–His_6, the binding ratio (R = fluorescence intensity of bound S15–GFP(S65T)–His_6/ total fluorescence intensity) at each concentration is obtained. The value

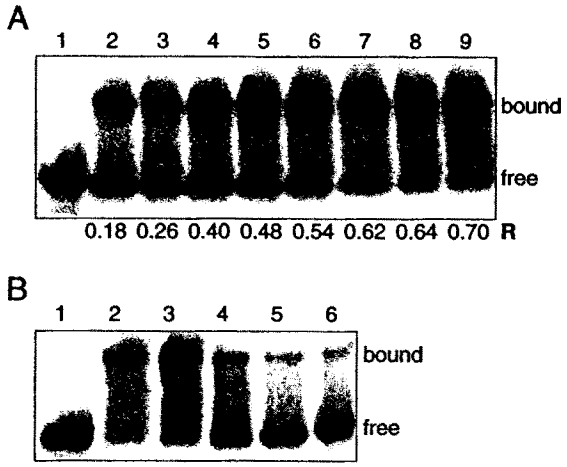

FIG. 3. Gel retardation assay of a protein–protein interaction. (A) Gel retardation assay of the interaction of S15–GFP(S65T)–His_6 with varying amounts of S-Protein. Lanes 1–9, 1 μM S15–GFP(S65T)–His_6 and 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 μM S-protein, respectively. The relative mobilities of free and bound S15–GFP(S65T)–His_6 are 0.72 and 0.47, respectively. The value of R is obtained for each lane, and values of K_d are calculated by using Eq. (1) with the average being $K_d = (6 \pm 3) \times 10^{-8}$ M. (C) Gel retardation assay demonstrating that S-peptide competes with S15–GFP(S65T)–His_6 for interaction with S-protein. Lane 1, 1 μM S15–GFP(S65T)–His_6 and no S-protein or S-peptide. Lanes 2–6, 1 μM S15–GFP(S65T)–His_6, 1 μM S-protein, and 0, 0.3, 1.0, 3.0, and 10 μM S-peptide, respectively.

of K_d for the complex formed in the presence of different S-protein concentrations is calculated from the values of R and the total concentrations of S-protein and S15–GFP(S65T)–His_6 [see Eq. (1)]. The average (±SE) value of K_d is $(6 \pm 3) \times 10^{-8}$ M.

A competition assay is used to probe the specificity of the interaction between S15–GFP(S65T)–His_6 and S-protein. S15–GFP(S65T)–His_6 and S-protein are incubated to allow for complex formation. Varying amounts of S-peptide are added and the resulting mixture is incubated further, and then subjected to native gel electrophoresis. As shown in Fig. 3B, the addition of S-peptide converts bound S15–GFP(S65T)–His_6 to the free state. Thus, S15–GFP(S65T)–His_6 and S-peptide bind to the same region of S-protein.

Protocol

Purified fusion proteins are quantified by using the extinction coefficient (ε = 39.2 mM^{-1} cm^{-1} at 490 nm[14]) of S65T GFP. S-protein (Sigma, St. Louis, MO) is quantified by using its extinction coefficient (ε = 9.56 mM^{-1} cm^{-1} at 280 nm[27]). To begin the gel retardation assay, purified S15–GFP(S65T)–His_6 (1.0 μM) is incubated at 20° with varying amounts of S-protein in 10 μl of 10 mM Tris-HCl buffer, pH 7.5, containing glycerol (5%, v/v). After 20 min, the mixtures are loaded onto a native polyacrylamide (6%, w/v) gel,[28] and the loaded gel is subjected to electrophoresis at 4° at 10 V/cm. For inhibition assays, S-peptide is added and the mixtures are incubated at 20° for another 20 min before electrophoresis. Immediately after electrophoresis, the gel can be scanned with a Fluorimager SI System (Molecular Dynamics, Sunnyvale, CA) at 700 V using a built-in filter set (490 nm for excitation and ≥515 nm for emission).

