Site-Dependent Excited-State Dynamics of a Fluorescent Probe Bound to Avidin and Streptavidin

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Abstract
Sensing protein environment: The femtosecond fluorescence dynamics of a molecular probe attached to avidin and streptavidin (see figure) depends markedly on the location of the probe and on the protein. These differences reflect not only the protein primary and tertiary structures but also the effect of the surrounding water molecules.

Reference

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Site-Dependent Excited-State Dynamics of a Fluorescent Probe Bound to Avidin and Streptavidin**


The excited-state dynamics of biotin–spacer–Lucifer-Yellow (LY) constructs bound to avidin (Avi) and streptavidin (Sav) was investigated using femtosecond spectroscopy. Two different locations in the proteins, identified by molecular dynamics simulations of Sav, namely the entrance of the binding pocket and the protein surface, were probed by varying the length of the spacer. A reduction of the excited-state lifetime, stronger in Sav than in Avi, was observed with the long spacer construct. Transient absorption measurements show that this effect originates from an electron transfer quenching of LY, most probably by a nearby tryptophan residue. The local environment of the LY chromophore could be probed by measuring the time-dependent polarisation anisotropy and Stokes shift of the fluorescence. Substantial differences in both dynamics were observed. The fluorescence anisotropy decays analysed by using the wobbling-in-a-cone model reveal a much more constrained environment of the chromophore with the short spacer. Moreover, the dynamic Stokes shift is multiphasic in all cases, with a ~1 ps component that can be ascribed to diffusive motion of bulk-like water molecules, and with slower components with time constants varying not only with the spacer, but with the protein as well. These slow components, which depend strongly on the local environment of the probe, are ascribed to the motion of the hydration layer coupled to the conformational dynamics of the protein.

1. Introduction

The excited-state dynamics of many fluorophores is strongly affected by their local molecular environment.[1–5] Relaxation processes such as the reorganization of the surroundings after photoexcitation, namely solvation, or vibrational cooling, or photochemical processes such as fluorescence quenching via electron transfer, proton transfer, or isomerization are largely dependent on the direct environment of the chromophore. As these processes take place on a time scale of a few tens of femtoseconds up to several nanoseconds, femtosecond-resolved spectroscopy is a powerful tool to investigate the nanoevironment of a probe.[4,5] Whereas the relaxation dynamics of chromophores in bulk solvents is fairly well understood, it is much less the case in heterogeneous environments or at interfaces. The highly organised nature of biological macromolecules such as proteins or nucleic acids makes biomolecular interfaces very special and despite the growing number of reports,[6] the available amount of information is too scarce for a full understanding of the influence of biological environments on the dynamics of excited chromophores to be achieved. A comprehensive picture would in turn enable the development of new routes to access very local information spectroscopically and gain deeper insight into selected molecular systems.

Despite the incomplete understanding of the protein–water interface, it is at the heart of life’s machinery. Interfacial water plays a very important role in the structure and the dynamics of proteins as it shapes the potential energy landscape that governs their folding, structure, stability, and function.[7–9] Protein dynamics seems to be slaved to the dynamics of water fluctuations.[10] On the other hand, protein activity is also related to its dynamics, so that gaining insight into the properties of interfacial water becomes crucial to understand the microscopic mechanisms of protein function.[9,11]

A large variety of techniques have been used to address the question of the structure and dynamics of interfacial water, also called hydration water,[7] over several time scales, from X-ray crystallography to dielectric spectroscopy (microseconds to 0.1 ns),[12,13] magnetic relaxation dispersion spectroscopy and terahertz spectroscopy (picoseconds),[14,15] inelastic neutron scattering (0.1–100 ps),[16] or fluorescence and photon-echo
spectroscopy (femtoseconds to nanoseconds).\[17,18\] Because the slower protein motions are slaved to fast solvent modes,\[19\] it is essential to capture the dynamics of the interfacial water on its intrinsic time scale, that is, femtoseconds to picoseconds. One way to experimentally access the diffusive dynamics of water is by measuring the solvation dynamics of a selected probe at the interface with a biological macromolecule. This has been mostly done using time-resolved fluorescence spectroscopy, by following the time-dependent Stokes shift of a fluorophore, or by photon-echo spectroscopy. Since the first experiment to probe solvation dynamics at a protein interface was performed in 1971 with 2-p-toluidinynaphthalene-6-sulfonate adsorbed to bovine serum albumin,\[19\] several protein systems have been investigated by spectroscopic and molecular dynamics (MD) tools.\[17,18,20–26\] The consensus picture, which emerges from these very heterogeneous investigations, is that the time-dependent Stokes shift observed at protein surfaces has been shown to be substantially slower compared to the bulk solvent. Essentially two time scales have been experimentally observed, one which is similar to that of bulk water (one up to a few picoseconds), and another which is at least one order of magnitude slower. The origin of this slower component is still being discussed,\[22,24,27\] although the results of recent MD simulations tend to indicate rather clearly that water motions coupled to protein fluctuations are responsible for it.\[25\] This long-time component seems to be most dependent on the nature of the investigated system and its interpretation should therefore be made cautiously. Another open issue is the absence of the ultrafast inertial solvation component in many of the studies, probably due to a lack of proper time resolution. Furthermore, besides one early report using two different chromophores,\[29\] and two others looking into the effect of a systematic variation of the same probe molecule at different positions of the same protein,\[28,29\] no experimental study describes a systematic variation in the same probe at variable distances from the protein surface.

Despite the large number of reported experiments, the exact mechanism of solvation at the interface with biological macromolecules is not fully understood in all cases. The diversity of the systems investigated has not favoured the emergence of a real general molecular understanding so far. Elucidation of the molecular mechanisms of solvation will only be possible if many more experiments with optimal control and systematic variation in the experimental conditions can be performed in parallel to an efficient modelling of the investigated systems.

Herein, we address some of these questions by presenting an investigation of the excited-state dynamics of the chromophore Lucifer Yellow (LY) in the close environment of the proteins avidin (Avi) and streptavidin (Sav) using femtosecond spectroscopy, in combination with MD simulations performed to get a better insight into the location of the probe. LY was selected as a probe because of its demonstrated sensitivity to its environment,\[30,31\] whereas Avi and Sav were chosen as proteins because they bind biotin, a small molecule which can easily be derivatised, very tightly. Biotin can indeed be covalently linked through its valeric acid moiety and a suitable spacer to almost any compound and remain available for specific and tight binding to (strept)avidin.\[32–36\] Avidin is found naturally in chicken egg white as a highly stable homotetramer of 4 × 128 residues (65.3 kDa).\[34,35\] The overall fold of each monomer is constructed of eight antiparallel β-strands which form a classical β-barrel.\[36\] Avidin binds up to four molecules of biotin in a non-covalent interaction. This is the highest known in nature between a protein and its ligand (affinity constant \(K_a = 1.7 \times 10^{15}\)).\[32,37\] Each biotin binding site is located near one end of a barrel-shaped avidin subunit and is easily accessible. Every subunit contains four Trp residues, two of them being located in the biotin binding site, one in the protein interior, while Trp110 is in a solvent-exposed loop and interacts with the biotin molecule bound to the neighbouring subunit. Streptavidin closely resembles avidin functionally and structurally.\[38,39\] Each of the four subunits is made of 159 amino acids (65.7 kDa in total), from which a core segment of 124 residues shares only approximately 32% amino acid identities when compared with the full chain of avidin. The conserved regions are only partially related to biotin recognition and binding. Among the six Trp residues contained in each streptavidin subunit, three interact with biotin at the end of the binding pocket, two are buried in the protein interior, whereas Trp120 is in a solvent-exposed loop and interacts with the biotin molecule bound to the neighbouring subunit similarly to Trp110 in Avd.

