Fluorescent Environment-Sensitive Dyes as Reporters of Biomolecular Interactions

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Abstract

Monitoring biomolecular interactions is a fundamental issue in biosensing, with numerous applications ranging from biological research to clinical diagnostics. Fluorescent dyes capable of changing their color and brightness in response to changes of their environment properties, the so-called environment-sensitive dyes, have recently emerged as reporters of these interactions. The most well established of these are dyes that undergo excited-state charge transfer showing red shift of their single emission band with increase in the solvent polarity. The other promising class are dyes of the...
3-hydroxychromone family that undergo excited-state intramolecular proton transfer and show solvent-sensitive dual emission. Examples of existing solvatochromic dyes and their biosensing applications are given, with particular focus on the 3-hydroxychromones. It is shown that solvatochromic dyes are powerful tools for monitoring conformation changes of proteins and their interactions with nucleic acids, proteins, and lipid membranes.

1. INTRODUCTION

Monitoring biomolecular interactions is a fundamental issue in biosensing, with numerous applications ranging from basic biological research to clinical diagnostics. Fluorescence techniques are particularly well suited for this purpose. The most established one is the Förster resonance energy transfer (FRET)-based approach, where interacting partners are labeled with donor and acceptor molecules. The interaction event results in an energy transfer between the proximal donor and acceptor, providing the analytical signal. Though the approach is robust, it requires double labeling, which is complicated and cannot be realized in many screening assays. Therefore, single fluorescence labeling techniques, where only one of the partners is labeled, are of high interest. The most well-established single-labeling approach is based on fluorescence anisotropy, which follows changes in the mobility of the fluorescent label that is grafted to one of the interacting partners. The other approach, which has emerged only recently, is utilization of environment-sensitive dyes.

Unlike “classical” dyes, environment-sensitive dyes can change their fluorescence properties, fluorescence intensity or emission color, in response to changes in the physicochemical properties of their molecular environment. While classical dyes are perfect markers of biological molecules, the environment-sensitive dyes are “smart molecules” that can be used as sensors for probing the local biological environment and monitoring biomolecular interactions. Within this approach, the interaction between the molecules changes the properties of the local site of interaction, which in turn affects the fluorescence properties (emission maximum or intensity) of the environment-sensitive labels (Fig. 2.1).

The response of environment-sensitive dyes to the environment is driven by excited-state reactions (conformational change, charge, electron and proton transfer, etc.) and noncovalent interactions with the surrounding, such as universal interactions (van der Waals, dipole–dipole, dipole–external electric...
field, etc.), and specific H-bonding interactions. Here, we do not consider pH- and ion-sensitive dyes as environment sensitive, as the response of these dyes is associated with changes in their chemical structure: protonation/deprotonation or formation of a complex with an ion.

Several types of environment-sensitive fluorophores are of particular interest for biomolecular applications: molecular rotors and solvatochromic fluorescent dyes. Molecular rotors are an interesting class of environment-sensitive dyes, which change their emission intensity in response to the change of the solvent viscosity. These dyes feature high rotational flexibility of their conjugated system so that they are poorly emissive in nonviscous environments (such as water or organic solvents). In viscous environments, such as biological membranes and biomacromolecules, their rotation mobility is restricted, which dramatically increases their fluorescence quantum yield. Thus, these dyes can turn on their fluorescence in response to interactions that rigidify their environment. Though these dyes were largely applied as probes in biological membranes, their application for detection of biomolecular interactions is still poorly explored and therefore will not be reviewed here. A much better established class comprises solvatochromic fluorescent dyes. They exhibit shifts in their emission maxima and sometimes change in their fluorescence quantum yield as a function of polarity and hydration of their environment. In these dyes, the dipole moment increases dramatically upon electronic excitation ($S_0 \rightarrow S_1$ transition) because of an intramolecular charge transfer from the electron-donor group to the electron-acceptor group (Fig. 2.2). Polar solvents relax efficiently the excited molecules to the $S_1^{\text{solv}}$ state as a result of polarization of the solvent dipoles around the fluorophore dipole. An increase in the solvent polarity decreases the energy of the $S_1^{\text{solv}}$ state, resulting in a red shift in the emission spectra (Fig. 2.2). In addition, protic solvents (which contain hydrogen atoms bound to oxygen (hydroxyl) or to nitrogen (amine, amide, etc.)) interact with the
fluorophore through H-bonding and thus can also decrease the energy of $S_1^{\text{solv}}$ state. Therefore, both dipole–dipole and H-bonding interactions in polar solvents can shift the emission of these dyes to the red. Water is a highly dipolar molecule as well as an exceptionally strong H-bond donor. Therefore, its effect on the emission color of the solvatochromic dyes is particularly drastic and can be used for detection of biomolecular interactions as shown in Fig. 2.1. An additional important property of most environment-sensitive dyes is their poor fluorescence intensity in water, because of intermolecular electron or proton transfer. Therefore, incorporation of these dyes into proteins and lipid membranes usually increases strongly their fluorescence intensity as a result of efficient screening of these molecules from bulk water.\textsuperscript{6,8,13} An unusual class is the two-band solvatochromic fluorescent dyes based on 3-hydroxychromone (3HC) derivatives. Because of excited-state intramolecular proton transfer (ESIPT), they show two emission bands, which change their relative intensities in response to solvent polarity.\textsuperscript{8} This chapter will briefly present the design and applications of single-band environment-sensitive dyes, with the main focus on the dyes based on 3HC. For more details on single-band solvatochromic dyes and their biological applications, the reader should see the excellent review by Imperiali \textit{et al.}\textsuperscript{6}

