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LETTER

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Recapture of GFP Chromophore Fluorescence in a Protein Host

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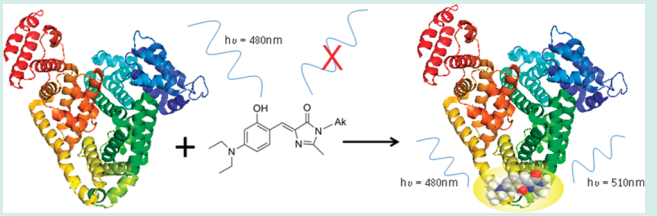
Supporting Information

ABSTRACT: When encapsulated by human serum albumin (HSA), certain derivatives of the green fluorescent protein (GFP) chromophore recover their fluorescence due to inhibition of torsional motion. These derivatives show remarkable sensitivity and selectivity as well as favorable spectroscopic properties toward HSA, thus providing selective probes for this and similar proteins and demonstrating the use of GFP chromophores as topological fluorophores.



KEYWORDS: human serum albumin, green fluorescent protein, selective fluorescent probes, topological fluorophores

The green fluorescent protein (GFP) and its derivatives have revolutionized molecular biology by providing a fluorescent tag that can be attached to other proteins using gene expression techniques. The marker itself, a 238-amino acid protein that maintains the chromophore within a restrictive β-barrel,[1] serves primarily as a probe without providing direct functional information. Moreover, the size of this 29 kD protein limits a number of applications, which has driven us to examine the use not of the protein, but only of its chromophore, derivatives of which, however, are invariably weakly fluorescent, an observation we have been able to attribute to the intervention of additional vibrational modes in the unbound state.[2]

Mimicking the effects of the β-barrel and restoring the fluorescence by rigidifying the otherwise conformationally flexible chromophore has been the subject of several studies. For instance, we showed that encapsulation within an octa acid cavitand results in a 15-fold fluorescence enhancement.[3] Could we obtain similar results using a protein host? If so, such a chromophore could act as a high-sensitivity "turn on" probe for proteins. Unlike other probes, for example, aminonaphthalenesulfonate (ANS), which respond to hydrophobic vs hydrophilic sites, such a probe would be a *topological* one, responding to the conformational rigidity of a host site

Using an unbiased high-throughput screening process, a variety of GFP chromophore analogs were tested against selected protein analytes, and those with 4-N(Et)$_2$ substituents showed selective emission quantum yield (EQY) enhancement for human serum albumin (HSA).[4] Using this lead, we now report an HSA probe with dramatically improved sensitivity and proof of concept that proteins can act to turn on fluorescence in arylmethyleneimidazolidinones (AMIs) when the guest is rigidified in a binding site.

HSA, the major protein component of blood plasma, is marked by an exceptional affinity that allows binding a myriad of ligands.[5] In fact, its twelve binding sites are probably a reason that albumin would be selected in a broad spectrum assay.

Although HSA is not a primary target for disease screening, microalbuminuria (MA), referring to a urinary albumin content of 30−300 mg/24 h, has become an important diagnostic and is linked to various diseases.[6] Thus a sensitive and selective determination of albumin concentration over this range is of medical significance. Moreover, such a protein serves as a test case for the use of AMIs in protein binding.

Currently, Albumin 580 (AB580) is commercially used as a fluorescent probe, given its selectivity for albumin over other proteins, its low susceptibility to environmental factors, and its favorable spectroscopic characteristics (λ_{abs} = 580 nm, λ_{em} = 610 nm).[7] Other assays used for HSA detection include immunoassays,[8] electrochemical assays,[9] and spectrophotometric assays.[10]

From an initial EQY screening showing enhancement for R_2 = Me, *n*-Pr, or pentyl, a number of 4-N(Et)$_2$-FP analogs were combinatorially synthesized using techniques highlighted elsewhere[11] to determine the maximum EQY achievable with a given substitution. These results are summarized in Table 1. Increasing turn-on ratios with increasing alkyl chain length substitution are readily rationalized by the increasing hydrophobicity of the chromophores coupled with the known hydrophobicity of the binding pockets,[12] which reach a limit, respective of alkyl chain length, with the heptyl group. Additionally, we speculated that substitution of an *ortho*-hydroxyl would provide additional sensitivity because of a cluster of polar residues located in each site.[13]

