Spectral Properties and DNA Targeting Features of a Thiazole Orange−Peptide Bioconjugate

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The molecular recognition features of a DNA-sensitive fluorescent bioconjugate capable of targeting a specific DNA sequence with high efficiency are described. The bioconjugate combines a polypeptide from the Tc3 transposase DNA-binding domain with the dsDNA-sensitive fluorophore thiazole orange. Fluorescence spectroscopy and circular dichroism reveal that the polypeptide moiety determines the DNA sequence specificity as the intercalating dye makes nonspecific contributions to binding affinity. The conjugated thiazole orange is able to intercalate and fluoresce when the peptide binds at concentrations where little fluorescence is observed from either the bioconjugate alone or the bioconjugate mixed with DNA lacking the target sequence. Fluorescence studies indicate this molecular probe is sequence specific, binds the native Tc3 DNA target sequence with nanomolar affinity ($K_D \approx 15$ nM), and is able to discriminate multiple point mutations in the cognate DNA site. The attachment of a sequence-specific binding peptide onto a functional probe provides a viable strategy for construction of synthetic enzymes and repressors, and facilitates dynamic studies of protein−DNA interactions.

Introduction

Ongoing efforts to design molecular probes of DNA to study molecular recognition events, identify cognate sequences, and quantify binding affinities have applied various strategies to localize functional groups to specific DNA sites. Such innovative targeting approaches include the minor groove binding polyamides,[1] DNA hybridizing peptide nucleic acids (PNA),[2] and the inherent sequence selectivity of DNA-binding proteins.[3,4] DNA-binding proteins have been shown to rapidly and selectively recognize a single high affinity site, and oftentimes the DNA-binding domain alone is able to bind the cognate site with high affinity.[5] Although recognition helices in the absence of other segments of the protein retain high specific to nonspecific DNA-binding ratios, they have binding affinities too low to be useful as molecular probes.[6−9] The use of intercalating dyes that not only report the binding state of the recognition helix, but also anchor the peptide at a specific DNA site has been shown to significantly enhance binding affinity.[6,9] Previous studies of a bioconjugate probe composed of a single zinc finger from the glucocorticoid receptor protein linked to a thiazole orange (TO) molecule concluded that DNA sequence specificities and affinities comparable to the native protein could be achieved.[10] Recent studies of the intercalating dye oxazole yellow linked to a segment from the Hin recombinase DNA-binding protein suggest that the intercalating dye makes nonspecific contributions to binding affinity that resulted in a decrease in the equilibrium dissociation constant by an order of magnitude.[11] The dye did not affect sequence specificity, and as such DNA site targeting was exclusively a function of the polypeptide moiety.

The Tc3 transposase DNA-binding domain used in these experiments is derived from residues 202−253 of the native Tc3 (Figure 1). Tc3 transposase of *C. elegans* is a member of the Tc1/mariner family of transposable elements (transposons).[12]

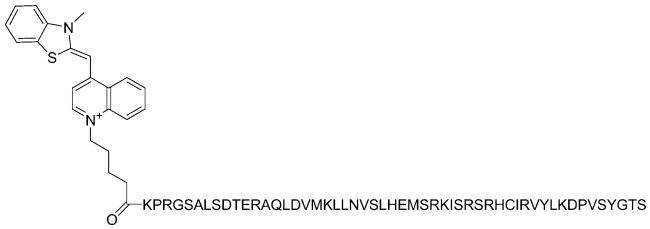

Figure 1. Peptide sequence and thiazole orange structure for the bioconjugate. The amino acid sequence derived from residues Pro202 to Ser253 of the native Tc3 transposase was tethered to the cyanine dye thiazole orange.

