Environment-sensitive Labels in Multiplex Fluorescence Analyses of Protein-DNA Complexes*

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Fluorescein is widely used for protein labeling because of its high extinction coefficient and fluorescence emission quantum yield. However, its emission is readily quenched by various pathways. We exploit these properties of fluorescein to examine the self-association of a DNA binding protein and determine the amount of the protein in gel-shifted complexes with specific DNA. A construct (HSFDT385S-Fl) of the heat shock transcription factor (HSF) was expressed that contains the DNA-binding and trimerization domains, residues 192–385 of HSF, with four additional COOH-terminal residues, GMLC, and then labeled at the COOH-terminal cysteine with fluorescein 5-maleimide to form HSFDT385S-Fl. The fluorescence increase accompanying the formation of heterotrimers on titration of HSFDT382S-Fl with HSFDT385SH led to an estimate of $3 \times 10^{-16}$ m$^3$ for the equilibrium constant for trimerization of HSFDT385SH. HSFDT fluorescence also increased 1.7-fold on binding to specific DNA, but not to nonspecific DNA. The protein and DNA content of the several gel-shifted complexes of HSFDT382S-Fl (4) with HSFDT385SH with specific DNA labeled noncovalently with the energy transfer heterodimer TOTAB ($\lambda_{\text{em}}$ 658 nm) were accurately determined by a two-color fluorescence emission assay with 488 nm excitation.

The “gel mobility shift” or “band-shift” assay is widely used to detect and analyze protein-DNA interactions. The most direct way to determine the stoichiometry of protein-DNA complexes resolved in such experiments is to measure independently the amounts of both DNA and protein in a band on a gel. Quantitation of DNA in the shifted complex is relatively straightforward with $^{32}$P end-labeling, but accurate measurement of protein can be quite challenging. Protein quantities in shifted complexes have been measured in double-labeling experiments using Coomassie staining, Western blot, and radioisotopic labeling of the protein with $^3$H, $^{125}$I, and $^{35}$S (1–7). Drawbacks of these methods include limitations on sensitivity, difficulty in ensuring accurately the specific activity of radioactive-labeled proteins, and the need to handle the gel after electrophoresis for staining or blotting, or to cut out gel bands for scintillation counting. Consequently, few accurate determinations of the molar ratios of protein-to-DNA have been reported.

We have shown that multiplex fluorescence analysis is a superior alternative method of characterizing protein-DNA complexes (8). In our earlier work, we described the first detailed analysis of the application of fluorescent polycationic intercalating dyes with high affinity for double-stranded DNA to the study of protein-DNA interactions. Such intercalating dyes, in conjunction with high sensitivity laser-excited, confocal, fluorescence scanning systems, allow detection of double-stranded DNA in agarose or acrylamide gels with a sensitivity comparable to that of autoradiography (9–12). We used such dyes in gel mobility shift assays to detect the multiple protein-DNA complexes formed by the heat shock transcription factor (HSF)$^1$ (8, 13, 14). Using a truncation of Kluveromyces lactis HSF that contains the DNA-binding and trimerization domains (HSFDT), we were able to detect fluorescent dye-labeled HSFDT-DNA complexes with a spatial resolution superior to that of conventional autoradiography, and therefore we were able to analyze multimer protein-DNA complexes that are not resolved by traditional methods. An analysis of the mobilities of the multiple HSFDT-DNA complexes, indicated that HSF forms multimeric complexes on DNA by the addition of trimeric units (8).

To measure the absolute quantities of protein and DNA in each complex, we developed a two-color mobility shift fluorescence assay with a mutant HSFDT engineered for site-specific labeling with fluorescein and target DNA labeled with thiazole orange-thiazole blue heterodimer (TOTAB), an “energy transfer” dye (15, 16). A technical difficulty was encountered in this work. The particular HSFDT-fluorescein derivative used in those experiments had a much lower affinity for DNA than the unlabeled protein. This property, presumably due to some form of steric interference by the fluorescein, resulted in poorer resolution of complexes and increased background. This factor decreased the precision of the quantitation of the DNA-bound protein.