The flexibility of the Avi–biotin system allows fluorescent dyes to be attached to biotin and the distance between biotin and the dye to be varied by adding a spacer and changing its length. Cross-linking or tagging with biotinylated molecules is further readily achieved under various experimental conditions, in vitro and in vivo. On that basis, this easily tuneable system seems particularly well-suited to look into the photophysics of a fluorescent probe located at a biomolecular interface, with the benefit that the position of the fluorophore can be varied and controlled, at least to some extent, by tuning the length of the spacer introduced between biotin and the probe (Scheme 1).

**Scheme 1.** Structure of the Lucifer Yellow derivatives used in this study.
2. Results

2.1. Steady-State Spectroscopy and Nanosecond Fluorescence Dynamics

The basic photophysical properties of the LY–spacer–biotin construct LYnb (n = 3, 13) in solution are essentially the same as those of Lucifer Yellow ethylenediamine (LYen), which have been discussed in detail previously. As illustrated in Table 1,

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Probe</th>
<th>( \lambda_{\text{abs}} )</th>
<th>( \lambda_{\text{fl}} )</th>
<th>( \phi_{\text{fl}} )</th>
<th>( \tau_{\text{rad}} )</th>
<th>( n^2 \tau_{\text{rad}} )</th>
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</thead>
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<td>H2O</td>
<td>LYen</td>
<td>428</td>
<td>536</td>
<td>0.20</td>
<td>5.7</td>
<td>65.9</td>
</tr>
<tr>
<td></td>
<td>LY3b</td>
<td>427</td>
<td>539</td>
<td>0.24</td>
<td>7.3</td>
<td>72.7</td>
</tr>
<tr>
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<td>538</td>
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<td>5.6</td>
<td>65.4</td>
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<td>537</td>
<td>0.23</td>
<td>6.9</td>
<td>72.5</td>
</tr>
<tr>
<td></td>
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<td>538</td>
<td>0.24</td>
<td>6.9</td>
<td>67.5</td>
</tr>
<tr>
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<td>539</td>
<td>0.40</td>
<td>11.5</td>
<td>67.7</td>
</tr>
<tr>
<td></td>
<td>LY3b</td>
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<td>539</td>
<td>0.41</td>
<td>12.7</td>
<td>72.5</td>
</tr>
<tr>
<td>DMSO</td>
<td>LYen</td>
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<td>520</td>
<td>0.46</td>
<td>10.6</td>
<td>74.2</td>
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<tr>
<td></td>
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<td>519</td>
<td>0.47</td>
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<tr>
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<td>LYen</td>
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<td>514</td>
<td>0.47</td>
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<td>70.5</td>
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<tr>
<td></td>
<td>LY3b</td>
<td>433</td>
<td>514</td>
<td>0.47</td>
<td>12.1</td>
<td>75.7</td>
</tr>
</tbody>
</table>


topics. Only a very small blue-shift (110 cm\(^{-1}\)) of the absorption band is noticed with Sav. With LY13b, a red-shift (240 cm\(^{-1}\)) of the absorption band is observed with both proteins. Additionally, a 10–20 % hypochromism is seen upon binding of LY3b to Avi and of LY13b to both Avi and Sav. The fluorescence band of LY3b exhibits a 250 cm\(^{-1}\) blue-shift upon binding to Avi only, whereas that of LY13b remains at the same position in all three environments. These small changes could indicate that the local environment experienced by the LY chromophore when bound to the proteins is not largely different from that in bulk aqueous solutions. However, the occurrence of several interactions with compensating effects cannot be excluded.

The fluorescence quantum yields of LYnb are listed in Table 2. With the exception of LY3b in Avi, protein binding leads to a decrease of \( \Phi_{\text{fl}} \). This effect being stronger with LY13b, especially in Sav, where \( \Phi_{\text{fl}} \) diminishes by a factor of almost three. The relatively small changes observed with LY3b can be accounted for by the variations in the fluorescence time constants measured by TCSPC. Indeed, the fluorescence decay of LY3b is monoexponential with a 6.9 ns time constant in PBS–EDTA and can be reproduced with a biexponential function with time constants \( \tau_{1} = 3.5 \text{ ns} \) and \( \tau_{2} = 8.3 \text{ ns} \) when

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Probe</th>
<th>( \lambda_{\text{abs}} )</th>
<th>( \lambda_{\text{fl}} )</th>
<th>( \phi_{\text{fl}} )</th>
<th>( \tau_{\text{rad}} )</th>
<th>( n^2 \tau_{\text{rad}} )</th>
</tr>
</thead>
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<td>PBS–EDTA</td>
<td>LY3b</td>
<td>428</td>
<td>537</td>
<td>0.23</td>
<td>6.9</td>
<td>69.9</td>
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<tr>
<td></td>
<td>LY3b– Avi</td>
<td>429</td>
<td>530</td>
<td>0.25</td>
<td>3.5</td>
<td>8.3</td>
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<tr>
<td></td>
<td>LY3b– Sav</td>
<td>426</td>
<td>537</td>
<td>0.17</td>
<td>2.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>LY13b</td>
<td>426</td>
<td>538</td>
<td>0.24</td>
<td>6.9</td>
<td>69.9</td>
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<tr>
<td></td>
<td>LY13b– Avi</td>
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<td>537</td>
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<td>7.1</td>
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<td>538</td>
<td>0.09</td>
<td>1.4</td>
<td>6.5</td>
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</tbody>
</table>

[a] Absorption and fluorescence maxima. [b] Fluorescence quantum yield. [c] Fluorescence lifetime measured by TCSPC (relative amplitude). [d] Amplitude-average of \( \tau_{1} \) and \( \tau_{2} \).
bound to Avi. This results to an amplitude-averaged lifetime, \( t_{78} \), of 7.5 ns, that is, notably larger than the lifetime of the free construct in aqueous solution. The occurrence of a biexponential fluorescence decay does not automatically imply the existence of two different emitting populations, each with its own lifetime. It could also originate from a Gaussian distribution of emitting populations with distinct fluorescence decay times.\[^{[42, 43]}\] This could be associated for example with a distribution of arrangements of LY3b in the protein, hence to a distribution of different environments. The 8.3 ns time constant found in Avi could possibly be due to a smaller exposure of some of the LY chromophores to water and thus to a less efficient H-bond-assisted deactivation. On the other hand, the 3.5 ns component probably arises from the opening of a new non-radiative deactivation pathway in Avi, for example an electron transfer (ET) with a protein residue. This is discussed in more detail herein. The fluorescence decay of LY3b in Sav can also be reproduced using a biexponential function, but with both time constants shorter than that of the free construct (Table 2). Therefore, the substantial shortening of the fluorescence lifetime, despite the probable limited access of water molecules to the probe, suggests the presence of new deactivation channels in Sav.