The transposase enzyme is itself encoded by the transposable element, which it translocates throughout the genome. Crystal structure data of the Tc3 DNA-binding domain complexed with cognate DNA show an α-helix containing the majority of noncovalent contacts for sequence recognition positioned within the major groove of DNA. Flanking this recognition helix, a short stretch of amino acids at the N-terminus are positioned within the minor groove, further stabilizing the protein−DNA complex by acting as a "thumb" to grip the DNA.[13] This N-terminal region was chosen as the location for TO conjugation to force the dye into a defined region within the minor groove and minimize the adverse affects of intercalation on DNA structure and peptide binding.

The cationic cyanine dyes, which include thiazole orange and oxazole yellow, have a large emission enhancement upon dsDNA intercalation (approximately 2000-fold), bind DNA with moderate affinity ($K_D \approx 10^{-6}$ M), and interact with DNA in a sequence neutral manner.[11,14−17] The generally accepted view is that the nonradiative decay of photoexcited cyanine dyes is controlled by the rate of rotation about the central methine bridge, which links the heterocycles (Figure 1). This mechanism also contributes to the nearly 50-fold increase in lifetime of the dye from 70 ps when free in solution to 3 ns upon intercalation within the DNA base stack.[15] The bioconjugate described here was designed with the highly fluorescent intercalating dye

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thiazole orange appended to the polypeptide in such a way as to use the inherent DNA affinity of the dye to anchor the polypeptide at the DNA target site.

The recognition helices from many DNA-binding proteins retain the majority of the sequence-specific contacts with DNA, and yet bind with an affinity too low to be useful as molecular probes. The appended intercalating dye makes a significant contribution to the binding affinity; yet because it has no inherent sequence preference, the dye does not adversely affect the specificity conferred by the polypeptide. Essentially, the dye compensates for the nonspecific contributions to protein−DNA complex stabilization imparted by other regions of the protein not present in the peptide bioconjugate. Here, the DNA targeting and photophysical properties of a fluorescent bioconjugate based on the Tc3 DNA-binding polypeptide and the intercalating dye thiazole orange will be characterized. The goal is to examine the binding modes of the peptide−thiazole orange bioconjugate for single and multiple site variants of the cognate TC3 binding site. If high selectivity and high binding affinity could be engineered into the probe, it could discriminate subtle sequence variations, including single nucleotide polymorphisms (SNPs).

Experimental Section

Materials. Lepidine, 5-bromovaleric acid, 3-methylbenzothiazole-2-thione, iodomethane, dioxane, anhydrous ethanol, and triethylamine were purchased from Aldrich (Milwaukee, WI) and used without further purification, except dioxane, which was distilled prior to use. F-moc amino acids and peptide synthesis reagents were purchased from Advanced Chemtech (Louisville, KY). The F-moc-Lys (Mtt)-OH was purchased from Anaspec (San Jose, CA).

Instrumentation. Peptides were synthesized on a 9050 peptide synthesizer (Millipore, Bedford, MA). Purification of synthetic peptides was performed using high performance liquid chromatography (HPLC). Characterization of peptide bioconjugate was performed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry using α-cyanohydroxycinnamic acid. Absorbance measurements were made on a Cary V spectrophotometer (Varian Instruments, Palo Alto, CA). Circular dichroism (CD) measurements were taken using an AVIV 60DS CD spectropolarimeter. Steady-state fluorescence measurements were performed on a Quantamaster-6/2003 spectrofluorometer in T-format, with a 75 W xenon lamp, and analyzed with Felix32 Fluorescence Analysis Software (PTI, Canada).