\textbf{Figure 2.2} Simplified diagram explaining the phenomenon of solvatochromism.
2. SINGLE-BAND SOLVATOCHROMIC DYES: DESIGN AND APPLICATIONS

Representative examples of single-band solvatochromic dyes are shown in Fig. 2.3. All these molecules bear an electron-donor and electron-acceptor group attached to a conjugated (usually aromatic) system. These dyes present a single emission band, which changes its position and sometimes intensity in response to changes in the environment polarity. Their spectroscopic and solvatochromic properties are shown in Table 2.1. As a general trend, it can be noticed that these dyes are characterized by an absorption in the violet or ultraviolet (UV) region as well as by a relatively low extinction coefficient, compared to the classical fluorescent dyes (rhodamine, fluorescein, cyanines, etc.). Two dye families, phenoxazine and nitrobenzoxadiazole (NBD) derivatives, present red-shifted absorption but are characterized by relatively weak fluorescence solvatochromism. Most solvatochromic dyes show relatively high fluorescence quantum yields in apolar solvents, which drop drastically in polar solvents, especially in water. From Table 2.1, it is clear that, though there is a large variety of solvatochromic dyes, there is none that could feature excellent

![Figure 2.3](image-url)

**Figure 2.3** Examples of fluorescent solvatochromic dyes. Schematic presentation of fluorophore structure: electron-donor and -acceptor groups are shown in red and blue, respectively. Group R indicates the common point of the dye conjugation.
To label biomolecules with solvatochromic dyes, their amino or thiol-reactive derivatives are commonly used. In solvatochromic labels, the fluorophore should be connected to the reactive group through the shortest possible linker, in order to localize it precisely at the labeling site of a biomolecule of interest. However, the most efficient method of site-specific protein labeling corresponds to the direct introduction of the amino acid derivative of the solvatochromic dye into the peptide. This can be achieved by solid-state peptide synthesis or by using cellular protein synthesis.

