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A designed protein binding-pocket to control excited-state intramolecular proton transfer fluorescence†

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Abstract

Excited-state intramolecular proton transfer involves a photochemical isomerization and creates the opportunity for the emission of two distinct wavelengths of light from a single fluorophore. The selectivity between these two wavelengths of emission is dependent on the environment around the fluorophore and suggests the possibility for ratiometric monitoring of protein microenvironments. Unfortunately, non-specific binding of ESIPT fluorophores does not often lead to dramatic changes in the ratio between the two wavelengths of emission. A protein binding pocket was designed to selectively discriminate between the two channels of emission available to an ESIPT fluorophore. This work is significant because it demonstrates that specific interactions between the protein and the fluorophore are essential to realize strong ratiometric differences between the two possible wavelengths of emission. The design strategies proposed here lead to an ESIPT fluorophore that can discern subtle differences in the interface between two proteins.

Introduction

The environment around a molecule can have a pronounced effect on its excited state and is frequently used in nature to control and manipulate photophysical outcomes. One example is the first step of vision, where the photoisomerization of retinal is governed by the microenvironment of opsin proteins.^{1,2} Another example is the fluorophore of green fluorescent protein that is only emissive when packaged inside of the folded protein.^{3,4} Because photochemistry is a zero-sum competition of the rates of different excited state processes, the manipulation of those rates by the environment can select which pathway is dominant. In particular, a photo-reaction known as excited-state intramolecular proton transfer (ESIPT) has attracted considerable attention because the ESIPT process affords the potential for two different wavelengths of light from the same molecule depending on local environmental conditions (Fig. 1).^{5–8} We sought to design a protein binding pocket that

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Conflicts of interest

ETP and JWK receive royalty payments from a patent for tafamidis.

could predictably and selectively distinguish between different excited-state pathways of the ESIPT process.

The result of this work is a fluorescent probe that exhibits a change fluorescence signal by detecting subtle changes in a protein-protein interface.

Consider the emission of 2-(2'-hydroxyphenyl)benzoxazole (**1**, Fig. 1). Upon absorbance of light, **1** forms the excited-state enol **1-E***. Emission from **1-E*** (enol emission) back to the ground state (**1**) can produce a photon of light, which is the “normal” fluorescence process of numerous dyes. Alternatively, the structure of **1** is such that an intramolecular isomerization can occur in the excited state to produce excited keto form **1-K*** from **1-E*** (Fig. 1, ESIPT step). Emission can then take place from **1-K*** (keto emission) to produce **1-K** in the ground state, which rapidly isomerizes back to **1**.

Several characteristics of the ESIPT process make it attractive as a tool in fluorescence detection:

- (i) The excited-state isomerization from **1-E*** to **1-K*** is ultrafast (~ps)—faster than fluorescence from the enol tautomer (ns)—leading to a predominance of keto fluorescence.⁸ Because the energy gap between the keto forms ($S_1' \rightarrow S_0'$) is smaller than for the enol forms ($S_1 \rightarrow S_0$), keto fluorescence typically leads to a >100 nm red-shift relative to enol fluorescence and the excitation wavelength. The ESIPT process therefore produces remarkably large Stokes shifts relative to “normal” fluorophores (*e.g.* fluorescein, rhodamine, BODIPY, *etc.*).
- (ii) The ESIPT process is also sensitive to environmental factors. Enol emission tends to dominate in polar-protic solvents, presumably due to hydrogen bonding that disrupts the intramolecular isomerization from **1-E*** to **1-K***. Alternatively, non-polar solvents or local desolvation from polar-protic solvents allows the excited-state isomerization to proceed—effectively turning on keto emission. The environmental dependence of the excited state isomerization process allows for a dual-emission readout. This multichannel output adds ratio-metric characteristics to ESIPT capable dyes which enhance assay sensitivity.⁹

The ratiometric difference between enol and keto emission was originally demonstrated by Sytnik and Kasha¹⁰ to interrogate the local desolvation effects upon nonspecific binding to human serum albumin. More recent studies have expanded on this work in various albumin proteins.^{11–15} ESIPT fluorophores have also been incorporated into non-natural amino acids to detect peptide/nucleic acid interactions,^{16,17} protein secondary structure,^{18,19} and detection of α -synuclein aggregation.^{20,21}