Peptide Bioconjugate Synthesis and Characterization. All labeled and nonlabeled peptides were synthesized on PAL-PEG-PS resin by automated solid-phase peptide synthesis using F-moc protection and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*^{9},*N*^{9}-tetramethyluronium hexafluorophosphate (HATU) coupling chemistry. An F-moc-Lys (Mtt)-OH was inserted at the N-terminal amino acid position of the polypeptide sequence (Figure 1) as previously described.[11,18] After Mtt deprotection, the carboxylic acid derivative of thiazole orange was coupled to the ϵ-amine of the lysine using HATU activated coupling chemistry. This dye attachment protocol was carried out either immediately after addition of F-moc-Lys (Mtt)-OH or after completion of the normal peptide synthesis. No differences in product yield or DNA-binding efficiency were detectible when comparing dye coupling to the α- or ϵ-amine of the N-terminal lysine, making this synthetic approach highly versatile for the synthesis of an array of sequence-specific DNA stains.[11,18] Peptides were purified by RP-HPLC on a Zorbax C8 column (Agilent Tech., Palo Alto, CA) using a flow rate of 1 mL/min and a water (0.1% TFA)−acetonitrile (0.1% TFA) gradient from 20% to 60% acetonitrile over 40 min. Peptide identity was confirmed by MALDI-TOF mass spectrometry. Stock solution concentrations of bioconjugate sample were determined using a Bradford Assay with BSA as a reference.

Oligonucleotide Sample Preparation. All oligonucleotides were made on a DNA synthesizer using phosphoramidite chemistry and gel purified on a 12% denaturing polyacrylamide gel. Bands were cut out and recovered by soaking the crushed gel pieces in 100 mM Tris (pH 8.0), 100 mM NaCl buffer for 4−6 h. Extractions were combined, and the urea and salts were removed by spin filtration on a Sephadex G-15 size exclusion column (Aldrich). Concentrations for purified single-stranded oligonucleotides were calculated from 260 nm absorbance using an extinction coefficient calculated from the nearest neighbor method.[19,20] Oligonucleotides were annealed in 20 mM Tris (pH 7.4), 50 mM NaCl buffer using the same molar equivalent of each oligonucleotide and its complementary sequence. The native and mutant oligonucleotide sequences used in these studies are derived from the cognate TC3 site shown in Table 1.

Table 1. DNA-Binding Affinities for TOTc3

sample	sequence[a]	K_D (nM)
TC3-1	CTATAGGACCCC	15 ± 1
TC3-2	CTATAGGATCCC	74 ± 1
TC3-3	CTATAGGACTCC	28 ± 1
TC3-4	CTATAGGATTCC	81 ± 3
TC3-5	CTATAAGACCCC	180 ± 10
TC3-6	CTATGGGACCCC	120 ± 10
TC3-7	CTATGAGACCCC	440 ± 60
TC3-8	CTATGAGATTCC	910 ± 70
TC3-9	TCGCGAAGTTTT	1400 ± 200

[a] Sequence changes are underlined.

Determination of Spectral Properties. Absorbance spectra were taken over a concentration range of 0.5−5 μM for all samples under the indicated conditions. Samples for the free dyes were prepared by dilution of a dimethyl sulfoxide (DMSO) stock solution into the indicated solvent or aqueous buffer. Measurements were made in polypropylene cuvettes to minimize surface adsorption of the cyanine dyes in aqueous solutions. Serial dilutions were taken to ensure adherence to the Beer−Lambert law. The calculation of the extinction coefficient was performed by plotting absorbance versus concentration for different dilutions giving the slope, which is equivalent to the extinction coefficient.

Samples for determination of fluorescence quantum yield contained 50−500 nM conjugate in 20 mM Tris (pH 7.6), 100 mM NaCl with 500 nM of the appropriate cognate DNA to ensure complete binding of the conjugate. Fluorescence quantum yields (Φ_F) for these samples were determined relative to fluorescein in 0.1 M NaOH ($\Phi_F = 0.90$) by integration of corrected emission spectra.[21] Solutions used for fluorescence quantum yield determination of the bioconjugate were made to have the same absorbance (ca. 0.03) at the absorbance maximum for each dye. The relative emission quantum yields were calculated as described by Demas and Crosby.[22]

