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Green fluorescent protein as a signal for protein–protein interactions

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Abstract

Green fluorescent protein (GFP) is autofluorescent. This property has made GFP useful in monitoring in vivo activities such as gene expression and protein localization. We find that GFP can be used in vitro to reveal and characterize protein–protein interactions. The interaction between the S-peptide and S-protein fragments of ribonuclease A was chosen as a model system. GFP-tagged S-peptide was produced, and the interaction of this fusion protein with S-protein was analyzed by two distinct methods: fluorescence gel retardation and fluorescence polarization. The fluorescence gel retardation assay is a rapid method to demonstrate the existence of a protein–protein interaction and to estimate the dissociation constant (K_d) of the resulting complex. The fluorescence polarization assay is an accurate method to evaluate K_d in a specified homogeneous solution and can be adapted for the high-throughput screening of protein or peptide libraries. These two methods are powerful new tools to probe protein–protein interactions.

Keywords: fluorescence polarization; fusion protein; gel retardation; green fluorescent protein; protein–protein interaction

Green fluorescent protein (GFP) from the jelly fish *Aequorea victoria* has exceptional physical and chemical properties, such as spontaneous fluorescence, high thermal stability, and resistance to detergents, organic solvents, and proteases. These properties endow GFP with enormous potential for biotechnical applications (Bokman & Ward, 1981; Ward, 1981; Ward & Bokman, 1982). To date, GFP has been used largely in vivo—as a marker for gene expression and a fusion tag to monitor protein localization in living cells (Chalfie et al., 1994; Inouye & Tsuji, 1994; Ren et al., 1996).

The cDNA that codes for GFP was cloned five years ago (Prasher et al., 1992). Since then, a variety of GFP variants have been generated in response to the demand for improved properties that could broaden the spectrum of its application (Cubitt et al., 1995; Delagrave et al., 1995; Ehrig et al., 1995; Heim et al., 1995; Crameri et al., 1996). Among those variants, S65T GFP is unique in having increased fluorescence intensity, faster fluorophore formation, and altered excitation and emission spectra than that of the wild-type protein (Heim et al., 1995). Recently, the groups of Phillips and Remington have determined the crystalline structures of wild-type (Yang et al., 1996) and S65T (Ormö et al., 1996) GFP by X-ray diffraction analysis. These studies indicate that the fluorophore is held rigidly within the protein. The wavelengths of the excitation and emission maxima of S65T GFP (490 nm and 510 nm, respectively) resemble closely those of fluorescein. The fluorescein-like spectral characteristics of S65T GFP enable its use with instrumentation, such as fluorescence-activated cell sorting (FACS) devices or fluorescence microscopes, that had been designed specifically for use with fluorescein.

Here, we demonstrate the use of S65T GFP as the basis of two new methods for exploring protein–protein interactions. The first is a fluorescence gel retardation assay. The gel retardation assay has been used widely to study protein–DNA interactions (Carey, 1991). This assay is based on the electrophoretic mobility of a protein–DNA complex being less than that of either molecule alone. In our fluorescence gel retardation assay, we use this principle, together with the fluorescent properties of S65T GFP, which is fused to one of the interacting proteins. The second is a fluorescence polarization assay. A complex between two molecules rotates more slowly than do the free molecules. The resulting increase in rotational correlation time gives rise to an increase in fluorescence polarization (LeTilly & Royer, 1993; Jameson & Sawyer, 1995). Fluoresence polarization assays usually rely on fluorescein as an exogenous fluorophore. In our fluorescence polarization assay, we show that S65T GFP can serve as well or better in this role.

Results

Monitoring protein–protein interactions

To demonstrate the potential of S65T GFP in exploring protein–protein interactions, we have chosen as a model system the well-characterized interaction of the S-peptide and S-protein fragments of bovine pancreatic ribonuclease (RNase) A. Subtilisin treatment

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of RNase A yields two tightly associated polypeptide chains: S-peptide (residues 1–20) and S-protein (residues 21–124) (Richards, 1955). Although the two individual polypeptide chains are inactive, full enzymatic activity is restored upon complex formation (Richards & Vithayathil, 1959). Because a truncated form of S-peptide (S15, residues 1–15) is necessary and sufficient to form an enzymatically active complex with S-protein (Potts et al., 1963), we used S15 in our studies. Specifically, we generated fusion proteins in which S15 is fused to the N or C terminus of S65T GFP.