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{Abs}}$ (nm) (MeOH)</th>
<th>$\varepsilon_{\text{max}}$ (MeOH)</th>
<th>$\lambda_{\text{Fluo}}$ (nm)</th>
<th>QY (%)</th>
<th>$\Delta\nu_{\text{Fluo}}$ (cm$^{-1}$)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoroprobe</td>
<td>308$^b$</td>
<td>12,000</td>
<td>695$^c$</td>
<td>$&lt;0.1^c$</td>
<td>476</td>
<td>46</td>
</tr>
<tr>
<td>FR0</td>
<td>396</td>
<td>43,000</td>
<td>570</td>
<td>19</td>
<td>434</td>
<td>98</td>
</tr>
<tr>
<td>Dapoxyl® derivatives</td>
<td>373</td>
<td>28,000</td>
<td>584</td>
<td>39</td>
<td>457</td>
<td>86</td>
</tr>
<tr>
<td>Prodan</td>
<td>361</td>
<td>18,400</td>
<td>498</td>
<td>51</td>
<td>417</td>
<td>55</td>
</tr>
<tr>
<td>3MC-2</td>
<td>445</td>
<td>29,000</td>
<td>597</td>
<td>1.4</td>
<td>485</td>
<td>68</td>
</tr>
<tr>
<td>6DMN</td>
<td>382</td>
<td>8000</td>
<td>589</td>
<td>1.2</td>
<td>491</td>
<td>21</td>
</tr>
<tr>
<td>Anthradian</td>
<td>456</td>
<td>12,100$^d$</td>
<td>604</td>
<td>41</td>
<td>507</td>
<td>58</td>
</tr>
<tr>
<td>4DMP</td>
<td>396</td>
<td>6500</td>
<td>534</td>
<td>12$^e$</td>
<td>457$^e$</td>
<td>62$^e$</td>
</tr>
<tr>
<td>Dansyl derivatives</td>
<td>335</td>
<td>4600</td>
<td>526</td>
<td>49</td>
<td>471</td>
<td>81</td>
</tr>
<tr>
<td>Nile Red</td>
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<td>45,000</td>
<td>632</td>
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<tr>
<td>NBD</td>
<td>465</td>
<td>22,000</td>
<td>541</td>
<td>$-$</td>
<td>529</td>
<td>$-$</td>
</tr>
</tbody>
</table>

$^a$$\lambda_{\text{Abs}}$, absorption maximum; $\varepsilon_{\text{max}}$, absorption coefficient (M$^{-1}$cm$^{-1}$); $\lambda_{\text{Fluo}}$, fluorescence maximum; QY, fluorescence quantum yield; MeOH, methanol; $\delta_{\text{Fluo}}$, band shift in response to change of solvent from toluene to methanol.

$^b$Data in cyclohexane.

$^c$Tetrahydrofuran.

$^d$Acetonitrile.

$^e$Dioxane.

Table 2.1 Properties of common solvatochromic fluorescent dyes

brightness and red-shifted absorption, together with high fluorescence solvatochromism.
machinery, where the genetic code is extended to an unnatural fluorescent amino acid.\textsuperscript{25,26} We will briefly present the most common classes of single-band solvatochromic dyes and examples of their applications.

2.1. Naphthalene sulfonic acid derivatives

One of the first solvatochromic dyes, which still remains an essential tool for protein and membrane studies, is 1-Anilinonaphthalene-8-Sulfonic Acid (1,8-ANS).\textsuperscript{13,27} In addition to the strong solvent-dependent shift in its emission spectrum, it shows a dramatic increase in fluorescence intensity on binding to biomolecules. In bulk water, the fluorescence of this dye is strongly quenched, while, on being bound to proteins or lipid membranes, it is efficiently screened from water, resulting in a strong increase in fluorescence. The other naphthalene sulfonic acid analogues that have found even more applications in biology are Dansyl derivatives (Fig. 2.3). The reactive derivative Dansyl chloride was commonly used to label amino groups in proteins and lipids. Similar to 1,8-ANS, the emission color and emission intensity of the Dansyl moiety are highly sensitive to solvent polarity.\textsuperscript{28,29} This fluorophore was one of the first used for protein labeling, but currently it is used only rarely because of its UV absorption and rather weak solvatochromism.

2.2. Prodan

Prodan is one of the best classical examples of solvatochromic dyes,\textsuperscript{17} and has found numerous biological applications because of its remarkable sensitivity to solvent polarity together with its relatively small size. Reactive derivatives of Prodan such as Acrylodan (6-acryloyl-2-dimethylamino-naphthalene)\textsuperscript{22,30} or Badan (6-bromoacetyl-2-dimethylaminonaphthalene)\textsuperscript{31} have been attached covalently to proteins via reaction with thiol groups. For instance, Acrylodan attached to the N-terminus of peptide ligands was used to monitor their interactions with the holecystokinin receptor.\textsuperscript{30} In another representative study, six cysteine-substituted sites of mouse acetylcholinesterase were labeled individually with Acrylodan and the kinetics of substrate hydrolysis and inhibitor binding were examined. While some sites of labeling located far from the active center did not show any spectral changes of Acrylodan on inhibitor binding, the sites located near the perimeter of the gorge showed blue shifts, reflecting the exclusion of solvent and creation of a hydrophobic environment by the associated ligand.\textsuperscript{32} This study is a nice demonstration how a conformation change upon enzyme–inhibitor interaction can be specifically “mapped” over all protein. To localize better the fluorophore on
the peptide backbone, an amino acid derivative of Prodan, namely, 6-dimethylaminonaphtoyl alanine (Aladan), was synthesized independently by two research groups. The probe allowed monitoring binding of S-peptide with ribonuclease S and estimation of the local dielectric constant of the B1 domain of the staphylococcal protein G at different sites. Later, this amino acid was successfully applied to the study of delta-opioid receptor antagonist binding.

