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Application of Fluorescence Resonance Energy Transfer to the GroEL–GroES Chaperonin Reaction

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Abstract

Fluorescence resonance energy transfer (FRET) is a sensitive and flexible method for studying protein-protein interactions. Here it is applied to the GroEL–GroES chaperonin system to examine the ATP-driven dynamics that underlie protein folding by this chaperone. Relying on the known structures of GroEL and GroES, sites for attachment of fluorescent probes are designed into the sequence of both proteins. Because these sites are brought close in space when GroEL and GroES form a complex, excitation energy can pass from a donor to an acceptor chromophore by FRET. While in ideal circumstances FRET can be used to measure distances, significant population heterogeneity in the donor-to-acceptor distances in the GroEL–GroES complex makes distance determination difficult. This is due to incomplete labeling of these large, oligomeric proteins and to their rotational symmetry. It is shown, however, that FRET can still be used to follow protein-protein interaction dynamics even in a case such as this, where distance measurements are either not practical or not meaningful. In this way, the FRET signal is used as a simple proximity sensor to score the interaction between GroEL and GroES. Similarly, FRET can also be used to follow interactions between GroEL and a fluorescently labeled substrate polypeptide. Thus, while knowledge of molecular structure aids enormously in the design of FRET experiments, structural information is not necessarily required if the aim is to measure the thermodynamics or kinetics of a protein interaction event by following changes in the binding proximity of two components.

Fluorescence spectroscopy is a powerful method for the study of biological molecules due to its exquisitely high sensitivity, specificity, and the wide variety of physical properties it can probe (1). For the analysis of protein–protein interactions, one very useful type of fluorescence spectroscopy is fluorescence resonance energy transfer (FRET). The specific utility of FRET rests in its ability to provide direct information on molecular proximity. In the most favorable instances, FRET can be used to accurately determine molecular distances over a range of 20–90 Å (2). In addition, dynamic alterations in these distances can be probed with extremely high time resolution. In general, FRET measurements are made by observing the transfer of excited state energy from one chromophore (the donor) to another (the acceptor). This “radiationless” transfer of energy is governed by long-range dipolar coupling between the transition state dipoles of the donor and acceptor, and the rate (or extent) of transfer is governed by a number of factors. These include the quantum yield of the donor, the extinction coefficient of the acceptor and the extent to which the acceptor’s absorption spectrum overlaps that of the donor’s emission spectrum, the relative orientation of the donor and acceptor transition dipoles, and the distance between the donor and acceptor chromophores (2). A number of outstanding reviews and monographs have been published in recent years covering the details of fluorescence theory, FRET, and its

applications (1–7), and the reader interested in greater technical detail than what is given here is referred to these sources. This article is intended as an introduction to the use of FRET for studying protein–protein interaction dynamics by providing a specific example of its application to the chaperone-mediated protein folding reaction of the *Escherichia coli* GroEL–GroES chaperonin system.

A GroEL–GroES protein folding reaction is a complex interplay of many protein–protein interactions (for review see Ref. 8). GroEL is composed of 14 identical 57-kDa subunits forming two heptamer rings stacked back to back, each with a large central cavity. The apical cavity surface of an open GroEL ring is lined with hydrophobic residues, which bind nonnative polypeptides, and collapsed protein folding intermediates that expose regions of hydrophobic surface (9–12). Subsequent binding of ATP and the cochaperonin GroES (a ring of seven 10-kDa subunits) to the same ring occupied by a nonnative protein displaces the substrate polypeptide into an enlarged and enclosed cavity and initiates folding (13–18). For substrates that strictly depend on GroEL and GroES to fold (so called “stringent” substrates), only a small fraction of the protein initially bound and encapsulated manages to fold to its native state before GroES and the polypeptide are dissociated from GroEL (19–21). This displacement reaction involves the coordinated binding and hydrolysis of ATP on the two GroEL rings (22–24). In general then, productive folding of an entire population of nonnative stringent substrates requires many rounds of polypeptide interaction with GroEL. The dynamic interactions between GroEL, GroES, and substrate polypeptide, and how ATP controls them, lie at the heart of how GroEL functions as a protein folding machine.

MATERIALS and METHODS

Design of Labeling Sites

GroEL and GroES do not possess intrinsic fluorophores that are useful for studying their interactions with one another or with substrate proteins. Neither protein has tryptophan residues, and while both proteins possess tyrosine, they are not well suited to study the specific interactions between GroEL and its ligands. It was therefore necessary to engineer versions of GroEL and GroES to which exogenous fluorophores could be coupled.