We pointed out that the problem was attributable to the properties of a particular fluorescent protein derivative and was not a feature of the fluorescence methodology as such (8). This view is supported by the studies described here. We have

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1 The abbreviations used are: HSF, heat shock factor; bp, base pairs, HSE, heat shock element; HSFDT, a portion of the K. lactis HSF containing the DNA-binding and trimerization domains; HSFDT385SH, an HSF truncation which contains HSF residues 102–384 and a COOH-terminal extension, Cys-Leu-Met-Asn; HSFDT382S-Fl, a derivative of HSFDT382S-Fl in which the Cys residue has been reacted with fluorescein 5-maleimide; HSFDT385S-Fl, an HSF truncation which contains HSF residues 191–385 and a COOH-terminal extension Gly-Met-Leu-Cys; HSFDT382S-Fl, a derivative of HSFDT382S-Fl in which the Cys residue has been reacted with fluorescein 5-maleimide; TOTAB, thiazole orange-thiazole blue heterodimer.
overcome the earlier difficulties by creating a new HSF truncation, which is site-specifically labeled with a fluorescein at a different location than the protein truncation previously described (8). The new fluorescein-bearing construct shows the same affinity for target DNA as does HSF<sub>DT</sub>. This allowed accurate multiplex determination of the composition of protein-DNA complexes containing multimers of HSF<sub>DT</sub>. Moreover, the sensitivity of the fluorescein label to its environment also provided information about the oligomerization of the labeled protein and its interaction with the target DNA.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions—Oligonucleotide-mediated site-directed mutagenesis** (17) was used to change the sequence adjacent to the single Spal site of a pET3b (18) derived overexpression vector so that a gene cloned in-frame into this site produces a protein with the addition of a 4-residue sequence GMLC at its COOH-terminal end. A BamHI-SacI restriction fragment from the K. lactis HSF gene (19) was cloned into pBlueScript KS<sup>®</sup> (Stratagene). Oligonucleotide-mediated site-directed mutagenesis was used to create an NdeI site, changing amino acid residue 191 to a methionine, and to create a Spal site immediately after the codon for amino acid residue 385. The mutations were confirmed by restriction digests and sequencing. The NdeI-Spal fragment from this plasmid was cloned into the overexpression vector pET3b, to give HSF<sub>DT</sub>SH, with fluorescein 5-maleimide (Molecular Probes) incorporated in this site-specifically labeled with a fluorescein at a specific target DNA, as does HSF<sub>DT</sub>. This allowed accurate multiplex determination of the composition of protein-DNA complexes containing multimers of HSF<sub>DT</sub>. Moreover, the sensitivity of the fluorescein label to its environment also provided information about the oligomerization of the labeled protein and its interaction with the target DNA.

**Gel Retardation Assays—** HSF recognizes heat shock elements (HSEs), which contain multiple, inverted repeats of the 5-bp NGAAN sequence, where N can be any nucleotide (20, 21). Gel retardation experiments showed that the labeled HSF<sub>DT</sub>-DNA complex on the gel was then calculated by dividing this number by the molecular weight of the specific DNA fragment. Simultaneously, emission data were collected at 532 nm. The emission at this wavelength derives mainly from the fluorescein label with a small contribution from the TO emission of the DNA-bound TOTAB. Since the ratio of TOTAB emissions at 532 and 645–670 nm is known from measurements on the dX174 DNA.HaeIII-TOTAB standard ladder, the 532-nm measurement was corrected for the TOTAB contribution; the residual emission originates from the fluorescein label.

It was not possible to run standard amounts of HSF<sub>DT</sub>-<sup>385S-Fl</sup> on the gel used to resolve the HSF<sub>DT</sub>-<sup>385</sup>S-Fl-DNA complexes. The free protein smeared on electrophoresis under these conditions and could not be accurately quantitated. To establish a protein calibration curve, varying amounts (0.2, 0.5, 1, and 2 ng) of HSF<sub>DT</sub>-<sup>385S-Fl</sup> were combined in a final volume of 24 µl in each case with a 50-fold molar excess of a 143-bp DNA target fragment, to ensure complete protein binding. These mixtures were loaded into a microtiter tray and scanned at 532 nm with the confocal scanning system at the same time as the gel. The scanner settings and the sampled volume were identical for the gel and the microtiter plate (12). Appropriate background corrections were applied to both sets of data. The fluorescein signal was plotted versus moles of fluoresceinylated protein. The resulting curve provides the signal intensity per mole of HSF<sub>DT</sub>-<sup>385S-Fl</sup> when the protein is specifically bound to DNA. This calibration curve was used to calculate the total amounts of HSF<sub>DT</sub>-<sup>385S-Fl</sup> in the complexes resolved in the gel mobility shift experiments.