The reduction of the fluorescence quantum yield of LY13b bound to the proteins cannot be fully accounted for by the decay times measured by TCSPC. Indeed, the radiative time constant calculated as \( \frac{t_{78}}{\Phi_f} \) is markedly larger than that of the free probe. This points to the existence of faster decay components that are missed because of the limited time-resolution of the TCSPC experiment.

To ensure that the binding of the LYnb construct to the proteins specifically takes place through biotin in the biotin-binding pockets, two equivalents of LYen were added to an aqueous solution containing Avi. Neither the spectral features, nor the fluorescence quantum yield and lifetime of LYen were affected by the presence of Avi. This confirms that the binding of the constructs to the proteins is specific.

### 2.2. Ultrafast Fluorescence Dynamics

Figure 2 shows time profiles of the fluorescence up-conversion signal measured at nine different wavelengths throughout the emission spectrum of LY3b in Avi. A strong wavelength dependence of the fluorescence dynamics is immediately noticed. The same measurements have been carried out in PBS–EDTA and in Sav, and with LY13b in all three environments. Sets of time profiles like those depicted in Figure 2 were analysed globally using the convolution of the instrument response function with a trial function. For the latter, the sum of one Gaussian and three exponential functions had to be used in addition to the two exponential functions found by TCSPC, which were kept fixed during the analysis. The resulting amplitudes were then renormalized to match the steady-state fluorescence intensity, as explained in detail in ref. [44].

The global time constants obtained from the analysis are listed in Table 3, whereas the spectral dependence of the associated amplitudes obtained with LY3b in Avi are shown in Figure 3. Qualitatively similar amplitude spectra were obtained with the other systems.

The amplitude associated with the Gaussian function is negative at all wavelengths and its spectral dependence is close to the steady-state spectrum. The presence of this Gaussian component in the trial function is necessary to properly reproduce the rise of the signal intensity. Without this component, an unrealistically large instrument response function would have to be assumed. The origin of this component is not absolutely clear, but has already been observed when excitation is performed far from the 0–0 transition.\[^{[44, 45]}\] Therefore, this Gaussian component can be possibly associated with vibrational relaxation processes taking place from the initially Franck–

![Figure 2. Wavelength dependence of the early fluorescence dynamics of LY3b in Avi (traces are vertically offset for clarity).](image)

![Figure 3. Wavelength dependence of the amplitude factors obtained from the global analysis of the fluorescence dynamics of LY3b in Avi (are just guides for the eyes).](image)

| Table 3. Time constants [ps] obtained from the global analysis of the early fluorescence dynamics. |
|----------------------------------|---|---|---|---|---|---|
| \( t_1 \) | \( t_2 \) | \( t_3 \) | \( t_4 \) | \( t_5 \) | \( t_6 \) |
| LY3b | 0.20 | 0.9 | 3.3 | 71 | | |
| LY3b–Avi | 0.20 | 1.0 | 6.4 | 72 | | |
| LY3b–Sav | 0.21 | 1.7 | 13.0 | | 440 |
| LY13b | 0.24 | 0.8 | 3.6 | 55 | | |
| LY13b–Avi | 0.22 | 1.0 | 4.3 | | 32 |
| LY13b–Sav | 0.17 | 1.2 | | 8.1 | 100 |
| LYen | 0.20 | 0.9 | 4.1 | | | |

[a] Gaussian component \( \langle \exp(-t^2/t_1^2) \rangle \).
Condon region of the excited state down to the equilibrium.\[46,47\] A Gaussian component was also found in all the other systems investigated herein. As its width, $\tau_g$, and the wavelength dependence of its amplitude is essentially the same for all these systems, this component is not discussed further.

The various time constants obtained by global analysis can be sorted into two groups according to the spectral dependence of their amplitude. Those of the first group, $\tau_1$ to $\tau_4$, are associated with an amplitude that changes from positive to negative sign with increasing wavelength. These features thus correspond to red-shifts of the fluorescence band, that is, to dynamic Stokes shifts, most probably due to the orientational relaxation of the environment around the excited chromophore. One to three of such components have been found depending on the system (Table 3). The time constants belonging to the second group, $\tau_5$ and $\tau_6$, have an amplitude spectrum much like the stationary fluorescence spectrum. They can thus be assigned to processes leading to a decrease of the excited-state population, besides those already found by TCSPC, with the time constants $\tau_5$ and $\tau_6$ (Table 2). Such additional population decays were found with LY13b in Avi and with both constructs in Sav, the fastest being associated with a 8.1 ps time constant.

In order to properly analyse the dynamic Stokes shift, the time-resolved emission spectra (TRES) were reconstructed using the parameters obtained from the fit. A log-normal function was fitted to these spectra to determine their maximum. An example of such intensity-normalised TRES recorded with LY3b in buffer solution is displayed in Figure 4A. Although the number of measured wavelengths is relatively small, a dynamic Stokes shift can clearly be recognised. Figure 4B shows the temporal evolution of the TRES maximum determined with the LY3b systems. The sum of two to three exponential functions was required to reproduce the temporal shift of the emission maximum. The resulting time constants, $\tau_5$ to $\tau_6$ (Table 4), are qualitatively very similar to the $\tau_1$ to $\tau_4$ values obtained from global analysis and corresponding to spectral shifts, confirming their initial assignment. The 32 ps time constant found with LY13b in Avi and assigned to a population decay (Table 3) is very close to $\tau_5$. This similarity indicates that, for this system, both population dynamics and Stokes shift occur on a similar timescale and that, in this case, the assignment of the time constant according to its associated amplitude spectrum can be ambiguous.

### Table 4. Parameters obtained from the analysis of the dynamic Stokes shift.

<table>
<thead>
<tr>
<th>System</th>
<th>$\lambda_{\text{em}}^{(1)}$ (nm)</th>
<th>$\lambda_{\text{em}}^{(2)}$ (nm)</th>
<th>$\tau_1$ (ps)</th>
<th>$\tau_2$ (ps)</th>
<th>$\tau_3$ (ps)</th>
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<td>18482</td>
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<td>73</td>
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<td>73</td>
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<td>LY13b−Sav</td>
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<td>18396</td>
<td>1.2</td>
<td>9.1</td>
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</tbody>
</table>

[a] Initial and final emission band maximum. [b] Stokes-shift time constant (limit of error: ±5 %) and amplitude in cm$^{-1}$ in brackets (limit of error: ±5 %).