A number of considerations must be taken into account when designing sites for coupling exogenous probes to a protein. The first choice to be made is the nature of the coupling chemistry. In general, the most commonly used method to attach an exogenous compound to a specific site on a protein is through the thiol side chain of a cysteine residue. This is due to the high selectivity of the chemistry involved under aqueous conditions that are well tolerated by most proteins (25). One must therefore know the following: Is a cysteine residue at or near the desired site of protein–protein interaction, and does the protein possess cysteine residues that are reactive at other sites in the protein? If the answer to the first question is negative, then a mutation must be engineered into the protein at a site that satisfies at least two requirements: (1) it must be close (ideally within roughly 20–60 Å) to the site of protein–protein interaction and (2) substitution of cysteine at the chosen site should minimally perturb the stability of the protein and the interaction under study and, ideally, should not perturb any aspect of overall function. Finally, if the protein possesses other cysteine residues that can react with the chosen probe, either they must be removed by mutation (while minimally affecting overall protein structure and function) or conditions must be found that minimize or eliminate their reactivity.

Figure 1 shows the structure of the GroEL–GroES complex and the sites used on each protein for fluorophore attachment. GroEL has no cysteine residues in the vicinity of its interaction with GroES and the endogenous cysteine residues at positions 138, 458, and 519 are reactive to thiol-directed reagents. The production of a GroEL variant useful for FRET

experiments was thus conducted in two stages. First, the endogenous cysteines of wild-type GroEL were replaced with alanine (23) to generate a cysteineless version of GroEL (GroEL_{cys0}). GroEL_{cys0} is functionally equivalent to wild-type GroEL, showing only a small drop (30–40%) in steady-state ATPase activity. Then, based on the structures of unliganded GroEL and the GroEL–GroES complex, a number of positions were chosen that met the following criteria: (1) amino acids whose side chains were surface exposed, (2) side chains that did not appear to make important structural contacts, and (3) side chains that were close to the site of GroES binding. The coding sequence of each of these amino acids was changed to cysteine by standard techniques of cassette or polymerase chain reaction (PCR) mutagenesis. The replacement of a glutamate with a cysteine at position 315 was selected for further study since this substitution in the GroEL_{cys0} background caused no apparent perturbation in stability or function. These modifications resulted in a variant of GroEL (EL315C) with a single exposed cysteine on the back of each apical domain near the site of GroES and substrate binding (Fig. 1).

Similar logic led to the use of an already studied cysteine-substituted version of GroES, which was constructed by Yoshida and colleagues (26). Here a GroES variant was generated with one cysteine added to the C terminus of each subunit (ES98C; see Fig. 1.) For the substrate protein, ribulose-1,5-bisphosphate carboxylase–oxygenase (Rubisco) from *Rhodospirillum rubrum*, no alterations of the primary sequence were required since only one cysteine of this protein is surface exposed and reactive (at position 58) (14).

Protein Expression and Purification

Wild-type GroEL and EL315C were overproduced in *Escherichia coli* (BL21) from a *trc* promoter and purified essentially as described (9). GroES and ES98C were overproduced in *E. coli* (BL21DE3) from a T7 promoter and purified essentially as described (27). Great care was taken during the purification of EL315C and ES98C to maintain the samples under reducing conditions to prevent intermolecular disulfide crosslinking between the exposed cysteine side chains. Buffers always contained fresh 3–5 mM dithiothreitol (DTT) or 5–7 mM TCEP at each step in the purification and concentrated protein samples were always maintained under Ar. Following purification, aliquots of the proteins were flash-frozen in liquid N₂ and were stored for long periods at –75°C. On thawing, samples were also stored for short periods (3–4 weeks) at 4°C without apparent loss in stability or activity.

Rubisco was expressed at 20°C in BL21DE3 from a T7 promoter without IPTG induction and was purified as described (14). The purified Rubisco was divided into small aliquots and flash-frozen in liquid N₂. Samples were stored at –75°C and, once thawed, were maintained at 4°C for up to 2–3 weeks.