A limitation of the above studies was that only relatively subtle changes in the enol and keto fluorescence ratios were observed. This general lack of sensitivity arose from a common theme of ESIPT schemes reported to date: (i) The assay relied on non-specific binding of the ESIPT chromophore to the target of interest. (ii) When the ESIPT reporter was conjugated to a molecule that provides selectivity for the intended target, this construct meant that the environmentally sensitive ESIPT fluorophore did not directly interact with the target. Thus, any environmental changes experienced by the ESIPT fluorophore were largely an outer-

sphere effect. Furthermore, conjugating a chromophore onto a specific binding ligand can often lead to a decrease in binding affinity, selectivity and sensitivity to the target.²³ The goal of this study was to design a protein that could bind an ESIPT fluorophore and selectively alter the enol and keto fluorescence signals through the specific interactions that take place between the small molecule and protein binding partner.

We elected to use the well-studied tetrameric transthyretin (TTR) protein (Fig. 2A).^{24,25} To facilitate the discussion, we will employ a symbol convention to distinguish between the two kinds of TTR proteins under consideration.

TTR□ denotes the WT-TTR protein subunit.

TTR■ denotes the A108G mutant TTR protein subunit.

TTR was chosen as a model system for the following reasons:

- (1) WT TTR□ displays excellent affinity for derivatives of benzoxazole (**1**) which are common scaffolds in ESIPT fluorophores.²⁶ An example of this is the drug, tafamidis,²² which binds at the binding pocket formed by the dimer–dimer interface between WT TTR□ homotetramers (Fig. 2B). Theoretically, **2** can function as an ESIPT fluorophore when an –OH group is installed at the 2' position to give **3** (Fig. 2C). In methanol, **3** displays an enol emission at 450 nm (Fig. 3, blue trace). Alternatively, a red-shifted keto emission at 520 nm appears in the nonpolar solvent dichloromethane (Fig. 3, green trace). This red-shift in emission in nonpolar solvents is characteristic of the ESIPT process. A small positive solvatochromism can be observed in the enol emission wavelength between methanol and phosphate buffer—likely due to differences in the protic state of the carboxylic acid in **3** between these two solvents (Fig. 3, purple trace).
- (2) Analysis of the binding interaction of **2** and WT TTR in Fig. 2B, reveals that the –OH group at the 2'-position in **3** would clash with the A108 residue. This steric clash would prevent binding, but also indicates the necessary mutation that needs to be made to WT TTR□ in order to allow binding of an ESIPT fluorophore based on **3**. Therefore, we hypothesized that an A108G mutation would remove the steric imposition of the A108 side chain to create room for the –OH group in **3**.
- (3) Because of the symmetry of the TTR homotetramer interface, however, an A108G mutation will alter both the 'top' and 'bottom' interfaces of the binding pocket (Fig. 4A, next page). Thus, we designed the more symmetric **4** to bind both the 'top' and 'bottom' A108G TTR■ interfaces. We therefore predicted that, in free solution, **4** would be restricted to enol fluorescence. Upon specific binding to the hydrophobic binding pocket of the A108G mutant TTR■, however, keto emission would be enabled.

Compound **4** also presented another advantage compared to **3**. The two OH groups in **4** have been shown to decrease rotational disorder across the biaryl due to hydrogen bonding from

the OH to both the N and O of the benzoxazole.²⁷ The result was an overall increase emission intensity, Stokes' shift, and sensitivity.

Results and discussion

We tested our design hypothesis described above against WT TTR□ and A108G TTR■ homotetramers and evaluated the slate of benzoxazole derivatives (**3–8**) listed in Scheme 1. The design rationale for **3** and **4** has already been discussed above. Compounds **7** and **8** were designed to improve the emission intensity through substitution of the Cl groups with isosteric²⁸ Me groups—because halogen atoms can increase the efficiency of non-radiative processes (*i.e.* intersystem crossing, “heavy-atom effect”²⁹). Finally, **5**, **6** and **8** are isomeric in the position of the carboxylic acid.