### 2.3. Fluorescence Polarisation Anisotropy (FPA)

Figure 5A shows time profiles of the FPA measured with LYen and LYnb in aqueous buffer solutions. All three profiles can be reproduced by a single exponential function decaying to zero with time constants increasing with the size of the molecule (Table 5). This decay can be unambiguously assigned to reorientational diffusion of the chromophore. The time constants are indeed consistent with those found with other chromophores of similar size in protic solvents.\[48,49\] The increase in $\tau$, by going from LYen to LY3b and to LY13b, can be perfectly well accounted for by the molecular volume, which increases by a factor of about 1.5 and 2, respectively.

![Figure 4](image-url)  
**Figure 4.** A) intensity-normalised time-resolved emission spectra of LY3b in PBS-EDTA buffer between 0.3 and 100 ps after excitation (----- represents the steady-state emission spectrum), and B) temporal evolution of the band maximum of LY3b emission in various environments and best multieponential fit (-----).

![Figure 5](image-url)  
**Figure 5.** Time profiles of the fluorescence polarisation anisotropy measured A) in bulk buffer solution and B) in proteins, and best fits for both cases.
performed by up-conversion did not allow a reliable determination of the whole protein. The time window of the FPA measurements was too short to determine \( r_d \) with TCSPC. As values between 33.5 and 36 ns have been reported for the reorientation of Avi, [52, 53] the value of \( r_d \) was fixed at 35 ns. In the second approach, Equation (3) was fitted instead of Equation (1):

\[
r(t) = r_0[(1 - S) \exp(-t/r_c) + S \exp(-t/r_d)]
\]

Expression (3) just assumes that the effect of tumbling on the anisotropy decay is negligible within the time window of the measurement. Finally, in the third one, Equation (3) with \( r_c \) fixed at the value measured for the free construct was used. All three methods gave a very good fit. Those obtained from the last one (\( r_c \), fixed) is shown in Figure 5B. The best-fit parameters are compared in Table 5. An important outcome is that the value of \( S \) is independent of the fitting procedure.

### 2.4. Transient Absorption and Fluorescence Quenching

The most probable origin of the reduced fluorescence lifetime of LY1b in Avi and especially in Sav is the occurrence of photoinduced ET from a protein residue to the LY chromophore. Trp, Tyr and His are among the amino acids present in Avi and Sav that exhibit the best electron-donating properties. Indication of ET quenching of LYen by Trp has already been reported. [30]

In water, both static and dynamic quenching have been found: the rate constant of bimolecular quenching was measured to amount to \( k_q = 3.2 \times 10^9 \text{M}^{-1} \text{s}^{-1} \), that is, not very far from the rate constant of diffusion in water of about \( k_d = 6.5 \times 10^8 \text{M}^{-1} \text{s}^{-1} \). On the other hand, ultrafast static quenching with a time constant of 720 fs and ascribed to ET in a LYen/Trp complex was measured by fluorescence up-conversion. Single-wavelength transient absorption (TA) measurements performed at 580 nm, within an absorption band of Trp, showed a 800 fs rise of the TA signal, followed by a 2.6 ps decay, ascribed to charge recombination. [30]

In order to have a stronger indication of ET between LY and Trp, TA measurements using broadband detection have been performed with LY13b. Figure 6 shows the TA spectra obtained with free LY13b in buffer solution, with Trp and in the presence of Sav. The TA spectra of LY13b alone exhibit negative bands around 440 and 540 nm, due to the bleaching of the ground-state absorption and to stimulated emission (SE), respectively, and positive bands below 400 nm and above 640 nm, that can be assigned to \( S_1 \), excited-state absorption (ESA). Apart from an initial red-shift of the SE band, the shape of the TA spectra remains constant over the time-window of the measurement and its amplitude decays slowly, in agreement with a 6.9 ns fluorescence lifetime. Global analysis with multieponential functions shows that the red shift of the SE band is associated with a 3 ps time constant, in very good agreement with the 3.6 ps time constant observed in the early fluorescence dynamics (Table 3).

In the presence of 80 mM Trp, the TA spectra recorded at short time delays differ completely and exhibit a narrow positive band centred at 440 nm and a broad one extending from 480 to more than 700 nm (Figure 6B). The band at 440 nm can be assigned to LY13b + and because the radical anion of a related naphthaleneimide has been shown to absorb in this region as well, [30] Furthermore, the other band coincides well


### Table 5. Parameters obtained from the analysis of the decay of the fluorescence polarisation anisotropy.

<table>
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<tr>
<th></th>
<th>( r_t )</th>
<th>( r_c ) [ps]</th>
<th>( r_d )</th>
<th>( S )</th>
<th>( \theta )</th>
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<td>0</td>
<td>0</td>
<td>90°</td>
</tr>
<tr>
<td>LY3b–Avi</td>
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<td>0.28</td>
<td>0.83</td>
<td>20°</td>
</tr>
<tr>
<td>2b</td>
<td>0.34</td>
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[a] Using Equation (1) with \( r_d = 35 \text{ ns} \). [b] Using Equation (3). [c] Using Equation (3) with \( r_c \), fixed at 161 ps (LY3b) or 227 ps (LY13b).
with the absorption spectrum of Trp$^+$ generated by two-photon ionisation in water. These two bands decay within a few tens of picoseconds and the subsequent TA spectra are similar to those measured with LY13b alone.

Figure 7 A shows the time profile of the TA signal intensity at 440 nm in the LY13b/C$^+$ band. The continuous line is the best fit of a triexponential function with a 450 fs time constant for the initial rise and 7.3 and 316 ps decay time constants. The rise time can be ascribed to an ultrafast ET between LY and Trp occurring through static quenching, and the shortest decay time to recombination of the ensuing ion pair. This agrees rather well with the single-wavelength TA measurements performed with LYen. The fact that the late TA spectra are very similar to those recorded without Trp can be explained by the dynamic quenching that is slow relative to the ultrafast charge recombination. Because of this, the instantaneous ion population is too small to contribute significantly to the TA spectra. Thus the 316 ps time constant essentially reflects the ionic population originating from dynamic quenching.

The TA spectra recorded with Sav show a fast decrease of the SE intensity (Figure 6 C), which agrees with the fast fluorescence decay component observed by up-conversion (Table 3). Moreover, the shape of the negative band due to the bleach of the S$^0$–S$^1$ absorption differs markedly from that shown in Figure 6 A, especially during the first 200 ps. Indeed this negative bleach band culminates at ~460 nm and exhibits a shoulder at about 430 nm. As time proceeds, this shoulder vanishes and the negative band broadens to finally resemble that recorded with free LY13b. This effect is illustrated in Figure 7 B, which compares the temporal evolution of the TA signal intensity at 430 and 450 nm. The solid lines are the sum of four exponential functions fitted globally to the time dependence of the TA signal throughout the whole spectral window. The first component with a 1.75 ps time constant accounts for the initial decay of the TA intensity and can be ascribed to a spectral shift. The second component is associated with an 11 ps time constant and is responsible for the build-up of the shoulder at 430 nm and for a partial decrease in the SE band. This component is ascribed to an ET process between LY13b and Sav, leading to a decrease in the excited-state population, hence of the SE band, and to the formation of LY13b$^+$, which absorbs around 440 nm and is at the origin of the shoulder. The third component, with a 170 ps time constant, is related to the disappearance of the shoulder and to a partial decay of the ground-state bleach. This component can thus be ascribed to the recombination of the ion pair population to the neutral ground state. Once this process is over, the residual TA spectrum is very similar to that of LY13b alone. Its shape remains constant within the time-window of the measurements and its intensity decays with a $>$3 ns time constant. The time constants found herein are in good agreement with those obtained from the analysis of the time-resolved fluorescence measurements (Table 3).