Other Reagents

ATP and ADP were obtained from Boehringer-Mannheim (Indianapolis, IN). 5-(((2-Iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAE-DANS), fluorescein-5-maleimide (F5M), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were obtained from Molecular Probes (Eugene, OR). Anhydrous *N,N*-dimethylformamide (DMF), TPCK-treated trypsin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma–Aldrich (St. Louis, MO). All other basic reagents were obtained from Sigma–Aldrich.

Protein Labeling

For labeling of EL315C with IAEDANS, a sample of the protein was exchanged into 100 mM potassium phosphate buffer (pH 7.4) and concentrated to approximately 5–10 mg/ml in a final volume of 10–15 ml. The sample was prereduced with 5 mM TCEP for 30–45 min, followed by addition (with stirring) of a 20- to 25-fold molar excess of IAEDANS (relative

to double rings). All dye stocks were prepared in anhydrous DMF immediately prior to use from dry powder. The reaction was allowed to proceed for 2 h in the dark at 23°C and was then quenched by addition of 5 mM glutathione. The labeled EL315C (EL315-D) was separated from unreacted dye by four rounds of dilution and concentration in a Centrprep 30, followed by gel filtration over a PD-10 desalting column (Pharmacia Piscataway, NJ). Alternately, dialysis can be used prior to sample concentration and gel filtration.

Labeling of ES98C and Rubisco with fluorescein-5-maleimide to produce ES98-A and Rub-A, respectively, was conducted essentially as outlined above. For a typical labeling reaction, the protein concentrations were approximately 10 mg/ml and the fluorescein-5-maleimide was added in 3- to 5-fold molar excess (relative to rings for ES98C or monomers for Rubisco).

Labeling ratios were estimated by absorption spectroscopy in 6 M GdmHCl at pH 7.4, using the following extinction coefficients for the conjugated dyes: IAEDANS, $5400 \text{ M}^{-1} \text{ cm}^{-1}$ at 336 nm; fluorescein-5-maleimide, $74,500 \text{ M}^{-1} \text{ cm}^{-1}$ at 492 nm. The extinction coefficients used for protein absorbance are (Rubisco monomer) $67,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm; (EL315C monomer) $9300 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm; and (ES98C monomer) $1200 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm. Protein concentrations can theoretically be established by correcting for the 280-nm absorbance of the conjugated dye alone under a standard set of conditions. However, if a labeled protein has a low extinction coefficient at 280 nm (like GroEL and GroES) and the conjugated dye has a significant absorbance at 280 nm, the protein concentration can be difficult to establish accurately by this method. For more exact determinations of labeling ratios, two additional procedures were used. First, the Bradford assay (Bio-Rad, Hercules, CA) was used to establish protein concentrations, because neither of the dyes employed in this study significantly interferes with this assay. This is not always the case, however, and must be checked for any dye used. For greatest accuracy, known concentrations of the unlabeled proteins were used to calibrate the Bradford assay.

The second method of examining the protein-labeling ratio used denaturing ion-exchange chromatography in 6 M urea with a MonoQ 5/5 ion exchange column (Pharmacia; see Fig. 2). Samples of the derivatized proteins were denatured in fresh 8 M urea and 4 mM DTT for 1 h at 25°C prior to loading onto a column that was equilibrated in Buffer E (50 mM Tris, pH 8.0, 2 mM DTT, 6 M urea). The column was developed with a linear NaCl gradient. Because both EDANS and fluorescein carry a formal negative charge at basic pH, protein subunits derivatized with either dye molecule tend to shift to higher elution positions in the salt gradient. The ratio of the peak areas of the labeled and unlabeled subunits can then be used to establish the labeling ratio.

For EL315-D, an average labeling of 3–4 dye molecules per tetradecamer was routinely achieved (Fig. 2). For ES98-A, labeling ratios of approximately 2 dye molecules per heptamer were typical. For Rubisco, labeling ratios of one fluorescein to one Rubisco monomer were obtained. All labeled proteins were stored in a manner identical to that for unlabeled proteins and displayed similar stabilities. EL315-D demonstrated ATPase activity identical to that of GroEL_{cys0} and, in conjunction with either wild-type GroES or ES98-A, refolded Rubisco in a GroES- and ATP-dependent manner that was identical (in both refolding yield and rate) to a wild-type GroEL–GroES reaction. Rub-A also showed wild-type enzymatic activity and was refolded by GroEL–GroES in an ATP-dependent manner that was identical to that of the unlabeled protein.