Compounds **3–8** were tested at 10.0, 5.0, 2.5 μ M concentrations against 5.0 μ M solutions of WT TTR□ and A108G mutant TTR■. According to our design hypothesis, we predicted **4** would bind A108G TTR■ to enable keto emission. Unfortunately, this was not the case, as **4** displayed little change in emission characteristics in the presence of the TTR proteins (Fig. S14[†]). Gratifyingly, however, **5** showed selectivity for the A108G TTR■ mutant interface (Fig. 5A) in preference to WT TTRD. Both in buffer and in the presence of WT TTR, **5** displayed the expected signal for enol emission at 450 nm indicating that it was excluded into bulk solution. In the presence of A108G, however, **5** exhibits pronounced keto emission at 520 nm and the original 450 nm band diminishes. These data indicate that the turn-on of keto emission is likely not due to nonspecific binding to TTR, but instead indicates specific binding in the designed A108G pocket wherein the protein increases the efficiency of emission *via* the keto *versus* the enol channel.

Unfortunately, all other derivatives (**3**, **4**, **6–8**, Scheme 1) did not exhibit a change in emission between buffer, WT TTR□ and A108G TTR■ tetramers (Fig. S13–18,[†] in fact **4**, **6–8** did not exhibit emission in free solution). It is important to recognize, however, that a lack of a change in the emission signal does not inform on the ability of a given molecule to bind the TTR binding pocket. It is possible that an ESIPT fluorophore, may bind the TTR pocket with high affinity, but the protein is unable to increase the efficiency of keto emission (*vide infra*).

The failure of **4** and **6** was surprising given their symmetrical design was anticipated to bind the symmetrical pocket of A108G TTR■. The behaviour of **5** and **3** was equally surprising: (i) with **5** displaying selective binding of the symmetric binding pocket of A108G TTR■, (ii) while **3** displayed no binding preference for TTR (Fig. 5B). Inspired by the unsymmetrical nature of **5** and **3**, however, we were curious if these compounds might display greater affinity for an unsymmetrical binding pocket (Fig. 4B)—where a higher affinity was anticipated to lead to an even greater turn-on in keto emission.

To create an asymmetric binding pocket within TTR, we capitalized on the well-characterized dissociative equilibrium of TTR between its tetrameric and monomeric forms.

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Mixed TTR tetramers can be generated by mixing an equimolar concentration of WT TTR□ homotetramers and A108G TTR■ homotetramers to give a statistical distribution of mixed interfaces (1:2:1 TTR□/TTR□ : TTR□/TTR■ : TTR■/TTR■).³¹ Thus, an asymmetric interface designed to bind **5** or **3** could be realized (Fig. 4B). In a similar manner as for **4** described above (Fig. 5B), **5** and **3** were anticipated to produce enol emission in bulk solution but elicit keto emission upon specific binding to the asymmetric TTR□/TTR■ interface. Following this logic, asymmetric TTR□/TTR■ interfaces were generated by allowing a 5.0 μM solution of WT TTRD and A108G TTR■ to equilibrate at 25 °C for seven days. Probes **5** and **3** were then administered (Fig. 5).

Intriguingly, the keto emission for **5** diminished in the TTR□/TTR■ mixture (Fig. 5A). This observation is consistent with a lower affinity for the TTR□/TTR■ mixed interface relative to the A108G TTR□ homo-tetramer. Of course, the TTR□/TTR■ mixture still contains a statistical population (25%) of TTR■/TTR■ homo-interfaces within the mixed tetramers. Thus, binding of **5** to this smaller fraction of A108G TTR■ homo-interfaces likely contributes to the observed keto emission signal.

Alternatively, **3** did not display any notable selectivity or change in enol *versus* keto emission intensity across any of the TTR tetramers (Fig. 5B). This result is unexpected given the structural similarities of **3** and **5** (differing only in the position of the carboxylic acid). For example, tafamidis (**1**) can bind the WT TTR□ binding pocket with *C*₂ symmetry (*i.e.* The CO₂H group can orient towards either the ‘top’ or ‘bottom’ interface, PDB 3tct). Thus, it is surprising that the subtle regioisomerism of the CO₂H between **3** and **5** can lead to significant differences across the TTR tetramers in Fig. 5.