Dynamic quenching of LYen by Tyr and His was also investigated. Because of the lack of solubility of Tyr in neutral aqueous solutions, N-acetyl-L-tyrosine (AcTyr) was used instead. Figure 8 A shows that the fluorescence decay of LYen is accelerated upon addition of AcTyr, while Figure 8 B is the corresponding Stern–Volmer plot. From the slope of a linear fit to these data, a quenching rate constant of $k_q = 1.8 \times 10^9$ m$^{-1}$s$^{-1}$ is obtained. This is about twice as small as the quenching rate constant found with Trp. Fluorescence quenching of LYen by His

This was followed by MD simulations and further analysis. To this end, LY13b and LY3b were docked into the X-ray structures of Sav to obtain the structural data and specifically the position of the LY moiety relative to the surrounding key residues. Knowledge of the relative position of the LY probe with respect to the aromatic residues is of crucial importance for the interpretation of the observed shortening of fluorescence lifetime. The aromatic residue that is the closest to the LY extended aromatic system is TrpA120, located on the L7,8 loop. However, the position of this residue on the most remote side of this loop results in a distance between the two aromatic systems beyond 9 Å. The structure optimized from the average geometry based on the last 50 ps of the simulation (Figure 9) indicates 10.5 Å. This value is in good agreement with the mean value of 10.8 Å obtained from the trace of the LY–TrpA120 distance during the last 4 ns of the MD simulations (Figure 10 and Table 6). Moreover, the optimized structure reveals the presence of LysA121 between LY and TrpA120. Such intercalation of LysA121 is reinforced by a hydrogen bond between this residue and the SO₃⁻ group of LY and by the cation–π interaction with LY on one side, and with TrpA120 on the other side.

The second-closest aromatic residue is HisA87, for which the distance towards LY fluctuates around 12.4 Å during the simulations with an identical value of 12.4 Å obtained from the optimized geometry. However, although a more pronounced fluctuation of the distance, due to the partial rotation of the imidazole ring, is detected during the simulation, this residue itself remains at the same position. TyrA83 is even more remote from LY with a mean value amounting to 24.4 Å during the last 4 ns and with a distance of 22.2 Å in the optimized structure of 1 (Figure 9A,B and Table 6).

In order to probe the possibility of closer contact between LY and aromatic residues such as Trp, the second series of MD simulations was performed, where constraints bringing the two aromatic systems closer together were applied. This NMR refinement technique, followed by an unrestricted production period of the MD simulation, results in the rearrangement of the L7,8 loop, which allows closer contact between LY and TrpA120 in the resulting optimized geometry of the complex 2. During the last stage of the simulation, the LY–TrpA120 distance fluctuated within a narrow range around 4.1 Å, reaching a value of 3.8 Å in the optimized structure, the typical interplanar distance for π–π stacking (Table 6). However, inspection of the traces for the interplanar distance over the whole 7 ns of the simulation reveals a short-lived spike of approximately 250 ps, during which the distance oscillates around 6.7 Å. This short digression from the otherwise quite stable interplanar distance of 4.1 Å is due to the displacement of the L7,8 loop, and consequently of TrpA120, to a more remote position. This
type of loop displacement remains rather unique to 2, since among the numerous MD simulations performed, it only occurred in this particular case. This indicates that, in the rearranged complex 2, the L7,8 loop adopts two conformational states: a preferred one where the interplanar LY–Trp A120 distance of approximately 4 Å warrants π-π stacking, and a second one, clearly less favourable, with a 6.7 Å distance. It should be stressed that in the latter geometry, the larger distance is due to the displacement of the L7,8 loop and not to the motion of the LY moiety. According to the optimized structure of 2, the distances of the LY aromatic moiety relative to His A87 and Tyr A83 remain quite large with 11.1 Å and 15.7 Å, respectively (Table 6). The MD simulations using the NMR refinement technique for 2 demonstrate that the π-π stacking between the aromatic part of the LY probe and Trp A120 is only possible at the cost of a rearrangement of the L7,8 loop. In the resulting rearranged complex 2, the conformation of the L7,8 loop is, however, slightly more labile than in the non-rearranged complex 1. To rearrange 1 into 2, we imposed the conformational changes in order to facilitate the π-π interactions between Trp A120 and LY moiety of the later. It should be reminded, however, that the conformation of 1 resembles the X-ray structure of the monomer more closely.

MD simulations of the Sav monomer-LY3b complex (3) were also performed. Its optimized structure reveals good anchoring of the biotin moiety in its binding pocket. Since the spacer is distinctly shorter, LY is positioned clearly in a different environment as compared to the LY13b complexes. The amide group, which is part of this connecting chain, is again hydrogen bonded to Asn A49. Although this chain is fully extended, the LY part of the ligand is buried at the surface of the protein. This contrasts with the other LY13b complexes, where the kink of the connecting chain is necessary for the LY moiety to interact with the residues at the protein surface. As a consequence of the shorter spacer, a longer distance of 12.2 Å is observed between the central atom of LY moiety and Tyr A120. However, the shorter chain positions LY closer to Tyr A83 (14.2 Å) and, particularly, to His A87 with a surprisingly short distance of 8.5 Å (Table 6). Several functional groups of LY interact with the surrounding residues, with one of the SO3 groups forming a hydrogen bond directly with His A87 (Figure 9D). This explains the short LY–His A87 distance. However, these interactions prevent the coplanar orientation of the extended aromatic system of LY relative to the aromatic residues in the vicinity.

To have a deeper insight into the location of the LY probe and of its distance relative to other aromatic residues, MD simulations of the complex between the Sav tetramer and LY13b have also been carried out. The same procedure as described above with the monomeric complex 1 was used. Prior to the MD simulations, LY13b was docked into the subunit A of the tetrameric protein. Whereas the positioning of LY on the protein surface appears to be quite constant during the production phase of the MD simulations for 1–3, the situation was different for the tetramer 4. Indeed, the LY moiety remained lo-
about 1 /C138 closer to TrpA120 than in monomer is indeed located in the vicinity of the L7,8 loop, where LY is from the last 50 ps shows that the aromatic part of the probe 4 also reveals that several aromatic residues belonging to the D subunit are at comparable interplanar distances to those found for the A subunit. This is namely the case for TrpD120 and His287, which are located at 16.2 Å and 18.4 Å respectively, from the central atom of the LY aromatic moiety (Table 6).