2.3. Improved Prodan analogues

The key weakness of Prodan is its absorption in UV (360 nm), which limits its cellular applications. In order to shift the absorbance of Prodan to the red, Lu et al. synthesized its benzo-analogue, 2-propionyl-6-dimethylaminoanthracene, Anthradan. This dye showed the desired red-shifted absorption (around 430 nm), but its brightness was limited because of its low absorption coefficient. Recently, we extended the electronic conjugation of Prodan by substituting its naphthalene core with fluorene. The obtained fluorene derivative, FR0 (Fig. 2.3), showed a

Figure 2.4 Absorption (dash) and fluorescence (solid) spectra of Prodan (A) and FR0 (B) in organic solvents of different polarities. Absorption spectra were recorded in toluene. ET(30)—empirical polarity index. Data from Ref. 15.
red-shifted absorption (close to 400 nm) (Fig. 2.4), with twice as large an absorption coefficient and a manifold larger two-photon absorption cross section (400 Goeppert-Mayer) compared to Prodan. Moreover, studies in organic solvents have revealed a much stronger dependence of its emission maximum on solvent polarity (Fig. 2.4), which is connected with its twice as large change in the dipole moment (14 D).

2.4. 4-Aminophthalimide analogues

The other important family of solvatochromic dyes, which attracted attention only recently, is 4-aminophthalimide analogues. The simplest of them, 4-N,N-dimethylaminophthalimide (4DMP), can be considered as one of the first chromophores for polarity sensing. This relatively small and rigid molecule presents a very strong solvent sensitivity. However, similar to Prodan, it shows absorption in the UV region (380–390 nm) and a very low absorption coefficient. To improve its properties, the 4DMP fluorophore was extended, giving a new environment-sensitive dye 6-N,N-dimethylamino-2,3-naphthalimide (6DMN) (Fig. 2.3). This fluorophore exhibits interesting fluorescence properties with emission in the 500–600 nm range and combined (fluorescence intensity and position of the maximum) response to changes in the environment polarity, though its absorption properties are not considerably improved. In this respect, a recently developed analogue of 4DMP, 4-N,N-dimethylamino-1,8-naphthalimide (4DMN), constitutes a significant improvement, showing a shifted absorption maximum at 440 nm. It should be noted that, similar to 1,8-ANS, 4DMP and all its analogues are nearly nonfluorescent in water but become highly fluorescent in aprotic media. These properties are important for intensiometric detection of molecular interactions. However, their poor fluorescence in water makes them inefficient in the cases where the label shows significant water exposure at all steps of interaction.

To apply the 4DMP fluorophore for protein studies, Fmoc-protected amino acid bearing this fluorophore was synthesized and introduced into an octapeptide using standard solid-phase synthesis. The label was able to sense phosphorylation-dependent binding of the synthesized peptide to the 14-3-3bp protein. Peptides labeled with a 6DMN-based amino acid were used for monitoring protein–protein interactions, as exemplified in studies with the SH2 phosphotyrosine binding domains. The same labeled amino acid was used for sensing peptide binding to proteins of a major histocompatibility complex at the cell surface. Furthermore, an amino acid
analogue of 4DMN, featuring improved spectroscopic properties and chemical stability, was introduced into a peptide that is recognized by calmodulin. Remarkably, the interaction event between these two molecules yielded >900-fold increase in the fluorescence intensity of the dye.36

2.5. Fluoroprobe

In the search for advanced environment-sensitive dyes, a bichromophoric dye Fluoroprobe (Fig. 2.3) was developed.14 This dye exhibits a charge transfer through space, which generates an exceptional change in the dipole moment (27 D) and, thus, solvent sensitivity. For the moment, Fluoroprobe remains the most solvatochromic fluorescent dye. However, it has found no applications in biology, because of the extremely strong quenching of its fluorescence in polar media, UV absorption (308 nm), and very low extinction coefficient (Table 2.1).