The contrasting behaviour of **3** and **5** is even more surprising because titration curves measuring absorbance *vs.* concentration of **3** and **5** in the presence of TTR have similar forms. While it was not possible to derive binding constants with confidence using this method (since absorbance is not very sensitive at concentrations low enough to obtain accurate *K*_d measurements, given that the *K*_ds are expected to be in the low-to-mid nanomolar range), this information does indicate that **3** and **5** have commensurate binding affinities with A108G TTR■. Thus, if both **3** and **5** bind with similar affinity but display different emissive behaviour, it suggests that specific interactions between the protein and the fluorophore are essential to realize strong ratiometric differences between the two possible wavelengths of emission. Removal of the alanine side chain to create the A108G binding pocket (Fig. 4A) likely creates a ‘looser’ fit for **3** and **5**. Thus, it is reasonable to conclude that the contrasting behaviour of **3** and **5** stems from differences in the bond lengths and geometry of the oxazole that pose the CO₂H group differently for each molecule in the binding pocket. Finally, the position of the electron-withdrawing CO₂H may alter the efficiency of emissive pathways once bound.

Conclusions

This work describes the first demonstration of a rationally designed protein receptor that selectively binds and alters the photochemical pathways of ESIPT fluorophores. More broadly, this work demonstrates that specific interactions between the protein and the

fluorophore are essential to realize strong ratiometric differences between the two possible wavelengths of emission in ESIPT fluorophores.

The ESIPT process has the potential to allow for two wavelengths to be read out from a single molecule in a ratiometric fashion, but previous systems have primarily observed only subtle changes in the wavelength ratio due to nonspecific binding. The system we describe here exploits the environmental dependence of the ESIPT process where bulk water disrupts the efficiency of proton transfer in the excited state. The protein receptor was designed to selectively bind and desolvate an ESIPT fluorophore within a hydrophobic pocket. The result is a dramatic difference and inversion of the proportion of enol and keto emission. Finally, we note that benzoxazoles and benzothiazoles are privileged scaffolds³² in medicinal chemistry as they are found in numerous therapeutics displaying possess anticancer, antimicrobial, antiviral and anti-inflammatory properties, among others.^{33–35} Thus, we envision benzoxazoles may be utilized in diagnostic assays based on ESIPT fluorescence.³⁶ We therefore anticipate this report to lay the groundwork towards the development of future ESIPT fluorescence based bioimaging technologies and monitoring of protein–protein interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig.1.

Excited-state intramolecular proton transfer (ESIPT) cycle of o-phenol-benzoxazole. Hydrogen bonding in bulk water disrupts the intramolecular hydrogen bond in **1-E***, constraining the molecule to “normal” fluorescence.

Fig. 2.
(A) X-ray crystal structure of WT-transthyretin (WT-TTR) with bound tafamidis (PDB 3tct).
22 (B) Zoom-in of the binding pocket of WT-TTR and tafamidis in PDB 3tct showing close-
contact residues. (C) Grafting of the ESIPT scaffold onto TTR-binding molecules.

Fig. 3. Emission traces of **3** in varying solvents. **E*** emission occurs 410–450 nm whereas **K*** emission is seen at 510 nm. Phosphate buffer consists 10 mM sodium phosphate (pH 7.6), 100 mM KCl, and 1 mM EDTA.

Fig. 4. Proposed binding models of TTR mutants to ESIPT fluorophores. (A) An A108G TTR homotetramer displays an interface that can accommodate the phenolic moiety necessary for ESIPT. (B) A mixed WT-A108G heterotetramer interface (TTR/TTR) designed to bind 3 and 5. Images were prepared using the structure editing tools available in UCSF Chimera³⁰ on the original protein structure (PDB 3tct).

Fig. 5. Emission traces of (A) **5** and (B) **3** at 2.5 μ M in buffer with 5.0 μ M TTR (total protein concentration). Buffer = 10 nM sodium phosphate (pH 7.6), 100 nM KCl, 1 mM EDTA.

Scheme 1.

Benzoxazole candidates tested to selectively bind TTR to enhance ESIPT fluorescence.