Purification and detection of S65T GFP fusion proteins

DNA encoding S15 and six histidine residues (His_6; Hochuli et al., 1988) was added to the 5′ and 3′ ends of the cDNA encoding S65T GFP. The two resulting proteins, His_6~GFP(S65T)~S15 and S15~GFP(S65T)~His_6 (Fig. 1A), were produced in *Escherichia coli* strain BL21(DE3) and purified by affinity chromatography using a Ni^{2+}~NTA column (Fig. 1B). The presence of an intact and functional S15 sequence on the fusion proteins was confirmed by zymogram electrophoresis in a poly(C)-containing gel, which was then incubated with S-protein (Fig. 1C) (Kim & Raines, 1993, 1994). Both His_6~GFP(S65T)~S15 and S15~GFP(S65T)~His_6 remain fluorescent after electrophoresis in a native polyacrylamide gel (Fig. 2A). 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 ≥0.1 ng (data not shown), which is comparable to that of an immunoblot using an anti-GFP antibody (Colby et al., 1995).

Purified His_6~GFP(S65T)~S15 migrates as two distinct species during SDS-PAGE (Fig. 1B), zymogram electrophoresis (Fig. 1C), and native PAGE (Fig. 2A). No unexpected mutations are present in the gene encoding His_6~GFP(S65T)~S15. The results of Ni^{2+}~NTA affinity chromatography and zymogram electrophoresis indicate that both the N and the C termini of His_6~GFP(S65T)~S15 are intact (Kim & Raines, 1994). Apparently, two isoforms of His_6~GFP(S65T)~S15 exist that migrate differently during electrophoresis, even in the presence of SDS (Fig. 1B).