2.6. Dapoxyl® derivatives

It is also worth mentioning a Dapoxyl® dye (Fig. 2.3) showing remarkable fluorescent solvatochromism (up to 200 nm red shift from hexane to water/acetonitrile (4/1) mixture), as well as high fluorescence quantum yield and extinction coefficient.16 However, its absorption in the UV range remains a disadvantage. Moreover, its applications are limited so far to only a few examples, such as biosensor development39 and FRET-based assays in peptides.40

2.7. NBD

An important example of red-shifted environment-sensitive dye is NBD. Its small-sized fluorophore and absorption around 480 nm are very convenient for biological applications. However, its solvatochromism is very small, which limits its applications to the cases where the changes in the environment are drastic. NBD was one of the first environment-sensitive dyes (together with Dansyl) used as a protein label. NBD helped revealing a mechanism for the transduction of ligand-induced protein conformational changes24 through the distinct maltose-dependent fluorescence response observed with three individual cysteine mutants of *Escherichia coli* maltose-binding protein, covalently labeled with NBD. The results provided insights for designing fluorescent biosensors.

2.8. Phenoxazine derivatives

A representative example of phenoxazine-based dyes is Nile Red.23 It combines red-shifted absorption (around 530 nm) and emission together with high brightness. Nevertheless, its solvatochromism is moderate, being larger
than that of NBD, but smaller than that of the UV/blue analogues described above (Table 2.1). In the recent years, a variety of Nile Red derivatives for biomolecule labeling have been reported.\textsuperscript{41,42} Thiol-reactive Nile Red derivatives were successfully applied for labeling a galactose/glucose binding protein. The obtained conjugate was validated as a fluorescence biosensor for glucose detection.\textsuperscript{43} Phenoxazine derivatives, which are more compact analogues of Nile Red, were also used for designing the thiol-reactive probe, aminophenoxazone maleimide (APM), which was used for reporting protein conformational changes.\textsuperscript{44} APM has a short linker between the probe and the protein, ensuring that it can closely follow the motions the water-exposed domain of the β2 adrenergic receptor during its interactions with ligands. In addition, APM was shown to sense the conformational changes underlying voltage sensing in the Shaker potassium channel.\textsuperscript{44}

2.9. New advanced solvatochromic dyes

This overview on the existing environment-sensitive dyes shows that there is a strong need for new solvatochromic fluorescent dyes presenting both strong solvatochromism and good fluorescence properties. Most of the dyes described above (except Nile Red and NBD analogues) absorb in the UV range (Table 2.1), which is generally not suitable for biological applications. Excitation at longer wavelengths would decrease significantly the photodamage of the biological samples and decrease their autofluorescence. The photostability of most of environment-sensitive dyes is also limited so that they are not compatible with some modern techniques, such as single-molecule fluorescence detection.\textsuperscript{45} Moreover, the low fluorescence quantum yield in aqueous media of most of the described dyes limits their application for the investigation of small peptides, where the label is exposed to water. Finally, sensitivity to solvent polarity of these dyes is frequently not enough to detect subtle changes in the environment of the biomolecule of interest. Therefore, the research in this field is now focused on improving the solvent sensitivity of the dyes, shifting their emission wavelength to the red and increasing fluorescence brightness and photostability. For example, the 2-dicyanomethylene-3-cyano-2,5-dihydrofuran family of fluorophores (see DCDHF in Fig. 2.3), featuring red-shifted absorption and good solvatochromism, appears promising for single-molecule experiments because of their very high photostability.\textsuperscript{46,47} The recently introduced 3-methoxychromones (3MC-2) appear also of interest, as in addition to good photostability and fluorescence quantum yields, they
present very high solvatochromism.\textsuperscript{18} We should also mention polymethine dyes displaying near-IR emission and solvent-dependence of their lifetime,\textsuperscript{48} as well as BODIPY derivatives, capable of switching on–off their fluorescence in response to solvent polarity changes.\textsuperscript{49} Although these fluorophores have not yet been applied for biomolecular studies, they appear as attractive building blocks for future high-performance polarity-sensitive labels of biomolecules.