Labeling and Conformational Heterogeneity

GroEL and GroES are each large multimeric protein complexes and therefore present a set of unique problems for FRET experiments based on fluorophores coupled exogenously as

above (Fig. 3). Since both EL315C and ES98C are sevenfold symmetric, each protein ring has seven potential labeling sites and any labeling reaction that does not proceed to completion will result in a variety of partially labeled species (Fig. 3A). For the double-ring GroEL structure, this problem is even more significant. Assuming that conjugation on any given site does not affect coupling at neighboring sites, then the distribution of species is a simple statistical function of the extent of the reaction (Fig. 3B). Additionally, even with uniquely labeled variants of GroEL and GroES, the rotational degeneracy of the GroEL–GroES complex generates four distinct donor–acceptor populations (Fig. 3C).

Labeling and rotational heterogeneity would seriously complicate the use of FRET to measure specific donor-to-acceptor distances with GroEL and GroES. Fortunately, these problems can be ignored when FRET is used, not to measure specific molecular distances, but to extract thermodynamic or kinetic information about a protein–protein interaction. In this sense, the FRET signal is being used simply as a proximity sensor (28) and the donor–acceptor distance heterogeneity can be neglected. This results from the following considerations. Consider two different molecules that bind to one another: one labeled with a donor chromophore (D) and one with an acceptor chromophore (A). For simplicity, assume that in the complex there exists a unique distance between D and A. Under conditions of steady-state fluorescence excitation and at chemical equilibrium (or an enzymatic steady state), the extent of fluorescence quenching observed at the donor emission wavelength (I_0) will be constant and reflect the number of DA pairs:

$$I_0 = \frac{F_{DA}}{F_D} = \alpha[DA]. \quad [2]$$

Scheme [1] illustrates the steady-state fluorescence excitation of a donor–acceptor complex at equilibrium, where D^* and A^* reflect the excited states of each chromophore and $h\nu_1$ and $h\nu_2$ represent the excitation and emission photons, respectively. In Eq. [2] F_{DA} is the fluorescence of the donor in the presence of the acceptor, F_D is the fluorescence of the donor alone, α is a proportionality constant, and $[DA]$ is the concentration of the complex. Again, for simplicity, assume that the dissociation of the complex is a simple reaction. Then disassembly of the complex will cause the donor-labeled molecule to diffuse away from the acceptor molecule and the donor fluorescence will increase with time as the population of complexes falls apart:

$$I(t) = \alpha[DA]e^{-kt} = I_0e^{-kt}. \quad [4]$$

Now consider a solution with a distribution of donor and acceptor distances. The extent of total quenching observed is the sum of all the donor chromophore signals:

$$I_{\text{tot}} = \sum_{i=1}^n I_i. \quad [5]$$

Since any given quenched donor chromophore is brought within FRET distance of an acceptor by the same molecular binding event as all others, the time dependent behavior of each subpopulation is governed by the same chemical dynamics:

$$I(t) = \sum_{i=1}^n \alpha[\text{DA}]_i e^{-kt} = \sum_{i=1}^n I_i e^{-kt}, \quad [6]$$

$$I(t) = e^{-kt} \sum_{i=1}^n I_i = I_{\text{tot}} e^{-kt}. \quad [7]$$

Thus, the population heterogeneity of the donor-to-acceptor distance distribution can be neglected for the purposes of studying the time-dependent behavior of the reaction. Note that the same arguments outlined above can also be applied to changes in the acceptor fluorescence and to binding events. While the arguments above are framed for simple reactions, in principle they can be extended to chemical reactions of arbitrary complexity provided the observed FRET signal can be cleanly assigned to specific molecular events. For example, elegant methods have been devised to follow the chemical kinetics of enzyme reactions using FRET between an enzyme and its substrate (29, 30).

Establishing Unique Labeling

Once a protein has been derivatized with a fluorescent probe, it is important to establish that conjugation has taken place with the intended labeling site. This is critical if the derivatized proteins are to be used for FRET experiments in which distance information is to be extracted. However, even if the FRET experiment is designed only to provide proximity information, as detailed above, unique labeling is still an important consideration since dye conjugation on unintended sites can introduce complicating signals that are best avoided at the outset. Considering that even when a unique cysteine labeling site has been introduced, coupling chemistries like those based upon α -haloacetamides or maleimides can sometimes display reactivity toward other amino acid side chains (25), it is best to establish that a designed labeling site is unique.