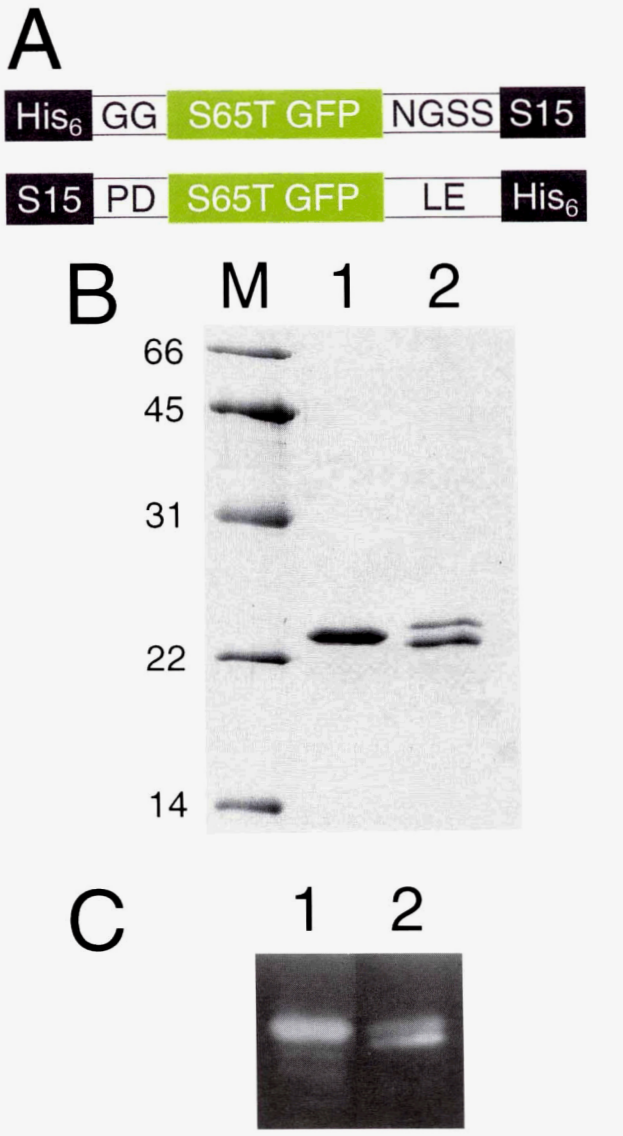

Fig. 1. A: PFP fusion proteins used in this study. In His_6~GFP(S65T)~S15 (top), six histidine residues are fused to the N terminus and S15 is fused to the C terminus of S65T GFP. In S15~GFP(S65T)~His_6 (bottom), S15 is fused to the N terminus and six histidine residues are fused to C terminus of S65T GFP. Residues that link the tags to GFP(S65T) are also indicated. **B:** SDS-PAGE analysis of purified GFP fusion proteins. Lane M, molecular mass markers (14.4, 21.5, 31, 45, and 66 kDa); lane 1, S15~GFP(S65T)~His_6; lane 2, His_6~GFP(S65T)~S15. **C:** Zymogram electorphoresis analysis of purified GFP fusion proteins. Lane 1, S15~GFP(S65T)~His_6; lane 2, His_6~GFP(S65T)~S15.

Fluorescence gel retardation assay

Gel mobility retardation is a popular tool for both qualitative and quantitative analyses of protein–nucleic acid interactions (Carey, 1991). The fluorescence gel retardation assay shown in Figure 2B is the first to apply gel retardation to the study of a protein–protein interaction. In this assay, free and bound S15-tagged S65T GFP were resolved and visualized in a native polyacrylamide gel. As shown in Figure 2B, only the slower migrating isoform of His_6~GFP(S65T)~S15 was shifted upon binding to S-protein during native PAGE, indicating that only this species has an accessible S15. The S15 portion of the faster migrating species is inaccessible to S-protein, perhaps because it becomes buried inside the GFP moiety during the folding process. We therefore believe it to be prudent to construct GFP fusions in which the target protein is fused to the N terminus of GFP, rather than to the C terminus. All subsequent experiments were performed with S15~GFP(S65T)~His_6.

The fluorescence gel retardation assay was used to quantify the interaction between S-protein and S15~GFP(S65T)~His_6. A fixed quantity of S15~GFP(S65T)~His_6 was incubated with a varying quantity of S-protein prior to electrophoresis in a native polyacrylamide gel. After electrophoresis, the gel was scanned with a fluorimager and the fluorescence intensities of bound and free S15~GFP(S65T)~His_6 were 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 was obtained. The dissociation constant (K_d) of the complex formed in the presence of different S-protein concentrations was calculated from the values of **R** and the total concentrations of S-protein and S15~GFP(S65T)~His_6. The average (±SD) value of K_d is $(6 \pm 3) \times 10^{-8}$ M.