Not only the position of the LY moiety and its orientation in 1 and 4 are apparently quite alike, but the biotin anchoring within the binding pocket and the conformation of the connecting chain are also very similar for both complexes. Even the amide group located on the connecting chain of the probe in 4 forms hydrogen bonds towards the same residues in 1 and 4. Such striking similarities in the location of the ligand show that the simulations with the monomeric Sav already give a reliable picture of the location of the probe.

3. Discussion

3.1. Differences in the Excited-State Lifetime

Apart from LY3b in Avi, the excited-state lifetime of the probe is shortened when inserted into the proteins. As already mentioned, the longer lifetime of LY3b in Avi can be ascribed to a smaller exposure to water molecules and hence to an inhibition of the hydrogen-bond assisted non-radiative deactivation of the excited state. The presence of shorter decay components in the other systems does not imply that the chromophore is more exposed to water, but rather points to the existence of additional decay channels that are not operative with LY3b in Avi. The TA data unambiguously reveal the occurrence of ET quenching of LY13b in Sav. While the TA spectra indicate the presence of LY13b−, no precise information on the location of the hole, that is, on the electron donor can be obtained. To identify the possible quenchers of LY fluorescence, the driving force of photoinduced ET has to be considered according to Equation (4):

\[ \Delta G_{ET} = -E_{ox} - E_{red}(A) + E_{ox}(D) \] (4)

where \( E_{ox} \) is the energy of the reactive excited state, and \( E_{red}(A) \) and \( E_{ox}(D) \) are reduction and oxidation potentials of the electron acceptor and donor, respectively. The correction factor that accounts for the electrostatic interaction between the charged moieties has been omitted as it is generally accepted to be very small in polar environments. From the steady-state absorption and emission spectra, the \( S_1 \) energy of LY3b in Sav amounts to 2.59 eV, whereas the reduction potential of LYen has been estimated to be on the order of 1 V vs NHE. Consequently, ET quenching of LY13b requires a donor with an oxidation potential smaller than about 1.65 V vs NHE. This is the case for Trp, Tyr and His with \( E_{ox} = 1.03 \), 1.02, and about 1.28 V vs NHE respectively. According to Equation (4), the ET driving force with these amino acids should range from about −0.3 to −0.55 eV. Such exergonicity is in good agreement with

9.6 Å interplanar distance from this aromatic residue (Figure 11). This closer proximity occurs at the cost of longer distances relative to the other two aromatic residues of subunit A, that is, His287 and Tyr283 (Table 6). The optimized structure of ET quenching of LY13b in Sav. While the TA spectra indicate the presence of LY13b−, no precise information on the location of the hole, that is, on the electron donor can be obtained. To identify the possible quenchers of LY fluorescence, the driving force of photoinduced ET has to be considered according to Equation (4):

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Table 6. Interplanar distances [Å] between the central atom of the LY moiety and relevant aromatic residues. Normal font: Distances in the optimized geometry. Italic: Average distances of the last 4 ns of the simulation.

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cated more or less at the same location during the first part of the simulation, but after 5 ns it moved closer to the L7,8 loop, where it stabilized during the second half of the simulation. For this reason the duration of the simulation was extended to 10 ns for the complex 4. The optimized structure extracted from the last 50 ps shows that the aromatic part of the probe is indeed located in the vicinity of the L7,8 loop, where LY is about 1 Å closer to TrpA120 than in monomer 1. The aromatic moiety of LY adopts a slipped and coplanar orientation with a
Figure 11. Optimized structures of complex 4 shown as (A) an overall representation together with (B) a close-up on the LY location. The Sav backbone is color-coded in grey (cartoon) and the LY13b ligand represented as a ball-and-stick model. The relevant aromatic residues are highlighted in orange.

the close to diffusion-limit quenching of LYen by Trp and with the slower quenching with His. The quenching rate constant measured with AcTyr is smaller than expected from the oxidation potential of Tyr. However, this discrepancy could be due to the use of AcTyr instead of Tyr. It should be noted that self-quenching of the probe due to occupation of multiple binding sites by LYb molecules on the same protein can be excluded, as the photophysics is independent of the ligand/protein ratio.

The strongest reduction of fluorescence lifetime is observed with LY13b in Sav and in Avi. Avi and Sav contain four and six Trp residues per subunit, respectively, most of which are unavailable for ET either because they are directly located in the binding pocket where they interact with bion or because they are in a region of the protein which is not accessible to LY even with the long spacer. The closest available residue is TrpA110 in Avi and TrpA120 in Sav, located in the subunit where the probe is bound (subunit A). The LY–TrpA120 distance found by MD simulations (Table 6) seems too large to account for the 8.1 ps decay component of LY13b in Sav and would better agree with the 100 ps component. On the other hand, the other potential quenchers are even more remote. A rather fast decay component (32 ps) was also observed in Avi, which lacks the Tyr and His residues of Sav. Consequently, it appears that TrpA110 in Avi and TrpA120 in Sav should be the major actors in the ET quenching of LY13b.

MD simulations of 2 show that a rearrangement of the L7,8 loop allows a 4 Å LY–TrpA120 distance to be achieved. However, the 8.1 ps quenching component is most probably not related to this rearranged structure. Indeed, such short interplanar distance could be expected to lead to much faster ET, similar to that observed with the static quenching of LY13b by Trp in bulk solution with a 450 fs time constant.

For 1 and 4, the MD simulations reveal the presence of LysA121 between the LY moiety and TrpA120. This residue could substantially enhance the electronic coupling between the ET partners via superexchange. This could account for a fast ET quenching at relatively long distances. Moreover, the decay of the FPA of LY13b also reveals that the LY moiety still has some degree of freedom for motion, indicating that the mutual orientation and distance between LY and Trp are not constant, but fluctuate. In fact, the MD simulations indicate that thermal fluctuations can bring LY and Trp120 to as little as 8 Å apart for a few picoseconds (Figure 11).

The dependence of the ET rate constant is usually expressed by Equation (5):[71]

\[
  k_{et}(d) = k(d_0) \exp\left[-\beta(d - d_0)\right]
\]

where \(d\) is the centre-to-centre distance between the ET partners, \(d_0\) the contact distance and \(\beta\) a decay constant for electron tunnelling. Assuming that ET at contact distance (\(d_0 = 4\ Å\)) occurs with a rate constant of 450 fs\(^{-1}\) as the TA measurements suggest, ET at \(d = 8\ Å\) with a rate constant of 8.1 ps\(^{-1}\) would require a \(\beta\) value of 0.75 Å\(^{-1}\). This is smaller than the \(\beta\) value of 1 Å\(^{-1}\) reported for ET in proteins, but not totally unrealistic.[72,73] Thus, the 8.1 and 100 ps found in Sav could in fact reflect a distribution of distances between the LY moiety and Trp120.

Finally, Sav contains more aromatic residues than Avi, thus more potential quenchers of LY. The existence of several relatively inefficient parallel ET pathways could also result in a significant reduction of the fluorescence lifetime.[74] This could account for the difference observed with LY3b in both proteins.