Steady-state fluorescence measurements were performed as reverse titrations using 1−5 nM of the peptide−dye bioconjugate and varying the concentration of nonlabeled DNA until the maximum fluorescence was attained. Excitation was at 510 nm, and emission detection was at 530 nm. Measurements were carried out in 20 mM Tris (pH 7.6), 100 mM NaCl. Total fluorescence counts for each data point were calculated and plotted versus DNA concentration using a two-state model of binding to attain the equilibrium dissociation constant (K_D) for bioconjugate binding to the unmodified DNA. The data are the average of at least three independent measurements and fit using a nonlinear least-squared algorithm (SigmaPlot).[18] Plots of the fluorescence titrations are represented as fraction of the bioconjugate bound by the indicated DNA sequence ($F_i - F_{min}/F_{max} - F_{min}$). The titration midpoint for individual determinations represents the equilibrium dissociation constant. For fluorescence spectra, excitation was at 508 nm and emission spectra were taken from 520 to 600 nm with 1 nm bandwidth on the excitation and emission monochromator.

Circular Dichroism Measurements. Measurements from 400 to 600 nm were the average of at least three scans taken at room temperature with a scan rate of 1 nm/s, and the response time was 2 s. The spectropolarimeter gives raw output in ellipticity, which is

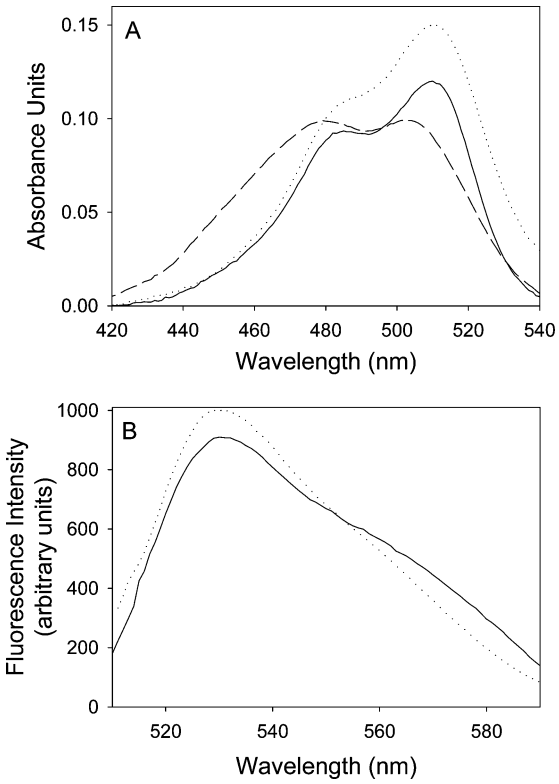

Figure 2. The effect of conjugation on the spectral properties for the TOTc3 bioconjugate in the presence and absence of the TC3-1 DNA sequence. (A) Absorbance spectra used a concentration of 2 μM for TOTc3 in the absence (− − −) and presence (−) of 3 μM DNA. (B) Fluorescence spectra of 50 nM of the bioconjugate (−) and 75 nM TC3-1 DNA. Unconjugated TO (· · ·) in the presence of DNA is shown in (A) and (B) for comparison. Excitation at 500 nm was used.

converted to molar ellipticity $[\theta] = \theta/(10 \times c \times l)$, where c is the molar concentration of the sample (mol/L) and l is the path length in cm. A quartz cell of path length 1 mm was used. The instrument was calibrated with 0.6 mg/mL D(+)-10-camphorsulphonic acid, which shows a molar ellipticity of 7800 deg cm^2 dmol^{-1} at 290.5 nm in nanopure water.[23] DNA-binding studies were performed at 25 °C using 5 μM TOTc3 and 7.5 μM (1.5-fold molar excess) of the indicated DNA sequence.