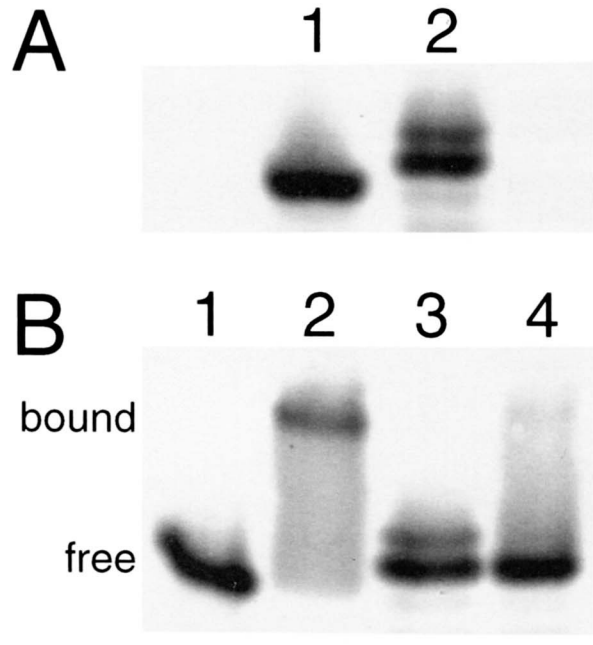

Fig. 2. A: Fluorimager analysis of purified GFP fusion proteins after native PAGE. Lane 1, S15~GFP(S65T)~His_6; lane 2, His_6~GFP(S65T)~S15. **B:** Gel retardation assay demonstrating that S15~GFP(S65T)~His_6 and His_6~GFP(S65T)~S15 interact with S-protein. A GFP fusion protein was incubated with S-protein in 10 mM Tris-HCL buffer, pH 7.5, containing glycerol (5% v/v) for 20 min at 20 °C in a volume of 10 μL. Mixtures were then electrophoresed in a native 6% (w/v) polyacrylamide gel at 4 °C at 10 V/cm and scanned by a fluorimager using an internal filter set. Lane 1, 1 μM S15~ GFP(S65T)~His_6 and no S-protein; lane 2, 1 μM S15~GFP(S65T)~His_6 and 1 μM S-protein; lane 3, 1 μM His_6~GFP(S65T)~S15 and no S-protein; lane 4, 1 μM His_6~GFP(S65T)~S15 with 1 μM S-protein.

A competition assay was 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 were incubated to allow for complex formation. Varying amounts of S-peptide were added and the resulting mixture was incubated further, and then subjected to native gel electrophoresis. As shown in Figure 3B, the addition of S-peptide converts bound S15~GFP(S65T)~His_6 to the free state. We conclude that S15~GFP(S65T)~His_6 and S-peptide bind to the same region of S-protein.

Fluorescence polarization assay

The fluorescence gel retardation assay is a convenient method to visualize a protein–protein interaction as well as to estimate the K_d of the resulting complex. Still, gel retardation assays have an intrinsic limitation in measuring accurate 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. Furthermore, 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 could be inaccurate.

Fluorescence polarization was used to quantify the interaction between S-protein and S15-tagged S65T GFP. In this assay, the formation of a complex is deduced from an increase in fluorescence polarization, and the dissociation constants are determined in a homogeneous aqueous environment. Most applications of fluorescence polarization assay have used fluorescein as a fluorophore (LeTilly & Royer, 1993; Radek et al., 1993; Wittmayer & Raines, 1996). We reasoned that the complex between S15~GFP(S65T)~His_6 and S-protein is likely to rotate more slowly and therefore to have a higher rotational correlation time than does free S15~GFP(S65T)~His_6. Such an increase in rotational correlation time results in an increase in fluorescence polarization, which can be used to assess complex formation. In contrast to a gel retardation assay, a fluorescence polarization assay is performed in a homogeneous solution in which the conditions can be dictated precisely.

Fluorescence polarization was used to determine the effect of pH on complex formation. As shown in Figure 4A, the K_d values obtained were 1.4×10^{-8} M, 1.1×10^{-8} M, and 1.0×10^{-8} M at pH 7.5, 8.0, and 8.5, respectively. The interaction between S-protein and S15~GFP(S65T)~His_6 was not affected significantly by changing the pH by 1.0 unit. The insensitivity of K_d values to the pH change (pH 7.5 to pH 8.5) was not unexpected, as none of the amino acid side chains involved in the interaction is known to change its protonation state in this pH range. The K_d (1.4×10^{-8} M) at pH 7.5 is approximately fourfold lower than the