To establish that EL315C, ES98C, and Rubisco were correctly and uniquely labeled on the intended sites, the labeled proteins were fragmented by protease treatment and the peptides analyzed by reversed-phase chromatography. Samples of the proteins were diluted to 0.3 mg/ml in 1 ml Buffer F (100 mM NaHCO_3 , pH 8.5, 1 mM CaCl_2) and supplemented with 0.4–0.5 mg/ml trypsin (a fresh 10 mg/ml stock was made immediately prior to use in 1 mM HCl, 20 mM CaCl_2). The samples were incubated overnight in the dark at 37°C. One-half of the reaction was treated with 1 mM PMSF and the remainder of the sample was frozen at –20°C. The PMSF-treated sample was supplemented with guanidinium hydrochloride (GdmHCl) to 1 M and dried down using a Speed Vac. The sample was then reconstituted in a small volume of Buffer G (10 mM sodium phosphate, pH 6.0), to a final GdmHCl concentration of approximately 7 M. The mixture was injected onto a C_{18} reversed phase column (218TP54; Vydac, Hesperia, CA) equilibrated in buffer G and the column was developed with a linear gradient using Buffer G, supplemented to 70% acetonitrile as the second buffer. Both absorbance and fluorescence were used to monitor the eluted peptide fragments (Fig. 4). A single peak in the fluorescence channel is a good indication of unique labeling, which can be further confirmed by sequencing or mass spectrometry of the identified peptide.

Instrumentation

The stopped-flow apparatus was constructed using as a template a design from the laboratory of Beechem (31). It consists of a Bio-Logic SFM/4 stopped-flow head (Molecular Kinetics, Pullman, WA), coupled to a 75-W mercury arc lamp and monochromator (Photon Technology International, South Brunswick, NJ) through a fused silica fiberoptic bundle

(Fiberguide Industries, Sterling, NJ) designed to provide a slit-shaped (3×0.66 mm) excitation beam directly to the face of the stopped-flow cuvette (FC.15). The two emission channels are collected in T-format through a pair of non-fiber, large-core-diameter, high-numerical-aperture (NA 0.47) light guides (Oriol; Stratford, CT) placed directly against the stopped-flow cuvette to maximize light collection. The collected fluorescence is spectrally filtered and detected with a pair of Hamamatsu PMTs (R4457P; Bridgewater, NJ) operated in photon-counting mode. Current pulses from the PMTs are amplified with an SR445 DC-300 MHz amplifier (Stanford Instruments, Sunnyvale, CA) and then discriminated and shaped with an SR400 two-channel photon counter (Stanford Instruments). The rectified output pulses from the photon counter are then detected with a pair of MCS-II multichannel scalar cards (Oxford Instruments, Oak Ridge, TN) in a Pentium II PC. The data acquisition sweep of the MCS-II cards is coordinated with the stopped-flow run by a triggering pulse from the stopped-flow controller. For a typical experiment, between 10 and 25 stopped-flow “shots” were sequentially summed in the MCS-II cards to enhance the signal-to-noise ratio. For FRET experiments with IAE-DANS and fluorescein, the excitation wavelength was set at 336 nm and both detection channels were fitted with long-pass barrier filters (KV370; Schott). The donor channel was configured with a blue separation filter (SWP filter, Reynard Corp.) and the acceptor channel with a green separation filter (Reynard). All stopped-flow experiments were conducted at 25°C in 50 mM Hepes (pH 7.6), 5 mM KOAc, 10 mM Mg(OAc)₂, and 2 mM DTT.

All analytical chromatography was conducted with a Waters HPLC system connected to an in-line ABI 980 fluorescence detector (Perkin-Elmer, Norwalk, CT). All fluorescence spectra were acquired with a PTI Quanta-master spectrofluorometer using a temperature-jacketed 1×1 -cm quartz cuvette.

EXAMPLE APPLICATIONS

Observation of GroEL-GroES Complex Formation by FRET

The ATP-dependent binding of GroES to a GroEL ring is central to the GroEL protein folding reaction. This interaction should be readily observable as a quench of the EL315-D signal and an enhancement in the ES98-A signal when the two proteins are mixed together in the presence of ATP. Figure 5 shows the spectra of EL315-D and ES98-A individually and mixed with and without ATP. The fluorescence decreases around 475 nm (the donor peak) and increases around 520 nm (the acceptor peak) only in the presence of ATP. In the absence of other spectral shape changes, this demonstrates that the binding of GroES to GroEL can be followed by FRET.