Results

Photophysical Features. Absorbance spectra of TOTc3 in the presence and absence of DNA are compared to those of unconjugated TO (Figure 2). The spectral features of the TOTc3 peptide bioconjugate in the visible region are attributed exclusively to the TO moiety. The visible absorbance spectrum of purified TOTc3 in the absence of DNA shows two roughly equivalent peaks at 480 and 503 nm. Incubation with DNA containing the TC3-1 sequence causes the TOTc3 absorbance maxima to red-shift to 510 nm with a much less pronounced shoulder to the blue side of the peak. The unconjugated TO shows a similar red-shift upon DNA intercalation to 512 nm and a less prominent shoulder. This red-shift upon DNA binding was previously observed and attributed to a change in the dye environment.[24] Figure 2 shows comparable effects between the TC3 conjugated and unconjugated TO bound to DNA, suggesting the dye is intercalated in both the unconjugated and the bioconjugate forms.

On the basis of the observed differences in the absorbance spectra of unconjugated and peptide conjugated TO, extinction coefficients for the TOTc3 bioconjugate were determined in the absence and presence of DNA containing the TC3-1 sequence. The extinction coefficient changes from 50 800 to 62 800 M^{-1} cm^{-1} upon DNA binding, representing nearly a 25% increase. This increase in the oscillator strength accompanies the 503 to 510 nm red-shift that occurs upon DNA intercalation by the dye (Figure 2). For comparison, the extinction coefficient of unconjugated TO shows nearly a 20% increase in the extinction coefficient from 63 200 to 77 000 M^{-1} cm^{-1} upon DNA intercalation.

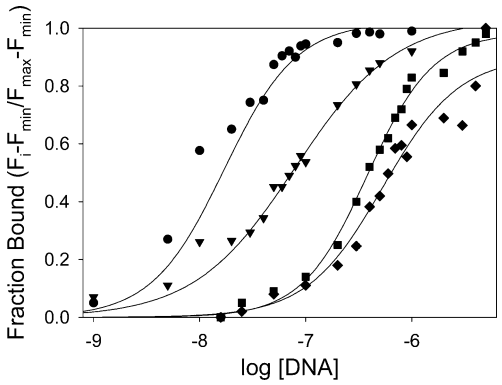

Figure 3. Comparison of the total fluorescence of TOTc3 bioconjugate due to sequence specificity at native, mutant, and nonspecific DNA sequences. Fluorescence measurements are given as a fraction of total fluorescence ($F_i - F_{min}/F_{max} - F_{min}$). Total fluorescence in the presence of TC3-1 (●), TC3-4 (▼), TC3-8 (■), and TC3-9 (◆). Measurements were performed using dye or bioconjugate concentrations of 1 nM in 20 mM Tris (pH 7.6), 150 mM NaCl.

The effects of conjugation on fluorescence were examined to characterize the photophysical effects of dye−peptide linkage. Fluorescence measurements taken after 15 min incubation with TC3−DNA show the spectra for the unconjugated and peptide conjugated dye are comparable (Figure 2). TO and TOTc3 samples measured at 530 nm in the absence of DNA had fluorescence counts that were indistinguishable from background (data not shown). The fluorescence quantum yields for TOTc3 ($\Phi_F = 0.20$) and unconjugated TO ($\Phi_F = 0.26$) were determined in the presence of TC3-1 DNA. This is comparable to previously determined values for the unconjugated TO in the presence of CT-DNA ($\Phi_F = 0.25$).[15]

DNA Targeting Properties. Previous studies comparing the binding affinity for different peptide bioconjugates[10,11] for a specific and a random DNA sequence indicate that each bioconjugate reproduces native-like site specificity. Because these molecular probes were not examined for sequence-dependent changes in binding affinity, it was unclear whether these bioconjugates retained the ability to discriminate point mutations and other subtle sequence variations as observed for native DNA-binding proteins. Here, the equilibrium dissociation constants (K_D) for the peptide−TO bioconjugate were determined as a measure of site specificity. To accomplish this, the binding of TOTc3 to cognate DNA was compared to a series of mutations within the cognate site using steady-state fluorescence spectroscopy. The mutant DNA sequences used in this study (Table 1) were designed on the basis of structural information from the DNA-bound Tc3 protein[11] with the goal of disrupting the peptide−DNA binding interface. Reverse titrations were performed to keep the concentration of the dye constant, such that the change in fluorescence signal for each DNA sequence is proportional to the fraction of bioconjugate bound. Figure 3 shows representative binding curves for the titration of the TOTc3 with the indicated TC3−DNA sequences. Binding by TOTc3 shows affinities ranging from 15 nM to 1.4