**Fluorescence Analyses of Protein-DNA Complexes**

**RESULTS**

**Binding and Fluorescence Properties of HSF<sub>DT</sub>-<sup>385S-Fl</sup>—** Our previous analysis of HSF<sub>DT</sub>-<sup>385</sup>-DNA-TOTAB complexes (8) indicated that fluorescein methodology could be used to determine the absolute content of protein and DNA. However, the accuracy of our earlier determinations of the amount of protein in the complex was limited by the much lower affinity of HSF<sub>DT</sub>-<sup>385</sup> relative to that of HSF<sup>385</sup> for the target DNA. We have addressed this problem by changing the position of the fluorescein label. The previous HSF truncation, which we have renamed HSF<sub>DT</sub>-<sup>385S</sup>-DNA, contained K. lactis HSF residues 102–394, as well as a carboxyl-terminal extension of four residues, Cys-Leu-Met-Asn, with the fluorescein attached to the Cys (Fig. 1A). For our new construct, HSF<sub>DT</sub>-<sup>385S-Fl</sup> contained K. lactis HSF residues 102–394, with a fluorescein attached to the Cys (Fig. 1B). This new truncation was quantitatively labeled at the COOH-terminal cysteine residue by reaction with fluorescein-5-maleimide (Fig. 1C), to give HSF<sub>DT</sub>-<sup>385S-Fl</sup>. Comparison of labeled and unlabeled protein showed that the fluorescein label did not decrease the affinity of the protein for DNA. Figure 2). This suggests that the

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*R. Peteranderl, personal communication.*
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![Diagram of protein structures]

**Fig. 1. Comparison of the HSF\textsuperscript{394S-Fl} and HSF\textsuperscript{385S-Fl} truncations.** Both proteins, HSF\textsuperscript{DT}\textsuperscript{394S-Fl} and HSF\textsuperscript{DT}\textsuperscript{385S-Fl}, contain the DNA binding and trimerization domains of \textit{K. lactis} HSF, with different COOH-terminal truncations. The four residues added to the carboxyl terminus of each protein are shown with the single engineered cysteine residue highlighted. C, structure of the fluorescein 5-maleimide adduct with a cysteine residue.

fluorescein has no effect on the structure or function of the protein, and that this truncation and its labeled version, are superior to HSF\textsuperscript{DT}\textsuperscript{394SH} and HSF\textsuperscript{DT}\textsuperscript{394S-Fl}, respectively, for the study of protein-protein and protein-DNA interactions.

**Dependence of the Fluorescence Emission Intensity of HSF\textsuperscript{385S-Fl} on the Proximity of the Fluorescein Labels within the Trimer—**The fluorescence emission of native HSF\textsuperscript{385S-Fl} trimer in 0.25 M TBE was only 0.2 of that of the protein that had been boiled in 1% (w/v) sodium dodecyl sulfate-containing solution (data not shown). The fluorescence emission is thus severely quenched in the native trimer. The quenching may be due to the interactions of the fluorescein with protein residues, or to excimer interactions between the three fluorescein labels within a trimer, or to both types of interactions. We show here that a significant fraction of the quenching is due to fluorescein-fluorescein interactions.

The fluorescein tag in HSF\textsuperscript{385S-Fl} is located at the COOH-terminal end of the trimerization domain. Recent studies have suggested that the trimerization domain is a long \(\alpha\)-helical coiled-coil, with the individual COOH-terminal ends of each subunit within the trimer proximal to each other. This suggests that the reduced fluorescence quantum yield could in part be due to quenching arising from direct interaction between the fluorescein labels.