Dissociation of GroES from GroEL at Steady State

The observation of an ATP-dependent FRET signal between EL315-D and ES98-A suggested that these molecules could be used to directly follow the dynamics of the chaperonin reaction cycle. To confirm this, we measured the rate of GroES release from GroEL under conditions of steady-state ATP turnover. Previous experiments had indicated that this release reaction is limited by a single slow step (i.e., a single exponential decay law) with a well-defined rate constant (32). The FRET experiment we used to measure this reaction is outlined in Fig. 6A. A sample of EL315-D is mixed with ES98-A and a large excess of ATP. This mixture rapidly reaches a steady-state distribution of GroEL–GroES complexes that should bring the donor and acceptor probes into close proximity. When this sample is rapidly mixed in a stopped-flow with a large excess of unlabeled GroES, the much larger pool of unlabeled GroES replaces the ES98-A. Consequently, the kinetics of ES98-A release from the EL315-D should be measurable. As shown in Figs. 6B to 6E, when the unlabeled GroES is added, the fluorescence of the donor channel drops and the acceptor

channel rises. These changes not only track the expected trends for disruption of FRET in the GroEL–GroES complex, but the measured kinetics match the anticipated reaction order and time constant derived from previous studies of the GroES release reaction.

In these FRET experiments it is important to remember that, even though the donor and acceptor signals are not being used to calculate distances, the FRET data must still be corrected for intrinsic changes in the donor and acceptor quantum yields that are not due to FRET. For both EL315-D and ES98-F, there are small changes in each fluorophore signal during the dissociation reaction that are not the result of FRET interactions (donor-only and acceptor-only traces in Figs. 6B and 6D). These changes likely result from environmental alterations that occur around each chromophore during the structural rearrangements that accompany GroEL–GroES complex formation. Under conditions where distance information is desired, the FRET signal is typically converted into a transfer efficiency (E) (2, 28). For the donor signal,

$$E = 1 - \frac{F_{DA}}{F_D} \quad [8]$$

For the acceptor signal, additional corrections must often be applied to account for both leakage of the donor fluorescence into the acceptor channel and the direct excitation of the acceptor. One simple correction for direct excitation of the acceptor is given by

$$E = \frac{\varepsilon_A}{\varepsilon_D} \left(\frac{F_{AD}}{F_A} - 1 \right) \quad [9]$$

where ε_A and ε_D are the extinction coefficients of the acceptor and donor fluorophores, respectively, at the excitation wavelength, and F_A and F_{AD} refer to the acceptor fluorescence in the absence and presence of the donor. The transfer efficiency derived from either the donor or acceptor signals can then be used to calculate a separation distance between the donor and acceptor. For the experiments discussed here, the donor and acceptor signals are not converted to a formal FRET efficiency since distance information is not being sought. Instead, the donor and acceptor signals due to FRET are calculated by taking the F_{DA}/F_D or F_{AD}/F_A ratio and then normalizing the result.

The relative merit of using donor quenching versus acceptor enhancement to follow FRET is an important concern (28, 33, 34), especially with IAEDANS and fluo-rescein. The signal-to-noise ratio and reliability of the data obtained from sensitized acceptor fluorescence are typically inferior to those obtained from the donor fluorescence unless great care is taken to correct the acceptor fluorescence signal (33, 34). However, even without extensive donor spectral correction, the acceptor channel still provides very useful confirmation of changes observed in the donor channel and in favorable cases, such as the experiment shown in Fig. 6, can also provide accurate quantitative results. In any case, failure to correct for non-FRET changes in both the donor and acceptor signals can introduce significant errors into kinetic data obtained from FRET experiments such as those described here.

Binding of GroES and Rubisco to GroEL

By simply altering the contents of the stopped-flow apparatus and the mixing sequence, the same reagents can be used to examine many aspects of the GroEL reaction cycle. An example of a binding experiment is illustrated in Fig. 7. This experiment was designed to measure the rates of GroES and denatured substrate binding to unliganded double-ring GroEL (Fig. 7A). For the binding of GroES, a sample of EL315-D is rapidly mixed with ES98-A and ATP (Fig. 7B). By monitoring the drop in the donor signal on complex formation the binding reaction can be readily followed. By denaturing a sample of acceptor-

labeled Rubisco (dRub-A) and mixing it with EL315-D at a sufficient ratio to dilute away the denaturant, the rate of denatured substrate binding to GroEL can also be measured (Fig. 7C). In both cases, binding is rapid and the rate is dependent on the concentrations of the reactants. Therefore, the binding reaction is governed by the bimolecular rate at which the proteins encounter one another in solution.