3. TWO-BAND SOLVATOCHROMIC DYSES BASED ON ESIPT

An alternative mechanism of solvent sensitivity can be realized by utilizing the so-called ESIPT. Particularly interesting ESIPT dyes are 3-hydroxychromones (3HCs), presenting dual emission originating from the normal excited state (N\textsuperscript{*}) and the ESIPT tautomer (T\textsuperscript{*}) (Fig. 2.5).\textsuperscript{50} The pathway for ESIPT in 3HCs is provided by the intramolecular H-bond through a five-membered cycle, which is much weaker than the six-membered cycle presented by other ESIPT systems. Therefore, it can be easily perturbed by H-bonding interactions, thus modulating the dual emission of 3HCs.

![Figure 2.5](image)

**Figure 2.5** Photophysical cycle of a 3HC derivative 4'-(N,N-diethylamino)-3-hydroxyflavone. On electronic excitation (N$\rightarrow$N\textsuperscript{*}), a charge transfer from the 4'-dialkylamino group to the 4-carbonyl takes place followed by an ESIPT process (N\textsuperscript{*}$\rightarrow$T\textsuperscript{*}). After T\textsuperscript{*}$\rightarrow$T transition, the proton remains at the 4-carbonyl group, producing a zwitterionic T state that rapidly converts into the stable N state.
Among the 3HCs developed so far, two derivatives are of particular interest: 4'-((dialkylamino)-3-hydroxyflavone (3HC-A) and 2-(2-furyl)-3-hydroxychromone (3HC-B) (Fig. 2.6). Owing to the 4'-dialkylamino group, the N* excited state of 3HC-A exhibits a large dipole moment, where the electronic charge is transferred from the dialkylamino group to the chromone moiety (Fig. 2.5).\textsuperscript{51} In contrast, the ESIPT product T* state exhibits much lower charge separation and, thus, lower dipole moment. Therefore, only the N* state shows a significant shift to the red on increase in solvent polarity (Fig. 2.6). This red shift is accompanied by an increase in the relative intensity of the N* band, because this state becomes energetically more favorable than the T* state.\textsuperscript{52} Therefore, the intensity ratio of the N* and T* bands, that is, N*/T*, is an important indicator of solvent polarity.\textsuperscript{51} However, 3HC-A shows dual emission only in the range of low-polar and polar aprotic solvents (Fig. 2.6). In polar protic solvents, including water, the ESIPT is efficiently inhibited so that the T* emission is no more observed.\textsuperscript{51} Therefore, this dye could be applied for probing biological environments of
relatively low polarity and hydration, such as, for instance, biological membranes (see below).

On the other hand, the 3HC-B dye is much more appropriate for highly polar media. Owing to its much weaker electron-donor 2-aryl group (2-furanyl vs. 4-dialkylaminophenyl in 3HC-A), the dipole moment of its N* state is relatively low. Therefore, this state cannot be stabilized even in highly polar aprotic solvents so that its emission is almost negligible (Fig. 2.6). Moreover, in contrast to 3HC-A, the ESIPT inhibition by protic solvents is not complete so that a clear dual emission depending on solvent polarity is observed. Thus, the 3HC-B dye is suitable for probing polar protic environments characterized by high hydration, which corresponds well to peptides and nucleic acids (see below).

We should note the key differences between single-band solvatochromic fluorescent dyes and 3HC dyes. While the former shift their emission maximum in response to solvent polarity, 3HC dyes may change both the positions of bands as well as their intensity ratio. This ratio is an additional channel of spectroscopic information, which allows more detailed (multiparametric) characterization of the probe environment. Moreover, as a result of ESIPT, 3HC dyes are focused in a narrower polarity range, where they can show higher sensitivity to properties of environment compared to single-band solvatochromic dyes.