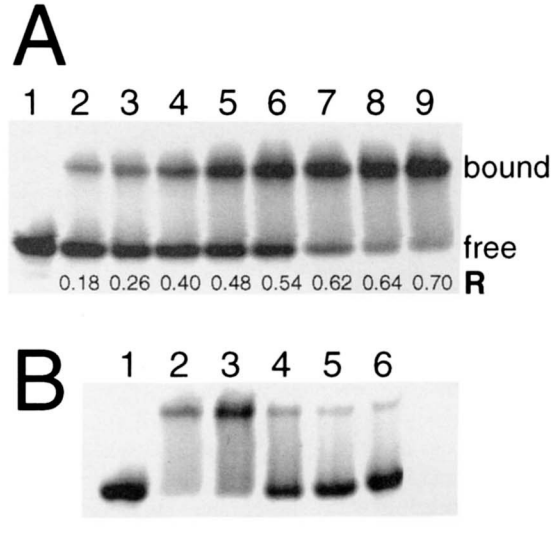

Fig. 3. A: Gel retardation assay of the interaction of S15~GFP(S65T)~His_6 with S-protein. Assays were performed as in Figure 2B. Lanes 1–9, 1 μM S15~GFP(S65T)~His_6 and 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 μM S-protein, respectively. Relative mobilities of free and bound S15~GFP(S65T)~His_6 were 0.72 and 0.47, respectively. The value of **R** was obtained for each lane, and values of K_d were calculated by using Equation 1, with the average being $K_d = (6 \pm 3) \times 10^{-8}$ M. **B:** Gel retardation assay demonstrating that S-peptide competes with S15~GFP(S65T)~His_6 for interactions with S-protein. S15~GFP(S65T)~His_6 was incubated with S-protein as in Figure 2B. After 20 min, S-peptide was added, and the mixtures were incubated for an additional 20 min at 20 °C. Mixtures were analyzed as in Figure 3A. 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.

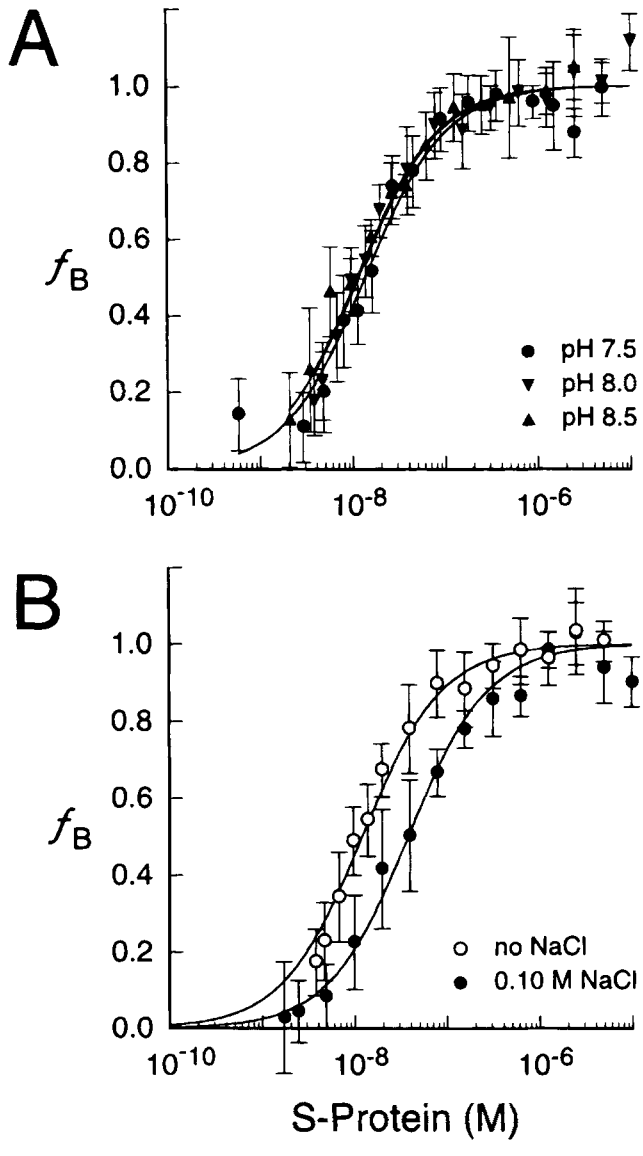

Fig. 4. Fluorescence polarization assay of the interaction of S15~GFP(S65T)~His_6 with S-protein. S-protein was added to 20 mM Tris-HCl buffer in a volume of 1.0 mL. Each data point is an average of 5–7 measurements. Curves were obtained by fitting the data to Equation 2. **A:** Effect of pH change on the interaction in the absence of added NaCl. Values of K_d at pH 7.5, 8.0, and 8.5 were 1.4×10^{-8} M, 1.1×10^{-8} M, and 1.0×10^{-8} M, respectively. Mean values of P_{min} and ΔP were (415.4 ± 3.0) mP and (17.2 ± 2.0) mP, respectively. **B:** Effect of NaCl concentration on the interaction at pH 8.0. Values of K_d in the presence of 0 and 0.10 M NaCl were 1.1×10^{-8} M and 4.2×10^{-8} M, respectively.