μM depending on the given sequence. These variations in binding affinity span nearly 2 orders of magnitude. TOTc3 clearly shows a strong preference for the TC3 cognate site having a nanomolar binding constant ($K_D \approx 15$ nM). The other sites are bound with a 5- to 60-fold weaker affinity. The binding isotherms for the interaction of TOTc3 with each of the different DNA sequences reveal important information about the sensitivity of the bioconjugate. The equilibrium dissociation constants were determined for each combination of TOTc3 and TC3 DNA sequence (Table 1). The large decrease in Tc3 binding affinity occurring as the TC3 site is progressively lost to mutations is the hallmark of sequence-specific DNA-binding proteins. For comparison, the Tc3 DNA-binding peptide alone has an equilibrium dissociation constant of approximately 100 nM in the presence of TC3-1 and 4 μM in the presence of TC3-9.[25]

The range of concentrations used in the binding analysis limits the amount of nonspecific binding, making the bioconjugate a very sensitive probe of sequence within the nanomolar concentration range. The unconjugated TO shows no variation in fluorescence intensity when incubated with any of the sequences examined in this study (data not shown). This lack of sequence specificity has previously been observed for the asymmetric cyanine dye monomers.[15] In the nanomolar concentration range, the dye is only able to intercalate DNA and emit fluorescence signal when the peptide is bound with high affinity to its native cognate site. The fluorescence from the bioconjugate incubated in the presence of DNA containing the target sequence is comparable in intensity (ca. 90%) to the same amount of the intercalating dye bound to DNA. The concentrations used here avoid self-quenching, which has been shown to occur for unconjugated dye fluorescence at high dye:bp ratios.[15,26] This implies that the fluorescence yield and the level of occupancy of the dye on the DNA in the assembled bioconjugate−DNA complex are similar to those observed for free dye interacting with DNA in solution.

Circular dichroism analysis of peptide-directed dye intercalation was performed in parallel to the fluorescence measurements to further understand the sequence-dependent effects of bioconjugate binding to DNA. Previous studies showed that the asymmetric cyanine dye YO has a strong CD signal upon intercalation, yet negligible signal when free in solution.[27] Studies were performed in the low micromolar range to yield sufficiently strong CD signal while retaining solution conditions that parallel the fluorescence measurements. Upon DNA addition, the unconjugated TO shows a signal decrease at 490 nm (Figure 4), which is characteristic of DNA intercalation.[21] The red-shifted minimum observed in the CD of the TOTc3 bioconjugate shows a 20% decrease to 494 nm as compared to that of the unconjugated TO signal.

The negative CD signal centered at approximately 494 nm corresponding to dye intercalation decreases in magnitude in parallel to the extent of which the DNA site is altered. As mutations accumulate, the negative CD at 494 nm decreases in magnitude by 50% and 70% in the cases of TC3-4 and TC3-8, respectively. In the concentration range examined here, the nonspecific site (TC3-9) shows a greater than 95% loss in CD signal, suggesting the Tc3 is not localized at a given site and the TO is unable to intercalate DNA. It should be noted that the unconjugated TO shows no difference in the fluorescence or CD measurements for all DNA sequences used in these studies, as well as calf-thymus DNA (data not shown). Because the fluorescence and CD signal intensities are a function of the DNA sequence in the TC3 site and unconjugated dye shows sequence-independent signal intensity, it can be stated that the peptide moiety drives DNA targeting and binding affinity.