4. APPLICATIONS OF TWO-COLOR DYES FOR MONITORING BIOMOLECULAR INTERACTIONS

4.1. Monitoring conformational changes of proteins

Conformational changes in proteins can result in significant changes in the site exposure to bulk water. This idea was validated using α1-antitrypsin (α1-AT), which, during the complex multistep inhibition of proteinases, undergoes dramatic changes in the tertiary structure (Fig. 2.7). As α1-AT contains only one cysteine (Cys-232), this residue was specifically labeled with a thiol-reactive 3HC-B derivative. The intensity ratio T*/N* of 1.18 for the labeled protein was between that observed for water (0.45) and for ethanol (2.22), suggesting that the label is partially screened from bulk water by the protein environment. The interaction of the labeled α1-AT with pancreatic elastase led to ~65% change in the T*/N* intensity ratio of the two emission bands, suggesting an increased exposure of the labeled Cys-232 residue to the bulk water on complex formation. Similar experiments with elastase and α1-AT conjugated to a NBD derivative
confirmed these results but led to much smaller modifications in the emission spectrum, indicating superiority of the 3HC label in terms of environment sensitivity. Stopped-flow studies of the reaction between the labeled \( \alpha_1 \)-AT and elastase showed, in addition to the well-described fast step, a new slow step of the inhibition process\(^{55} \) that is probably associated with a slow structural reorganization aimed at stabilizing the final inhibited complex.

### 4.2. Peptide–nucleic acid interactions

Monitoring interactions of peptides with nucleic acids can be achieved through a single labeling of the peptide partner with a solvatochromic dye. However, in this case, solvent sensitivity of these dyes should be optimized for polar environments and remain fluorescent in the DNA complex. 3HC-B fluorophore meets both requirements according to our previous

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**Figure 2.7** Conformational changes in \( \alpha_1 \)-AT on its reaction with elastase, as monitored by a 3HC probe. The graph A shows the X-ray structures of the free \( \alpha_1 \)-AT and the \( \alpha_1 \)-AT/ elastase complex.\(^{54} \) The labeled Cys is in yellow. The graph B shows the kinetics of this conformation transition monitored by our 3HC probe. The inset in this figure presents the fast part of this transition.\(^{55} \)
A carboxylic acid derivative of 3HC-B was attached to the N-terminus of the (11–55) sequence of the NCp7 protein from human immunodeficiency virus (HIV-1) using solid-state peptide synthesis (Fig. 2.8). This protein plays an important role in HIV-1 through interactions with the viral RNA and its DNA copies, and notably with cTAR, the complementary DNA sequence of the transactivation response element from the HIV-1 genome. The interaction of the labeled NCp7 fragment with cTAR changed...
dramatically the emission color of the 3HC probe. Different oligonucleotide (ODN) sequences have been tested and the obtained spectroscopic data were correlated with the known nuclear magnetic resonance (NMR) structure of the peptide–ODN complexes (Fig. 2.8). The results suggested that the 3HC label senses the proximity of the peptide-labeling site (N-terminus) to the ODN bases. This approach allowed us to determine the peptide–ODN binding parameters and distinguish multiple binding sites in ODNs, which is rather difficult using other fluorescence methods. Moreover, this method was found to be more sensitive than steady-state fluorescence anisotropy, in the case of small ODNs. Further synthesis of the analogues of 3HC-B presenting increased brightness and solvatochromism allowed us to significantly improve the sensitivity of this method. Currently, we are developing L-amino acids bearing 3HC fluorophores, which is an important milestone in the probing of peptide–DNA interactions at any desired peptide site.

4.3. Protein–protein interactions

Monitoring protein–protein interactions is of key importance in the development of peptide-based biosensors. These interactions result commonly in changes of the environment particularly at their interface and, therefore, can be detected by solvatochromic labels (Fig. 2.1). However, classical solvatochromic dyes (based on NBD, Prodan, 4DMP, etc.) working on the principle of the emission band shift and/or fluorescence quenching are not always so efficient in highly polar media. In this respect, ESIPT-based 3HC labels are attractive alternatives, particularly for applications with small peptides and proteins, where the polarity of the labeling site is high.