K_d (6×10^{-8} M) obtained by the fluorescence gel retardation assay performed at the same pH. The difference in the K_d values is consistent with an increase in complex dissociation during the course of the gel retardation assay.

Fluorescence polarization was used to determine the effect of salt concentration on complex formation. The value of K_d increased by 3.8-fold when NaCl was added to a final concentration of 0.10 M (Fig. 4B). A similar salt dependence for the dissociation of RNase S had been observed previously (Schreier & Baldwin, 1977). 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 (Baldwin, 1996). Finally, the value of K_d (4.2×10^{-8} M) that we observed in 20 mM Tris-HCl buffer, pH 8.0, containing NaCl (0.10 M) was similar (i.e., 2.6-fold lower) to that obtained by titration calorimetry in 50 mM sodium acetate buffer, pH 6.0, containing NaCl (0.10 mM) (Connelly et al., 1990).

Discussion

Methods to reveal and characterize the noncovalent interaction of one molecule with another are necessary to understand and control such interactions (Attie & Raines, 1995; Winzor & Sawyer, 1995). We have developed 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 shift 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 and less informative. In a typical method, protein **A** is fused to an affinity tag (such as glutathione S-transferase), 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 mixing a protein **A**~GFP fusion protein 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) challenges that of an immunoblot using an anti-GFP antibody (Colby et al., 1995). Moreover, the sensitivity of GFP detection is likely to improve as brighter GFP variants become available.

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 assay have focused on analyzing protein–DNA interactions, with fluorescein (linked to DNA) serving as the fluorophore. In our assay, a GFP fusion protein is titrated with another protein, and the dissociation constant is obtained from the change 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 is held rigidly within the protein (Ormö et al., 1996; Yang et al., 1996). 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 (Jolley, 1996).

Another advantage of these new methods is the ease with which a protein can be fused to GFP using recombinant DNA techniques

and the integrity of the resulting fusion protein. Traditionally, fluorophores have been attached to proteins by chemical modification with reagents such as fluorescein isothiocyanate (FITC). In this approach, additional purification steps are necessary to separate labeled protein from the reagent and 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 (Hochuli et al., 1988) or S15 (Kim & Raines, 1994). 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 (Heim et al., 1995). We suggest that the role of fluorescein as a fluorescent label can be replaced by S65T GFP in many biochemical analyses.

Materials and methods

His_6~GFP(S65T)~S15 construction

The His_6 tag and S65T mutation were introduced simultaneously into the cDNA that codes for wild-type GFP [TU#58 from Chalfie et al. (1994)] by PCR mutagenesis (Picard et al., 1994) using three primers: P39 (GGCATATGCACCACCACCACCACCACGGCG GTAGCAAAGGAGAAGAAC for the His_6 tag and an *Nde* I site), M5 (CCATGGCCAACACTGGTCACCACTTTCACCTATGGTG TTCAATGCTT for the S65T change), and P36 (GTGAATTCTT GTATAGTTCA TCCATGCCA for an *Eco*R I site). The resulting PCR fragment was digested with *Eco*R I and *Nde* I and inserted into an *Eco*R I/*Nde* I site of pET-29a (Novagen; Madison, Wisconsin) by the TIES method (Zeng et al., 1996) (which was used because of an internal *Nde* I site in GFP gene). The DNA fragment encoding S15 was generated from pET-29a by PCR using P37 (GGGAATTCCGGCGGCAAAGAAACCGCTGCT GCTAAA with an *Eco*R I site) and P38 (TGGTCGACTTAGCTGTCCATGTGCTGG CGTTCGA with a *Sal* I site) and inserted into *Eco*R I/*Sal* I site of the above plasmid to give pSH24.