To examine this possibility we have titrated the labeled protein with a large excess of unlabeled protein (molar ratio of labeled-to-unlabeled protein of 1000:1). In such a mixture, monomer exchange between labeled and unlabeled trimers ensures that no trimer will have more than one labeled monomer. Such “hybrid trimer” formation, leads to a 1.4-fold increase in fluorescence emission. Thus, approximately 10% of the decrease in the fluorescence emission of native versus denatured HSF\textsuperscript{385S-Fl} is attributable to the presence of multiple fluoresceins in this trimer. The fluorescence emission quantum yield of the labeled protein is high; consequently, a 10% change is easily detected.

**Quantitation of Trimerization Equilibrium Constant—**We can exploit the fact that we have a quantifiable way of differentiating homo- and heterotrimers to calculate the trimerization constant. The increase in fluorescence emission intensity upon titration of HSF\textsuperscript{385S-Fl} with HSF\textsuperscript{385Sh} is directly related to \(c\), the ratio of the concentration of labeled subunits bound in mixed trimers to the total concentration of labeled monomer. Two assumptions are made. First, we assume that HSF oligomerization involves the formation of trimers, with no significant population of dimeric intermediates. This assumption is validated by the analysis of sedimentation equilibrium data which show an excellent fit to a monomer to trimer equilibrium (data not shown). This means that the equilibrium constant can be defined simply as \(K\), which is equal to the cube of the free monomer concentration divided by the free trimer concentration.

The second assumption is that the monomer-trimer equilibrium constant is not affected by the presence of the label on HSF\textsuperscript{385Sh}. Although we have not measured this directly, the fact that the labeled HSF has the same apparent affinity for DNA as the unlabeled HSF supports this assumption. Given these two assumptions, the following equation, relating \(c\) to the equilibrium constant \(K\), can be derived as,

\[ c = \frac{(KU_i)^2}{K + (KU_i)^3} \]  

where \(U_i\) is the concentration of unlabeled trimers. Fig. 3 shows a plot of \(c\) (expressed as a normalized fluorescence change) versus the concentration of unlabeled trimer, \(U_i\). The best fit of these data is \(K = 3 \times 10^{-16} \text{ M}^2\), with a correlation coefficient of 0.995.

**Quantitation of HSF\textsuperscript{385S-Fl} Emission Change upon Interaction with Specific DNA—**The experiments described above indicate that the fluorescence of HSF\textsuperscript{385S-Fl} is sensitive to the mutual proximity of the fluorescein labels. To examine and quantitate any changes in the fluorescence of the labeled trimer that might take place upon binding to target DNA, we measured the fluorescence emission of known amounts of HSF\textsuperscript{385S-Fl} incubated with molar excess amounts of DNA.
fragment containing an HSE with four NGAAN inverted repeats. In Fig. 4A, we compare the relative fluorescence emission at 532 nm/mol of protein of HSFDT385S-Fl in the absence of DNA and in the presence of 10-, 20-, 50-, and 100-fold molar excess of DNA fragment. The fluorescein emission intensity increases by 60% with increasing DNA concentration up to a plateau at 50-fold molar excess DNA. No change in labeled protein emission was seen in the presence of nonspecific DNA. The protein concentration is in the range used in the gel shift assays and this plateau indicates the DNA concentration at which all of the protein is bound as trimers to the target DNA. As a validation of the calibration curve, one can estimate half-maximal binding at around a monomer concentration of $5 \times 10^{-8}$ M, which is similar to that seen in the gel shift assay (Fig. 2). The plot of fluorescence emission at a constant 50-fold molar excess of DNA over protein is linear with the HSFDT385S-Fl concentration over a 9-fold range in labeled protein concentration (Fig. 4B).

The increase in the quantum yield of the fluorescein emission of HSFDT385S-Fl on binding to specific DNA may be due either to greater constraints on the motion of the fluorescein labels when the trimer is bound to DNA, or the changes in the spacing and/or environment of the fluorescein labels, or to all of these factors. However, the observed fluorescence enhancement is intriguing since the labels are at a site distant from the DNA-interaction domain.

**Direct Determination of Protein-DNA Stoichiometry**—Protein-DNA stoichiometry was determined in two-color mobility shift experiments as described in detail under “Experimental Procedures.” Such an experiment is shown in Fig. 5. Here, target DNA labeled with TOTAB at 40 bp/dye, was titrated with increasing amounts of HSFDT385S-Fl. The fluorescein emission was detected in the “green” channel (500–565 nm), and the DNA-TOTAB emission in the “red” channel (645–700 nm). The contribution of the fluorescein emission to the red channel was negligible (for example, compare lanes 6 in Fig. 5, A and B). A correction for the contribution of the TOTAB emission to the green channel was determined by comparing the signal in the red versus the green channel from the φX174 DNA/HaeIII molecular weight ladder stained at 40 bp/dye with TOTAB and run on the same gel.