Calculation of Equilibrium Dissociation Constants

The fluorescence intensities of bound and free S15–GFP(S65T)–His_6 can be quantified by using the program ImageQuaNT 4.1 (Molecular Dynamics). The value of R (the fluorescence intensity of bound S15–GFP(S65T)–His_6/total fluorescence intensity) for each gel lane is determined from the fluorescence intensities, and values of K_d are calculated by using Eq. (1):

$$K_d = \frac{1 - R}{R}([\text{S} - \text{protein}]_{\text{total}} - R[\text{S15–GFP(S65T)–His}_6]_{\text{total}}) \quad (1)$$

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Fluorescence Polarization Assay

The fluorescence gel retardation assay described above is a convenient method by which to visualize a protein–protein interaction as well as to estimate the value of K_d for the resulting complex. Still, gel retardation assays have an intrinsic limitation in evaluating equilibrium dissociation constants. In a gel retardation assay, it is assumed that a receptor–ligand interaction remains at equilibrium during sample loading and electrophoresis. Yet, as samples are loaded and migrate through a gel, complex dissociation is unavoidable and results in an underestimation of the value of K_d. Moreover, if the conditions (e.g., pH, or salt type or concentration) encountered during electrophoresis differ from those in the incubation, then the measured value of K_d may not correspond to the true value.

In a fluorescence polarization assay, the formation of a complex is deduced from an increase in fluorescence polarization, and the equilibrium dissociation constant is determined in a homogeneous aqueous environment. Most applications of fluorescence polarization assay have used fluorescein as a fluorophore.[29–32] Like a free fluorescein-labeled ligand, a free GFP chimera is likely to rotate more rapidly and therefore to have a lower rotational correlation time than does a bound chimera. An increase in rotational correlation time on binding results in an increase in fluorescence polarization, which can be used to assess complex formation.[33] In contrast to a gel retardation assay, the fluorescence polarization assay is performed in a homogeneous solution, in which the conditions can be dictated precisely.

Example

Fluorescence polarization is used to determine the effect of salt concentration on the formation of a complex between S-protein and S15–GFP(S65T)–His_6. The value of K_d increases by fourfold when NaCl is added to a final concentration of 0.10 *M* (Fig. 4). A similar salt dependence for the dissociation of RNase S had been observed previously.[34] The added salt is likely to disturb the water molecules hydrating the hydrophobic patch in the complex between S-peptide and S-protein, resulting in a decrease in the binding affinity.[35] Finally, the value of $K_d = 4.2 \times 10^{-8}$ *M* observed in 20 m*M* Tris-HCl buffer, pH 8.0, containing NaCl (0.10 *M*) is similar (i.e.,

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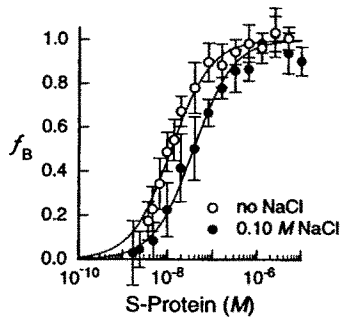

FIG. 4. Fluorescence polarization assay of a protein–protein interaction. S15-GFP(S65T)-His_6 with S-protein. S-protein is added to 20 m*M* Tris-HCl buffer, pH 8.0, in a volume of 1.0 ml. Each data point is an average of five to seven measurements. Curves are obtained by fitting the data to Eq. (3). The values of K_d in the presence of 0 and 0.10 *M* NaCl are 1.1×10^{-8} and 4.2×10^{-8} *M*, respectively.

threefold lower) to that obtained by titration calorimetry in 50 m*M* sodium acetate buffer, pH 6.0, containing NaCl (0.10 m*M*).[27]

Protocol

Fluorescence polarization (P) is defined as

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{2}$$

where $I_{\parallel}$ 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, being a ratio of light intensities, is a dimensionless number and has a maximum value of 0.5. Fluorescence polarization can be measured with a Beacon fluorescence polarization system (Pan Vera, Madison, WI).[36] Purified S15–GFP(S65T)–His_6 (0.50 n*M*) is incubated at 20° (±2°) with various concentrations of S-protein (20 μ*M*–1.0 n*M*) in 1.0 ml of 20 m*M* Tris-HCl buffer, pH 8.0, containing NaCl (0 or 0.10 *M*). Five to seven polarization measurements are made at each S-protein concentration.