CONCLUSIONS

FRET is a sensitive and versatile way to study many aspects of protein–protein interactions. In the most favorable cases, it can be employed to measure specific distances in a protein complex. However, even in circumstances when distance information is too difficult to obtain or is equivocal, FRET can be used as a powerful proximity sensor that can provide both thermodynamic and kinetic information about a protein–protein interaction. This utility of FRET as a simple molecular sensor has been greatly enhanced in recent years with the development of green fluorescent protein variants (GFPs) that are spectrally optimized for FRET and that can be easily fused to proteins of interest (35, 36). While this provides a simple method for tagging a protein of interest, and further permits a protein interaction to be studied *in vivo*, GFPs are folded protein domains of significant size (approximately 27 kDa). Not all proteins or their functions will tolerate such an addition, and thus chemical attachment of small fluorescent probes will remain a useful tool for studying many protein–protein interactions by FRET.

A number of considerations must be taken into account when using chemically coupled fluorophores for FRET experiments, and the example(s) described above benefited enormously from having available the crystal structures of the proteins under study. Knowing the molecular structure of a protein certainly permits a more rational approach to the design of innocuous and useful sites for the attachment of fluorescent probes. However, if measurement of specific distances is not being attempted, significant heterogeneity in the donor-to-acceptor distance distribution can be tolerated. Consequently, FRET between extrinsic fluorescent probes can be applied to a wide variety of protein interaction problems even in the absence of a known molecular structure.

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FIG. 1. Positions of the fluorophore labeling sites in the GroEL–GroES complex. The positions of the donor chromophore attached to GroEL (EDANS) and the acceptor chromophore attached to GroES (F5M) were modeled into the structure of the GroEL–GroES complex (16) using InsightII (Molecular Simulations, San Diego, CA). While the exact orientations of the dyes are only speculative, their effective separation is constrained in the GroEL–GroES complex to within 35–40 Å. The GroES subunits are colored light green and the GroEL subunits are colored purple. *Inset:* Wider view of the entire GroEL–GroES complex.

FIG. 2.

The protein-to-dye labeling ratio can be examined by denaturing ion exchange chromatography. A sample of EL315C labeled with IAEDANS was purified and then denatured in 8 M urea. This sample was injected onto a Mono Q 5/5 column equilibrated in buffer containing 6 M urea, and the column was developed with a linear NaCl gradient (see Materials and Methods). The elution positions of the unconjugated EL315C subunits and those that have been labeled with IAEDANS (EL315-D) are indicated. Top: Observed absorbance at 229 nm; bottom: observed fluorescence at greater than 425 nm with excitation at 336 nm. Note that the protein peak corresponding to the EL315-D subunits is fluorescent.

The labeling ratio indicated by the peak areas in the upper panel is 0.3, or approximately 4 dyes per tetradecamer on average.

FIG. 3.

The oligomeric structures of labeled GroEL and GroES introduce significant complexity in the donor–acceptor distance distribution. (A) Labeling of a 7-subunit ring produces a wide variety of conjugated species if the reaction does not proceed to completion. (B) The theoretical distribution of labeled species from a 14-subunit protein is plotted as a function of the number of labeled sites. The distribution of labeled species is assumed to be Poisson and plots are shown for average labeling of 1, 4, 8, 10, and 12 labels per oligomer. A sample of GroEL–GroES complexes created from incompletely labeled proteins would possess a similar distribution of donor-to-acceptor distances. (C) The rotational symmetry of the

GroEL–GroES complex adds an additional layer of complexity to the donor–acceptor distance distribution.

FIG. 4.

The specificity of labeling is established by reversed-phase chromatography of a protease-fragmented sample. A sample of EL315C labeled with IAEDANS was digested with trypsin and then loaded onto a C_{18} reverse-phase column (see Materials and Methods). Top: Absorbance at 229 nm; bottom: fluorescence at greater than 425 nm with excitation at 336 nm. Note that a single peptide of the many generated by the EL315-D tryptic digest is fluorescent, indicating that the site of fluorophore conjugation is unique.