From the preliminary screening, **10** was chosen for more extensive studies to determine its application to albumin sensing in aqueous solution. For all experiments, phosphate buffered

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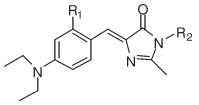
Table 1. FP Analogs and EQY Enhancements



	R_1	R_2	F/F_o[a]		R_1	R_2	F/F_o[a]
1	H	Me	3	**8**	OH	pentyl	36
2	H	*n*-Pr	11	**9**	OH	hexyl	67
3	H	pentyl	24	**10**	OH	heptyl	72
4	H	hexyl	20	**11**	OH	octyl	52
5	H	$C_3H_6CO_2H$	6	**12**	OH	undecyl	7
6	OH	Me	30	**13**	OH	$C_3H_6CO_2H$	28
7	OH	*n*-Pr	48				

[a] Intensity(bound)/Intensity(unbound) at λ_{em} for 7.5 μM (0.5 mg/mL) HSA solutions.

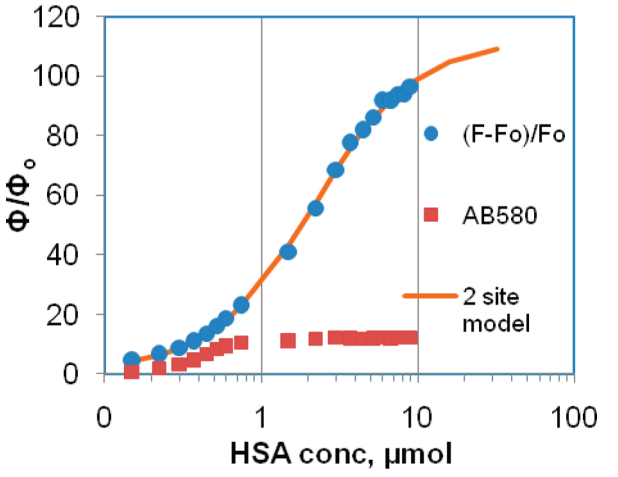


Figure 1. Emission response of **10** and **AB580** to HSA.

saline (PBS) at pH = 7.4 was used. Within PBS, **10** showed favorable spectral characteristics (λ_{abs} = 470 nm, λ_{em} = 510 nm), which shows some differences compared to the Green Fluorescent Protein (λ_{abs} = 398 nm, λ_{em} = 508 nm).[14] A Langmuir calibration curve for **10** required a two-site cooperative binding model[15] and showed a sensing range of 0.15 μM to 15 μM (10 mg/L to ~1000 mg/L) with two dissociation constants (K_d) of 0.24 and 0.45 μM, offering a higher sensitivity and greater sensing range than AB580 (Figure 1).[16]

Given the relative fluorescence yield of 72, which we obtain at saturation with the heptyl analog **10**, we also subjected the bound protein to competitive binding studies against **10** using warfarin and ibuprofen, which selectively bind Sudlow's Sites I and II (see Figure 2 and Supporting Information).[17] As noted from Figure 2, binding of **10** to HSA occurs at both binding pockets, each of which is displaced by one of the secondary binders. We attribute the initial increase in fluorescence to allosteric effects, a conclusion consistent with the two-site binding model. These results, mentioned earlier, are not surprising since secondary binding of ligands to HSA sites is common and well established.[18] Apparently, binding of one ligand, either warfarin, ibuprofen, or HSA itself, leads to tighter binding of the secondary site and an increased fluorescence.

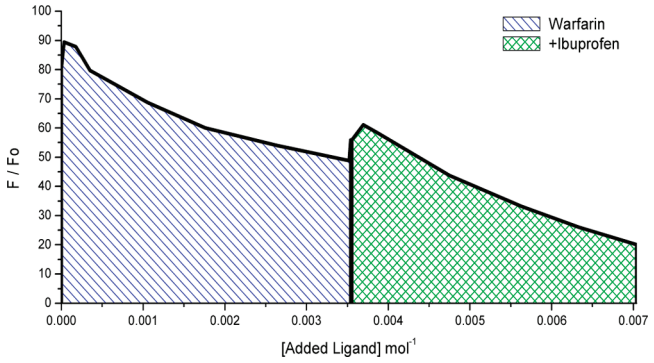


Figure 2. Competitive binding with ibuprofen and warfarin (for experimental details, see Supporting Information).