Calculation of Equilibrium Dissociation Constants

Values of K_d can be determined by using the program DeltaGraph 4.0 (DeltaPoint; Monterey, CA) to fit the data to Eq. (3):

[36] P. K. Wittmayer and R. T. Raines, *Biochemistry* **35**, 1076 (1996).

$$P = \frac{\Delta PF}{K_d + F} + P_{min} \quad (3)$$

In Eq. (3), P is the measured polarization, ΔP ($= P_{max} - P_{min}$) is the total change in polarization, and F is the concentration of free S-protein. The fraction of bound S-protein (f_B) is obtained by using Eq. (4):

$$f_B = \frac{P - P_{min}}{\Delta P} = \frac{F}{K_d + F} \quad (4)$$

The binding isotherms (Fig. 4) are obtained by plotting f_B versus F.

Prospectus

Methods to reveal and characterize the noncovalent interaction of one molecule with another are necessary to understand and control such interactions.[37–39] We describe two new methods for probing protein–protein interactions. The first method is a fluorescence gel retardation assay in which one protein is fused to GFP. The GFP fusion protein is incubated with the other protein, and the mixture is separated by native PAGE. The interaction between the two proteins is evident by a decrease in the mobility of the fluorescent fusion protein that results from complex formation.

The fluorescence gel retardation assay is a fast and convenient way to demonstrate interactions between two proteins, and in addition allows for an estimation of the value of K_d for the resulting complex. Conventional methods to demonstrate an interaction between two proteins (e.g., protein A and protein B) are more laborious or less informative (or both).[40–45] In a typical method, protein A is fused to an affinity tag [such as glutathione *S*-transferase (GST)], which is then used to immobilize protein A on a resin. Protein B is applied to the resin to allow for complex formation. The complex is eluted and detected by an immunoblot using an antibody to protein B. In contrast, the fluorescence gel retardation assay requires simply

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mixing a protein A–GFP chimera with protein B, separating the mixture by native PAGE, and scanning the gel with a fluorimager. The interaction between protein A and protein B is apparent from the shift of the protein A–GFP band that results from complex formation. The sensitivity of S65T GFP detection (≥0.1 ng; Fig. 2) approaches that of an immunoblot using an anti-GFP antibody.[24] Brighter GFP variants have become available. These variants show similar excitation and emission spectra but two- to eightfold stronger fluorescence intensity than the S65T variant.[46–48] Using these brighter variants in the gel retardation assay will improve further the sensitivity of the fluorescence gel retardation assay.

The second new method for probing protein–protein interactions, a fluorescence polarization assay, provides a more accurate assessment of the value of K_d. Most applications of fluorescence polarization have focused on analyzing protein–DNA interactions, with fluorescein (linked to DNA) serving as the fluorophore. Here, a GFP fusion protein is titrated with another protein, and the equilibrium dissociation constant is obtained from the increase in fluorescence polarization that accompanies binding. The interaction between the two proteins is detected in a homogeneous solution rather than a gel matrix. 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 (Fig. 1A) is held rigidly within the protein, as revealed by the three-dimensional structures of wild-type GFP and the S65T variant.[17,49] 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 effected by a protein–protein interaction. Finally, it is worth noting that this assay is amenable to the high-throughput screening of protein or peptide libraries for effective ligands.[50]

Another advantage of both of these new methods is the ease with which a protein can be fused to GFP, using recombinant DNA techniques, and the high integrity of the resulting chimera. Traditionally, fluorophores have been attached to proteins by chemical modification with reagents such as fluorescein isothiocyanate (FITC).[30,51,52] In this approach, additional purification steps are necessary to separate labeled protein from the reagent and

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unlabeled protein. Further, labeling the protein at a single site can be difficult or impossible. In contrast, labeling a protein with GFP is complete and generates a single species. Purification of that species can be facilitated by the incorporation of an affinity tag such as His_6[53] or S · Tag.[54] The success of S65T GFP as the fluorophore in fluorescence gel retardation assays and fluorescence polarization assays arises largely from the altered spectral characteristics and increased fluorescence intensity of S65T GFP.[14] The availability of brighter S65T variants makes these assays more promising tools with which to investigate and analyze protein–protein interactions *in vitro*. We suggest that the role of fluorescein as a fluorescent label can be replaced by S65T GFP or its variants in many biochemical analyses.

Acknowledgments

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