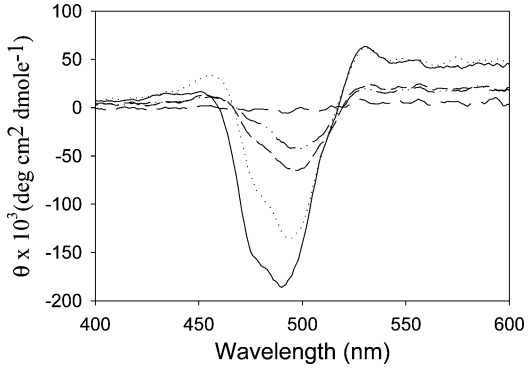

Figure 4. Circular dichroism spectra of the sequence dependence of cyanine dye intercalation. Steady-state measurements were performed at ambient temperature using 5 μM unconjugated or peptide conjugated TO and 7.5 μM of the indicated DNA sequence. The CD signal derived from thiazole orange is only observable when the asymmetric cyanine dye is intercalated in dsDNA. Unconjugated TO (solid) is used as a comparison to show the strong negative CD at 490−494 nm from fully intercalated dye. The TOTc3 bound at the TC3-1 (· · ·), TC3-4 (- - -), TC3-8 (— ·· — ·· —), and TC3-9 (— — —) dsDNA sequence as listed in Table 1. Here, the TC3-1 dsDNA sequence is used for the unconjugated TO, although no difference in the spectral features is observed between any of the TC3 sequences used in this study.

Discussion

The fluorescent intercalating dye thiazole orange has been tethered to the ϵ-amine of a lysine strategically positioned within the amino acid sequence of the Tc3 transposase DNA-recognition region, and the DNA sequence-specific targeting properties have been examined. TO has been shown to be several orders of magnitude more fluorescent upon DNA intercalation as compared to free dye in solution, making it a very sensitive probe of DNA binding.[14,24] Here, the DNA recognition features of the polypeptide from Tc3 transposase were used to control the fluorescence of the dye, which has no inherent sequence preference. The fact that the peptide conjugated dye intercalates DNA in a sequence-specific manner comparable to the native Tc3 protein with the TC3 site indicates the peptide moiety drives binding.[25] While covalent attachment of thiazole orange to the Tc3 transposase DNA-binding region has been shown to increase the DNA-binding affinity, it does not adversely affect the sequence specificity. These studies reveal three main points: first, the specificity of the probe reproduces recognition characteristics of the native protein; second, the dye is only able to intercalate DNA and fluoresce when the peptide is bound; and third, the conjugation to the peptide causes a modest decrease in fluorescence quantum yield of the dye. These combined elements make the fluorescent bioconjugate well suited to identify DNA target sequences and for mutational analyses.

The photophysical properties of the dye are comparable for the unconjugated TO and TOTc3 bioconjugate because the interaction of the dye with DNA is predominantly due to intercalation in both cases. The bioconjugate probe shows photophysical properties comparable to those of the thiazole orange itself, indicating a modest adverse affect upon conjugation to the peptide. The fluorescence quantum yield for the unconjugated TO in the absence of DNA was shown to be 1.4×10^{-4}.[15] Assuming the unbound bioconjugate exhibits the same

fluorescence quantum yield as the unbound dye, then the emission enhancement for the conjugate would be 1400 for TOTc3. For comparison, the emission enhancement of unconjugated TO bound to CT-DNA was determined to be 1800. Parallel to the difference in quantum yield of the dyes upon conjugation to the polypeptide, the emission enhancement of the conjugate is about 80% of that for the unconjugated dye. For comparison, fluorescein shows a decrease in its quantum yield of approximately 50% upon conjugation presumably due to a quenching mechanism.[28]