To evaluate the possibility of using our 3HC probe for sensing peptide–peptide interactions, the high-affinity model of interaction between the synthetic 18-amino acid peptide pTMVP and a recombinant antibody fragment, Fab57P, was used. The pTMVP peptide, which contains the Fab57P epitope of the tobacco mosaic virus coat protein, was functionalized with a thiol-reactive 3HC-B derivative. The dissociation constant $K_d$ of the interaction between pTMVP and Fab57P was largely preserved upon labeling, as evidenced by the surface plasmon resonance technique. The ratio of the two emission bands ($N^*/T^*$) of the pTMVP peptide labeled at its C-terminus was found to change by 40% upon interaction with Fab57P. These changes corresponded to a decrease in the
hydration of the peptide labeling site upon its interaction with the target peptide. Following a similar approach, another biosensor was developed (Fig. 2.9), in which peptides labeled at the N-terminus were used for specific detection of an antibody fragment scFv. Studies of different fluorescent peptide conjugates revealed that the response to the interaction event requires optimal distance between the fluorophore and the peptide interaction site. These works show the possibility of transforming a peptide, representing a minimized analyte binding site, into a ratiometric biosensor molecule by functionalization with a fluorophore.

Another important aspect is monitoring protein aggregation, which commonly leads to the dramatic changes in the protein environment at their interface. A current challenge in this field is to monitor the early and intermediate stages of α-synuclein aggregation, a process associated with Parkinson’s disease. To this end, an Ala→Cys mutant of α-synuclein was labeled with the thiol-reactive 3HC–B derivative and used as a sensor of the α-synuclein aggregation. Strong changes in the dual emission of the label resulted from protein aggregation, allowing continuous monitoring
of this process and detection of the aggregated structures much earlier than other techniques.

### 4.4. Peptide–membrane interactions

Monitoring interactions of proteins with biological membranes is of particular importance for studying membrane proteins and peptide-based toxins and for understanding the peptide transport through cell membranes. These interactions should lead to remarkable changes in the polarity of the peptide environment, as the rather polar peptide interface is substituted with the highly apolar lipid membrane environment. Therefore, the 3HC-A dye is more appropriate for these studies. Its carboxylic acid derivative was attached to the N-terminus of melittin and poly-L-lysine peptides, which interact with lipid membranes in a very different manner. Binding of these peptides to lipid vesicles induced a strong fluorescence increase, which enabled the quantification of the peptide–membrane interactions. Moreover, the dual emission of the label in these peptides correlated well with the depth of its insertion, measured by the parallax quenching method (Fig. 2.10). Thus, in melittin, which shows deep insertion of its N-terminus, the label presented a dual emission corresponding to a low-polar environment, while the environment of the poly-L-lysine N-terminus was rather polar, in line with its binding at the surface of the lipid head groups. Moreover, imaging of labeled peptides bound to giant vesicles gave some clues on the orientation of the label within the membrane, which could help estimating the peptide orientation.

This label was further successfully applied for monitoring the interaction of α-synuclein with model membranes, as this interaction was hypothesized to play a role in the pathological misfolding and aggregation of this protein during Parkinson’s disease. Systematic studies of α-synuclein labeled with 3HC-A fluorophore revealed the influence of charge, phase, curvature, defects, and lipid unsaturation on binding of α-synuclein to model membranes and its further conformational changes.

### 5. CONCLUSIONS

Solvatochromic dyes, because of their ability to undergo excited-state reactions (charge and proton transfer), can change their emission color and intensity in response to variation of solvent polarity. Though a number of
Solvatochromic dyes has already been developed, there is a clear need for new dyes presenting high solvatochromism, brightness, and photostability. 3HCs are particularly interesting examples of solvatochromic dyes because of the strong solvent sensitivity of their dual emission generated by ESIPT. Sensitivity of the dyes to the environment polarity and hydration can be applied for probing the interaction between biomolecules. Being covalently attached to peptides, they enable monitoring conformation changes of proteins and their interactions with nucleic acids, proteins, and lipid membranes. Therefore, solvatochromic dyes could become a universal tool for detecting almost any kind of biomolecular interactions. However, the further success of the approach will strongly rely on the development of new improved dyes.

Figure 2.10 Monitoring the interaction of melittin and polylysine labeled with the 3HC-A fluorophore with lipid membranes. (A) Fluorescence spectra of labeled melittin and polylysine bound to model lipid membranes. (B) Schematic presentation of the insertion of the peptides into the lipid membrane.66
REFERENCES


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