S15~GFP(S65T)~His_6 construction

The coding region of GFP(S65T) was amplified from pSH24 with P53 (TCAAGATCTTAGCAAAGGAGAAGAACTT with a *Bgl* II site) and P54 (GCCCTCGAGCTTGTATAGTTCATCCATGC with an *Xho* I site). The PCR fragment was digested with *Bgl* II and *Xho* I and inserted into *Bgl* II/*Xho* I site of pET-29b to give pSH41.

Expression and purification of the fusion proteins

His_6~GFP(S65T)~S15 and S15~GFP(S65T)~His_6 were produced from *E. coli* strain BL21(DE3)/pSH24 and BL21(DE3)/pSH41, respectively. Cells were grown at 37 °C in 0.5 L of LB medium until the absorbance at 600 nm was 0.5, IPTG was then added to a final concentration of 0.5 mM, and the cells were grown at 30 °C for an additional 4 h. The culture was harvested and resuspended in 25 mL of 50 mM HEPES buffer, pH 7.9, containing NaCl (0.3 M), DTT (0.5 mM), and PMSF (0.2 mM), and the cells were lysed by using a French pressure cell. The lysed cells were subjected to centrifugation at 18,000 × *g*. The supernatant was collected and loaded onto a Ni^{2+}~NTA agarose column (Qiagen; Chatsworth, California). The column was washed with 50 mM HEPES buffer, pH 7.9, containing imidazole (8 mM), NaCl (0.3 M), and PMSF (0.5 mM). GFP(S65T) fusion proteins were eluted in the same buffer containing imidazole (0.10 M). The green fractions were pooled and further purified by FPLC on a Superdex 75 gel filtration column (Pharmacia; Piscataway, New Jersey) with elution by 50 mM HEPES buffer, pH 7.9.

Gel retardation assay

Purified fusion proteins were quantified by using the extinction coefficient [ϵ = 39.2 mM^{-1} cm^{-1} at 490 nm (Heim et al., 1995)] of S65T GFP. S-protein (Sigma Chemical; St. Louis, Missouri) was quantified by using its extinction coefficient [ϵ = 9.56 mM^{-1} cm^{-1} at 280 nm (Connelly et al., 1990)]. To begin the gel retardation assay, purified S15~GFP(S65T)~His_6 (1.0 μM) was incubated at 20 °C 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 were loaded onto a native continuous polyacrylamide (6% w/v) gel (Laemmli, 1970), and the loaded gel was subjected to electrophoresis at 4 °C at 10 V/cm. Immediately after electrophoresis, the gel was scanned by a Fluorimager SI System (Molecular Dynamics; Sunnyvale, California) using a built-in filter set (490 nm for excitation and ≥515 nm for emission). The fluorescence intensities of bound and free S15~GFP(S65T)~His_6 were quantified by using the program Image QuaNT 4.1 (Molecular Dynamics; Sunnyvale, California). Values of **R** (= fluorescence intensity of bound S15~GFP(S65T)~His_6/total fluorescence intensity) were determined from the fluorescence intensities, and values of K_d were calculated from the equation:

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

Polarization assay

Fluorescence polarization was measured with a Beacon Fluorescence Polarization System (PanVera; Madison, Wisconsin) (Wittmayer & Raines, 1996). Purified S15~GFP(S65T)~His_6 (0.50 nM) was incubated at 20 °C (±2 °C) with various concentrations of S-protein (20 μM–1.0 nM) in 1.0 mL of 20 mM Tris-HCl buffer, pH 7.5, 8.0, or 8.5, containing NaCl (0 or 0.10 M). Five to seven polarization measurements were made at each S-protein concentration. Values of K_d were determined by using the program DeltaGraph 4.0 (DeltaPoint; Monterey, California) to fit the data to the equation:

$$P = \frac{\Delta P \cdot F}{K_d + F} + P_{\text{min}}. \quad (2)$$

In Equation 2, 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) was obtained from the equation:

$$f_B = \frac{P - P_{min}}{\Delta P}. \quad (3)$$

Acknowledgments

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