3.2. Polarisation Anisotropy

The above differences in ET quenching dynamics in both proteins show unambiguously that the environment of the LY chromophore is not the same with short and long spacer. This is further supported by all the other results described in Section 2, such as the FPA decay, the amplitude of the dynamic Stokes shift, as well as by the MD simulations.

First, the FPA decay results shown in Table 5 reveal very clear differences between LY3b and LY13b. Indeed, the residual anisotropy, \(r_0\), is about twice as large with the short spacer as with the long one. In terms of the WIC model, this translates into a larger order parameter \(S\) for LY3b, and hence points to a much more restricted motion of the chromophoric unit than with LY13b. This is in total agreement with the MD simulations that indicate an extended structure of the spacer in LY3b and a folded one in LY13b. The latter most probably allows more freedom for motion of the LY moiety than the former one. The LY mobility with LY3b is apparently the same in both proteins, whereas with LY13b it is markedly larger in Sav than in Avi. Such difference with LY13b could possibly also account for the dissimilarity in ET quenching dynamics observed with LY13b in...
these two proteins. Its higher mobility in Sav should give LY additional freedom to adopt more favourable distances and orientations relative to Trp and other potential quenchers. As the first electronic transition of LY has a charge-transfer-type character, its associated dipole moment should be aligned along a direction going from the imide nitrogen to the amino-nitrogen of the naphthalene substituent. Therefore, the depolarisation of the fluorescence should essentially arise from rotatory motion around the axes perpendicular to the long axis of the construct defined by the spacer. Such motion should thus lead to a variation of the LY–Trp distance.

Although Avi and Sav are very similar proteins in terms of structure and function, their amino acid compositions are very different, with about only 30% correspondence in their sequences. Therefore different interactions between the spacer and the protein have to be expected. For example, the MD simulations show the existence of hydrogen bonds between one amide group of the spacer and Asn in Sav. Whereas there is an equivalent to Ser in Avi (Ser), no equivalent to Asn is present. Such different anchoring of the long spacer to the protein should have an impact on the mobility of the LY unit.

3.3. Dynamic Stokes Shift

The parameters obtained from the analysis of the dynamic Stokes shifts (Table 4) also point to different environments of the probes, depending on the spacer and the protein.

If one considers the initial and final emission maxima, and one notices that, consistently with the steady-state spectra, LY3b in Avi differs markedly from the free and the Sav-bound constructs, both maxima being at higher energy. On the other hand, such a difference cannot be discerned with LY13b.

An additional feature concerns the amplitude of the fastest Stokes shift component, . For both constructs, it is noticeably larger in bulk water than when bound to a protein (except for LY3b–Sav). This can be interpreted as a shift due to a quickly reorienting polar environment, and should therefore essentially be due to the motion of surrounding water molecules. Indeed, a similar time constant has been observed with fluorescent probes attached to proteins and nucleic acids. The origin of this slow “solvation” component was also found in MD simulations performed on a tripeptide. These observations support our assignment of the slow Stokes shift component found with LY in water, which, with their two amide bonds, can be considered as small peptides as well.

As biotin is tightly bound to Avi and Sav, the longer Stokes shift component measured with the protein-bound constructs can obviously not be interpreted as conformational dynamics of LYN. Slow Stokes shift components with time constants of the order of ten to a few tens of picoseconds have already been observed with fluorescent probes attached to proteins and nucleic acids. The origin of this slow “solvation” component is still debated. It was first discussed in terms of “biological” water molecules, namely water molecules bound to the protein surface and forming a more rigid network than bulk water. This interpretation was later questioned by Nilsson and Halle on the basis of MD simulations of the Stokes shift of the single Trp of melolin, where the slow component was found to be due to protein dynamics, including self-motion of the chromophore. Further MD simulations by Golosov and Karplus indicate that the slow solvent relaxation does not represent the lifetime of so-called bound water molecules, but rather corresponds to the interconversion among protein conformations with different numbers of bound water molecules. This implies the existence of a coupling between hydration and conformational dynamics, which makes the interpretation of dynamic Stokes shift and decomposition into distinct contributions rather difficult.

This last interpretation seems to be the most adequate for discussing the dynamic Stokes shifts found herein. As mentioned, is most certainly dominated by the diffusive motion of bulk-like water molecules. On the other hand, the value found in Avi may reflect the motion of partially confined but not bound water molecules. The amplitude of this component is smaller with LY than with LY, in agreement with a reduced exposure to water. The value is substantially larger.
with LY3b than with LY13b. This component is most probably not directly connected to bound water molecules, as it is difficult to justify such largely different time constants, but rather reflects conformational dynamics of the protein. The smaller \( \tau_3 \) with LY13b could be explained by a locally looser protein structure and thus by faster motion, whereas its larger amplitude, \( \Delta \tau_p \), with LY13b is ascribed to the better exposition to water molecules. As this part of the protein is more hydrated than that probed by LY3b, structural changes involve the displacement of more water molecules, leading to a larger “solvation” energy of the excited probe.

On the other hand, the dynamic Stokes shift is on average substantially faster in Sav and only a single component with time constant, \( \tau_3 \), is required to reproduce its slower phase. However the \( \tau_3 \) values in Sav are larger than those in Avi, denoting a probable combined contribution of different processes, such as partially confined water molecules and fast protein motion. At first sight, such differences between Sav and Avi are rather surprising, given the very similar tertiary and quaternary structures of these two proteins. However, Avi and Sav differ strongly at the molecular level, that is, if their amino acid sequences are considered. Given the intrinsic molecular nature of the probes used here, dissimilarities in the dynamic Stokes shift should be expected, especially if they are associated with fast conformational dynamics.

Finally, it should be noted that these differences in “solvation” dynamics between Avi and Sav agree very well with those observed in the FPA measurements that point to a weaker confinement of the probe, hence to a looser environment in Sav.

4. Conclusions

The main objective of this investigation was to explore a new approach for studying water dynamics at the different locations of a protein, using the same probe. The results show that this strategy works rather well although there is still space for improvements. One of them could be to choose a better solvation probe than that used here. This is however not such a simple task, since such differences between Sav and Avi are rather surprising, given the very similar tertiary and quaternary structures of these two proteins. However, Avi and Sav differ strongly at the molecular level, that is, if their amino acid sequences are considered. Given the intrinsic molecular nature of the probes used here, dissimilarities in the dynamic Stokes shift should be expected, especially if they are associated with fast conformational dynamics.

Furthermore, because of the flexibility of the spacer used herein, the effect of distance from the protein surface on the water dynamics could not really be studied. Thus, a rigid spacer with the same length that as used with LY13b may have allowed this aim to be achieved. Despite this, different regions of the proteins could be probed using the flexible spacer, and substantial dissimilarities have been observed. These differences demonstrate rather unambiguously that the “solvation” dynamics sensed by the LY chromophore depends strongly on the local structure of the protein and thus most probably also reflects protein dynamics. Therefore, interfacial water and conformational protein dynamics are partially entangled and their contributions to the measured time-dependent Stokes shift cannot be dissociated.