For each protein-DNA mixture, we quantitated the amount of labeled DNA fragment in each HSFDT385S-Fl-DNA complex by comparing its emission in the red channel to the emission of known amounts of labeled DNA in the φX174/HaeIII ladder run on the same gel. The amount of HSFDT385S-Fl in each complex was quantitated from the calibration curve established in Fig. 4B. These two calibrations allowed us to determine directly the stoichiometry of the first three HSF-DNA complexes. The results (Table I) indicate that the first three complexes formed by HSF binding to an HSE contain, respectively, three, six, and nine HSF monomers per DNA molecule. It is difficult to quantitate accurately the fluorescence of complexes containing more than nine subunits of HSF, because the high concentrations of fluorescein-labeled protein used cause smearing in the gel and high background in the fluorescein channel. Our results confirm those of Sorger and Nelson (14), which showed indirectly that HSF binds DNA as trimer and our previous work, which showed that the larger multimeric complexes formed by HSF on DNA are multimers of trimers (8).

**DISCUSSION**

This study shows that accurate determination of the stoichiometry of prelabeled protein-DNA complexes in gel mobility shift experiments can be achieved by measuring simultaneously and independently the amounts of both DNA and pro-
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In a two-color multiplex experiment. The labeled HSF truncation we studied earlier (8), HSF\textsuperscript{[385S-Fl]}, showed impaired binding to specific DNA. Moreover, the decrease in binding affinity was directly attributable to the presence of the fluorescein label. This change in binding properties of the protein compromised attempts at two-color analysis. In the new truncation used here, HSF\textsuperscript{[385S-Fl]}, the label does not affect the DNA-binding properties of the protein. It appears that interference by protein fluorescent labels that might be encountered when this methodology is applied to other protein-DNA complexes can be readily overcome by varying the site of label attachment.

The fluorescence emission quantum yield of the fluorescein label varies with the number of fluorescein-labeled monomers in a HSF\textsuperscript{DT} trimer and changes when HSF\textsuperscript{[385S-Fl]} binds to specific DNA. These variations in fluorescence emission need to be taken into account in determining the stoichiometry of protein-DNA complexes. They also provide a valuable probe of protein-protein and protein-DNA interaction.

We developed a simple microtiter well assay to establish a calibration curve relating the fluorescence of native labeled protein in the presence of an excess of its specific DNA binding site against protein concentration. We found that the fluorescence emission for DNA-bound trimers was 40% greater than the fluorescence emission of the labeled protein. With this calibration curve, we could directly determine the stoichiometry of the first protein-DNA complex in the gel shift assay as three monomers per one DNA, in agreement with the results obtained indirectly from mixed subunit experiments (14). We also used the same calibration curve to calculate the HSF concentration in the larger protein-DNA complexes. These calculations may not be fully accurate because we do not know how binding of multiple HSF trimers to the same binding site may affect fluorescence emission. Nevertheless, the calculations gave values in agreement with previous results; that is, that HSF bound in higher complexes in multimers of trimers (8). This suggests that the changes in fluorescence emission are similar for the consecutive binding of labeled HSF trimers to form higher order complexes.

The sensitivity of the fluorescein label to the proximity of multiple labels and to protein conformation and dynamics is advantageous. Here, we have exploited the difference in fluorescence emission between a fully labeled trimer and a partially labeled trimer to determine the equilibrium constant for trimerization, 3 × 10\textsuperscript{-16} M\textsuperscript{−1}. With this value, less than 5% of the protein would be monomeric at 1 μM concentration, in agreement with analytical ultracentrifugation results on a similar preparation. We have not defined the cause(s) of the fluorescence increase, but point out that such fluorescence changes, linked to the formation of a specific protein-DNA complex, might form the basis of a highly sensitive homogeneous solution assay for the presence of a particular single- or double-stranded DNA sequence in the presence of large excess of nonspecific DNA.

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