The binding affinity and specificity of TOTc3 for single-site mutants range from a 2- to 12-fold decrease in the binding constant when compared to the TC3-1 cognate site (Table 1). The bioconjugate probe is able to distinguish point mutations in the binding site. The double and multiple mutant TC3 sites show a progressively lower binding affinity consistent with the loss of the cognate site. The double mutants (TC3-4 and TC3-7) range from a 6- to 30-fold decrease in the binding constant when compared to the TC3-1 cognate site. The loss in binding affinity is consistent with the loss of direct contacts at the binding interface as indicated in the Tc3 crystal structure, which shows the amino terminal helix interacting toward the 5′ end of the recognition sequence.[29] The TC3-7 double mutant exhibits a greater impact on binding than does TC3-4 due to the multiple contacts in the central region of the recognition helix with the mutated bases. The TC3-8 mutant, which is the combination of the two double mutants, loses a majority of direct contacts at the protein−DNA interface, resulting in a dissociation constant that is comparable to that for the nonspecific sequence (TC3-9). The results of the binding studies with different target sequences described above are qualitatively consistent with previous binding studies using a single zinc finger from the glucocorticoid receptor.[10] In that work, 40-fold increases in the dissociation constants were observed relative to the native GRE recognition sequence for sequences carrying point mutations.

For the TOTc3 bioconjugate studied here, there is a 6-fold contribution to the TC3 binding affinity by TO as determined by equilibrium binding studies. It should be noted that the dissociation constant of TOTc3 is higher than the product of the individual dissociation constants from the peptide and the dye, suggesting the binding of the dye and the peptide behaves in a non-cooperative manner. Structural analysis has shown the dye untwists the DNA approximately 20°.[30] This may cause a decrease in the ability of the peptide to stay bound if the intercalation site is sufficiently close to perturb the noncovalent interactions between the peptide and the DNA, resulting in suboptimal interactions at the interface. The peptide, on the other hand, alters multiple DNA helical parameters in the region where the peptide interacts,[31] perhaps limiting the positions and orientations available for dye binding to the DNA. Presumably, the two moieties find a low-energy configuration that permits both peptide targeting and dye intercalation.

Recent single molecule measurements of a thiazole orange−histone complex reconstituted into a nucleosome with DNA suggest that there may be multiple binding configurations of the tethered thiazole orange molecule.[32,33] A 10% decrease in the quantum yield has been attributed to the existence of dual binding modes for the dye.[11] Previous studies suggested that the bound peptide moiety might force the dye to bind by two modes: intercalation within the base-stack and external binding via electrostatic interaction with the backbone.[32] This external binding mode is known to be fluorescent with a lower quantum yield because rotation about the central methine bridge is more accessible than is the case for fully intercalated dye.[24] The relative population of each binding mode will determine the observed quantum yield and therefore the emission enhancement upon DNA binding.

Fluorescence analysis of the bioconjugate shows that the DNA recognition features of the polypeptide control the ability of the dye to interact with DNA under physiological conditions. The thiazole orange is able to intercalate and fluoresce when the conjugate binds, at concentrations where little fluorescence is observed from either the bioconjugate alone or the bioconjugate mixed with DNA lacking the target sequence. In the bound state, the dye associates with the dsDNA and fluoresces, but is essentially nonfluorescent in the unbound form. The TOTc3 bioconjugate targets the native TC3 sequence with a dissociation constant of about 15 nM. Lower binding affinities (10- to 100-fold) are observed for DNA site variants and nonspecific DNA. Peptide−intercalator bioconjugates have been shown to be highly sensitive probes of DNA sequence and represent a new technology in studying protein−DNA recognition features, as well as sequence-specific DNA stains. The tethering of a sequence-specific binding peptide onto a functional probe provides a viable strategy for construction of synthetic enzymes, repressors, and dynamic studies of protein−DNA interactions. The demonstration of high selectivity between native and mutant DNA sequences is an important advancement of knowledge in this area of fluorescent reporter biopolymers. This class of molecular probes has the potential to deliver an array of functional groups to specific DNA locations and could be developed to probe the structural and functional features of chromatin.

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