This investigation also shows that the effect of spacer length on the mobility of the fluorescent probe as well as on the “solvation” dynamics is qualitatively the same for both Avi and Sav. This reflects the similar tertiary structures of these two proteins. However, when the results obtained with a given spacer length in Avi and Sav are compared, substantial dissimilarities can be observed. This in turn points to the rather dissimilar primary structures of these proteins.

**Experimental Section**

Samples: Lucifer Yellow biocytin (LY13b) [Invitrogen] and the amino acids l-tryptophan (Trp), l-histidine (His) and N-acetyl-l-tyrosine (AcTyr) were of the highest commercially available purity and were used as received.

LY3b was synthesized by adding Lucifer Yellow ethenylenediamine (lYen, 0.02 g, 0.0407 mmol, in 5 mL water) to a DMF solution (1 mL) of biotin-N-hydroxysuccinimide (0.0153 g, 0.0448 mmol) containing N-methylmorpholine (2 drops). The resulting solution was stirred overnight at room temperature. Following solvent removal under vacuum, the crude product was purified by flash chromatography on silica gel using a methanol dichloromethane gradient (1:3 to 1:1 gradient) to afford analytically pure (by HR–MS, \(^1\)H and \(^13\)C NMR) LY3b (0.0238 g, 81 % yield).

For \( C_{24}H_{25}K_2N_5O_{10}S_3 \), Fw calc. = 717.88 g mol\(^{-1}\); HR–MS (ESI) m/z (I %), 640.4 (M\(^-\)2K\(^+\), 100 %), 320.3 ((M\(^-\)2K\(^+\))/2), 14 %, 656.2 (M\(^-\)2K\(^+\) + H\(_2\)O, 90 %).

\(^1\)H NMR (400 MHz, D\(_2\)O) \( \delta = 8.83 \) (d, 1 H, J = 1.6 Hz), 8.65 (d, 1 H, J = 1.6 Hz), 8.57 (s, 1 H, Ar), 4.35–4.32 (m, 1 H), 4.04 (d, J = 9.6 Hz, 1 H), 2.2 Hz, 3.35–3.46 (m, 2 H), 2.63 (dd, 1 H, J = 13.1 Hz, J = 5.0 Hz), 2.49 (d, 1 H, J = 12.9 Hz), 2.49–2.44 (m, 1 H), 2.03–1.93 ppm (m, 2 H).

\(^13\)C NMR (100 MHz, D\(_2\)O) \( \delta = 176.97, 165.42, 165.27, 165.00, 149.94, 140.63, 134.32, 131.46, 129.51, 127.50, 122.51, 121.51, 120.70, 107.76, 65.33, 62.22, 61.81, 60.39, 55.24, 39.92, 35.64, 27.93, 27.65, 25.27 ppm.

DMSO was from Fluka, phosphate buffer saline (PBS, composition: NaCl 137 mm, KCl 26.8 mm, Na\(_2\)HPO\(_4\), 8.1 mm, KH\(_2\)PO\(_4\), 1.5 mm) from Sigma, and ethenylenediaminetetraacetic acid (EDTA) disodium salt dihydrate from AppliChem. Egg-white avidin (Avi) was obtained from Belovo SA, Bastogne, Belgium. Recombinant T7-tagged mature streptavidin (Sav) was produced in E. coli according to Humbert et al.’s procedure. The average number of biotin sites available was determined using biotin-4-fluorescein as a probe according to Kada et al.’s protocol. For Avi (65.3 kDa) and Sav (65.7 kDa), it was 3.09 and 2.99 respectively. Accordingly, the molecular weight of the two proteins was rescaled so as to have a concentration in solution corresponding to four available sites per protein. The virtual molecular mass used was thus 84 530 Da with Avi and 87 990 Da with Sav and all concentrations were calculated on this basis.

Stock solutions (2 mm) of Lyen, LY3b, and LY13b were prepared in DMSO and stored in the dark at −20 °C. Lyophilised Avi and Sav as well as 100 µm stock solutions in water (according to their correct-
ed molecular weight) were stored in the dark at −20 °C. Unless specified, experiments in aqueous solution were performed in PBS supplemented with 1 mM EDTA (PBS–EDTA). All samples were freshly prepared from the stock solutions by adding the dye–biotin constructs from their stock solution to an Avi or Sav solution in PBS–EDTA. The solutions were equilibrated for a few minutes before any measurement was performed. As the dye stock solutions were made in DMSO, the DMSO content of the samples under investigation was of the order of 0.1–0.5 % for steady-state and TCSPC measurements, whereas it was around 5 % in up-conversion measurements. Control measurements in pure water indicated that the presence of DMSO has a negligible effect on the dynamics of all systems investigated. Except for the fluorescence up-conversion experiments, which were performed with 2 dye equivalents, all measurements were done with 1–4 dye equivalents. For the sake of simplicity however, only traces with 2 equivalents are shown, since the dye/protein ratio did not significantly affect the photophysics.

Steady-State Measurements: Absorption spectra were recorded on a Cary 50 spectrophotometer, and fluorescence spectra were measured on a Cary Eclipse fluorimeter. The latter spectra were corrected for the wavelength dependence of the detection. The fluorescence quantum yields of LY13b and LY3b ligands were optimized with the PBE1PBE/6-31G* density functional,[30,31] within the Gaussian 03 program.[32] Such optimized geometry of the appropriate ligand was then docked by means of AutoDock 3.05 package[33,34] into the Sav structure in order to generate the starting geometry of the ligand–Sav complexes, which were subsequently submitted to MD simulations. In case of the tetrameric structure, only one ligand molecule was docked into the A subunit of the Sav oligomer.

The MD simulations were carried out with the AMBER 9 package.[35] The protein–ligand complexes were solvated by a rectangular box containing 10,898 TIP3P water molecules[36] with a closeness parameter of 15 Å apart from the boundary of the protein. The long-range electrostatic interactions were treated with the particle-mesh Ewald method[37] (PME) and periodic boundary conditions were used.

Using the conjugate gradient algorithm, an energy minimization of 2000 steps was performed for solvated complexes. During the initial 50 ps of the simulation, the system was heated up to 300 K at constant volume, followed by 950 ps simulation at 300 K at constant pressure for achieving equilibration at 300 K and 1.0 atm. For certain simulations, a constraint, by the way of the NMR refinement technique,[38] was applied during the initial 50 ps stage for distances between LY moiety of the ligand and selected aromatic residues. Production runs were carried out at 300 K and 1.0 atm (under an NPT ensemble) typically up to 7 ns of the total simulation duration. A 2 fs time step was used and the structure was saved every 2 ps. At the end of the 7 ns MD simulation, the average structure of the complex was extracted from the last 50 ps and minimized to obtain the optimized geometry. The convergence criterion on the gradient was set to 0.05 kcal mol⁻¹ Å⁻¹. The binding energy involving the protein and the ligand was computed using the MM–PBSA program,[39,40] implemented within the AMBER 9 package. This energy was calculated over the last 100 ps of the MD simulation. The molecular structures issued from the MD simulations were represented with the VMD program.[41]

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