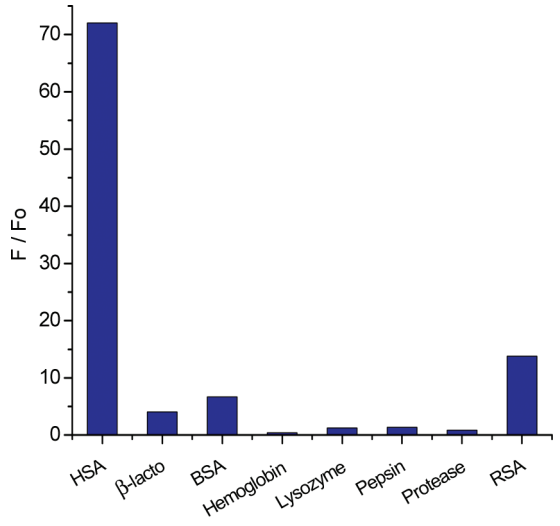


Figure 3. Emission response of **10** with proteins and macromolecules 7.5 μM (0.5 mg/mL) in PBS buffer.

HSA probes find application in the medical community as methods of quantification of albumin within biological samples. For these purposes, probes must be both selective and sensitive to quantify HSA as well to operate within an aqueous environment. To explore the selectivity of **10**, buffered solutions of proteins representative of those found in biological fluids were prepared with equimolar amounts of the probe. Additionally, the selectivity of **10** was explored by testing other albumins. The results of these interference studies are summarized in Figure 3.

The data from Figure 3 shows the selectivity of **10** to HSA compared to other analytes. One remarkable quality is the selectivity over albumins from other species. In PBS buffer, **10** showed a ~70-fold enhancement at 5 μmol HSA (for general reference, Φ = 0.25) compared to a 5-fold and a 13-fold enhancement for Bovine Serum Albumin (BSA) and Rat Serum Albumin (RSA), respectively. The similar responses of RSA and BSA were anticipated, due to the high degree of structural homology shared by HSA, BSA, and RSA.[19] Other proteins did not show such enhancements, and in one case exhibited emission quenching, providing a very robust probe that experiences little interference.

Mentioned earlier, AB580 has gained significant application as a fluorescent probe for the detection of albumin in biological

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samples.[20] To test the competitiveness of **10** against AB580, equimolar probe solutions were prepared and tested in PBS buffer. In Figure 1, **10** exhibits significantly higher fluorescence enhancement ratios compared to AB580 as well as a wider operation range for sensing. AB580 is generally used to accurately detect albumin from 0.15 μM to 3.0 μM (10−200 mg/L). In both cases, the probes exhibit weak unbound fluorescence followed by 10 to 100-foldstrong enhancements with binding to HSA. Given the relative modes optimization performed on the initial hit, we anticipate that further enhancements using combinatorial synthesis will produce even better probes.[21]

The efficient fluorescence of the chromophore in GFP has been ascribed to the uniqueness of the β-barrel. We have shown that such chromophores can be activated by a nonbarrel protein and can accommodate an aqueous environment. This study represents one of the first cases utilizing a FP chromophore as a probe, with the exclusion of metal sensing,[22] and provides motivation for this class of compounds to be considered for sensing. We note that other probes for HSA exist and are documented in the literature,[23] however unlike probes which depend upon changes in environmental (solvent) polarity, this is a topological probe, depending instead upon conformational freezing for its efficacy. Additional studies to thoroughly quantify selectivity in more complex biological samples are currently underway.

■ ASSOCIATED CONTENT

Supporting Information. Experimental information, synthetic details, characterization, and additional spectroscopic information. This material is available free of charge via the Internet at http://pubs.acs.org.

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