FIG. 5.

Changes in the fluorescence intensity of EL315-D and ES98-A in the presence of ATP demonstrate that complex formation can be observed by FRET. Top: Fluorescence spectra of EL315-D (500 nM) and ES98-A (500 nM), both individually and mixed together. Bottom: Spectra of EL315-D and ES98-A mixed together in the presence and absence of 5 mM ATP. Note the decrease in fluorescence around 425 nm and the enhancement of fluorescence around 520 nm when ATP is added to the mixture, indicating the transfer of excitation energy from the IAEDANS donor to the fluorescein acceptor in the GroEL–GroES complex. For all spectra, the excitation wavelength was 336 nm, the temperature was

maintained at 25°C, and the buffer used was 50 mM Hepes, pH 7.6, 5 mM KOAc, 10 mM Mg(OAc)₂, 4 mM DTT.

FIG. 6.

Dissociation of ES98-A from EL315-D during steady-state ATP turnover. (A) Schematic of experiment in which EL315-D is first mixed with ES98-A and ATP and allowed to come to a steady state, after which the sample is rapidly mixed in the stopped flow with an excess of unlabeled GroES (gray disk). The loss of energy transfer as ES98-A is replaced by unlabeled GroES is monitored as an increase in the donor fluorescence intensity and a decrease in the acceptor fluorescence intensity. For this experiment, 188 nM EL315-D was premixed with 175 nM ES98-A and 5 mM ATP and loaded into one stopped-flow syringe. The steady-state mixture was then mixed (4:1) with 7.5 μ M GroES in the stopped-flow. The rate of ES98-A release can be monitored as either the dequenching of the donor (B, C) or loss of

fluorescence of the acceptor (D, E). Control experiments in which one of the energy transfer partners was replaced with its unlabeled conjugate are shown in (B) and (D), where “D” designates EL315-D alone, “A” designates ES98-A alone, and “D+A” indicates the use of donor and acceptor labeled proteins together. For the “D”-only trace, the initial steady-state mix was made with EL315-D and unlabeled GroES, with unlabeled GroEL used as the competitor. For the “A”-only trace, the initial steady-state mix was made with ES98-A and unlabeled GroEL, with unlabeled GroES used as the competitor. Note that the plots in (B) and (D) have been scaled and offset so that trends in the donor-only and acceptor-only traces can be visualized on the same graph with the donor plus acceptor trace. As a result, the changes in the “D” and “A” traces appear exaggerated relative to the “D+A” traces. The corrected FRET signals are shown in (C) and (E). In both channels, the data are fit by a single exponential model (shown as a thin white line superimposed on each relaxation curve: for (C), $k_{\text{slow}} = 0.031 \pm 0.0001 \text{ s}^{-1}$ and for (E) $k_{\text{slow}} = 0.029 \pm 0.0001 \text{ s}^{-1}$). The measured rate constant does not change at different ratios of ES98-A to EL315-D, is not dependent on the salt concentration (up to 250 mM KCl), and is independent of the concentration of competitor GroES used (not shown). The data for this figure were taken from (23) with permission. © Cell Press 1999.

FIG. 7.

Binding of ES98-A and Rubisco-A to EL315-D. (A) Schematic of binding experiments used to monitor the rate of ES98-A and dRub-A association with unliganded GroEL. The promotion of FRET as an EL315-D ring binds either ES98-A or dRub-A results in a decrease in the donor fluorescence. (B) Binding of ES98-A to EL315-D at equimolar concentrations. In the upper trace, ES98-A and EL315-D were mixed at final concentrations in the stopped-flow of 50 nM each, and in the lower trace at 1 μ M; ATP in each case was 2 mM. The bimolecular rate constants derived from these traces are 5.7×10^7 and 2.9×10^7 $\text{M}^{-1} \text{s}^{-1}$ for the 50 nM and 1 μ M traces, respectively. (C) Binding of dRub-A to EL315-D. The upper curve was generated at final concentrations of 100 nM dRub-A and 100 nM

EL315-D. The lower was produced at a concentration of 1 μM in each component. Bimolecular rate constants were estimated from the 100 nM traces only ($k \sim 1 - 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), due to a competing aggregation reaction of the dRub-A at higher concentrations. The data for this figure were taken from (23) with permission. © Cell Press 1999.