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The Effect of Local Dynamics of Atto 390-labelled Lysozyme on Fluorescence Anisotropy Modeling

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Abstract

Fluorescence anisotropy decay is a popular optical technique to study the structure, size, shape and even functions of biomolecules. The method measures the time dependence of the depolarization of a fluorophore and is therefore sensitive to the changes in the rotational motion (e.g., aggregation and binding) or changes in the mobility of segments of biopolymers (such as the ones associated with tertiary structure changes). Fluorescence anisotropy decay often requires the use of fluorescent dyes that need to be covalently attached to the biomolecule. The location of the attachment on the biomolecule (e.g., a protein) and the linker used, affect the mobility of the dye and its anisotropy decay. With this study we have combined the experimental data with molecular dynamic simulations to offer a more correct interpretation of the fluorescence anisotropy decay of a popular fluorescent dye (Atto 390) attached to the N-terminus of Hen Egg White Lysozyme (HEWL). Our model shows how the use of relatively simple molecular dynamics computation to simulate the motion of the dye, provide a model to interpret the experimental fluorescence anisotropy decay that yields a better estimate of the hydrodynamic radius of HEWL. The improvement is due to a more detailed description of the segmental motion of the dye attached to the protein.

Introduction

The knowledge of the fundamental characteristics of biomolecules is important in the investigation of their biological functions. Size and structure of the molecules as well as local dynamics and affinities for certain ligands are among the most important features investigated by biophysics research. For many of these investigations fluorescence spectroscopy continues to provide a sensitive, targeted approach that offers a broad array of methods that can be used to characterize parameters in solution, *in vivo*, in films, etc.¹. One of these methods, time-resolved fluorescence anisotropy decay, yields information about the size and shape of biomolecules in solution by recording the time-dependent changes of the polarization of emitted light^{2,3}. This technique often employs a small fluorophore that is covalently tagged to the target biomolecule. The choice of fluorescent tag (e.g., emission wavelength, fluorescence lifetime decay, etc.) typically depends on the characteristics (e.g., size) of the biomolecule that one investigates^{1,4–6}. Care is typically taken to ensure that the fluorophore itself or the procedure to covalently link it to the biomolecule, does not modify the structural integrity or the function of the biomolecule^{5,6}. This approach poses the challenge of understanding the mobility of the fluorophore at the location of the covalent attachment so that a more precise interpretation of the fluorescence anisotropy decay data

can be carried out in order to decouple the diffusion that is due to the target biomolecule as a whole, from the (typically faster) motion of the tagged segment of the biomolecule (for instance a terminus or a mobile loop) or the (even faster) motion introduced by the rotation of the fluorescent tag itself⁷. The decoupling is intended as a way to resolve the faster dynamic of the fluorescent tag. Once this dynamic has been simulated, it can be combined to the overall rotational dynamic of the biomolecule. Thus, in order to better understand the complex motion of the fluorophore computational simulations should be considered as an integral contribution to anisotropy decay analysis.

As mentioned above, covalent tag attachment couples the motion of the fluorescent probe to the rotational diffusion of the biomolecule⁷. The type of linker that attaches the fluorophore to the biomolecule leads to different models of anisotropy decay analysis such as the rigid rotor¹ and segmental mobility⁸ models.

In order to demonstrate the importance of the local dynamics of the fluorescent tag on the model and the analysis of anisotropy decay data, we have simulated the motion of a fluorophore (Atto 390, a novel coumarin-based fluorescent dye⁹) covalently linked to the N-terminus of hen egg white lysozyme (HEWL). Atto 390 exhibits attractive properties for fluorescence anisotropy usage, such as high fluorescence quantum yield ($\Phi_f = 0.9$), large Stokes shift (~ 60 nm), and a rigid aromatic structure, which is normally associated with high fundamental anisotropy r_0 ⁹. On the other hand, HEWL is a well-characterized 129 residue, ~ 14 kDa anti-bacterial enzyme^{10,11}.

In our combined experimental and computational investigation Atto 390 is linked to the N-terminus of HEWL via a four-carbon acyclic aliphatic chain with four rotatable bonds, which enables the dye to extend from the protein surface and ensures its independent mobility. This configuration creates an excellent test for anisotropy decay models, as the motion of the Atto 390 tag will include components that are independent of the overall rotational diffusion of the protein (e.g., the motion of the 5-residue N-terminus of the protein, the rotation of the dye around its linker). In addition, Atto 390 has a single lifetime in solution (~ 5 ns), making it a sensitive probe for the typical rotational correlation times of globular proteins < 30 – 40 kDa¹.

The computational model of the motion of Atto 390 linked to HEWL is carried out using molecular dynamics (MD) simulation¹² which provides insight into the rotations of the covalently-labeled dye and allows the fitting of the measured anisotropy decays¹³.

Experimentally we carried out fluorescence spectroscopy, fluorescence lifetime and fluorescence anisotropy decay of the Atto 390-labeled HEWL using time-correlated single photon counting (TCSPC) and the experimental data was rationalized using the model generated by the MD simulations in order to correlate the experimentally-determined rotational diffusion with the segmental motion of the label and the overall rotation of the protein.

Since the relatively limited experimental resolution provided by fluorescence anisotropy decay often produces ambiguous data, this study shows that the combination of anisotropy decay experiments and MD simulations is useful to better interpret anisotropy decay data

that can be correlated with the conformation of the biomolecule. Moreover, since the fundamental anisotropy of Atto 390, to the best of our knowledge, has not been established, part of the study will also be dedicated to analyzing r_0 , using steady-state and time-resolved data.

Our results suggest that the proposed approach might be practically useful for experimental studies whereby one can better analyze the motion of the fluorescent tag instead of making assumptions about its mobility (or lack of thereof) and adopt a more correct model for the interpretation of the anisotropy decay such as establishing whether faster component to the anisotropy are due to motion of the tag or motions of segments of the protein or a combination of both.

Results

Ligand Dynamics

MD simulations show that the ligand covalently attached to the N-terminal of HEWL exhibits complex mobility. The time-window probed by the simulation spanned a 67 ns range. The choice of time window was a compromise between two necessities: i.) reasonable computational time (few days) and ii.) the ability to sample as much of the complete trajectory of the dye/biomolecule complex in a time scale comparable to the one recorded in the TCSPC experiments. Since the time interval recorded during the decay experiments is 50 ns, the 67 ns simulation time allows us to probe the motion of the fluorescence probe for a time longer than the time interval of the experiments and at the same time maintain a reasonable computational time for this system (~ 12 hours per 10 ns of simulation). In order to characterize the dynamics of the system through anisotropy decay, one has to consider the positioning and motion of the fluorophore independent of the rotation or translation of the protein. This is accomplished by root-mean-square fitting to the first reference frame of the trajectory so that the protein is always translated back to the position of the first frame. The transformation isolates the dynamics of the Atto 390 ligand from the rotation/translation of the entire protein.

The motion of the chromophore decoupled from the overall rotational motion of the protein, can be described by three Euler angles representing the rotations of the three-ring aromatic head group, as shown in Figure 1. The pitch angle, θ , represents the in-plane scissoring motion of the ring of Atto 390 in the direction perpendicular to the protein surface. The roll angle, ϕ , represents the rotation of the aromatic plane about its longitudinal axis. The yaw angle ψ represents the “spinning” of the polyaromatic ring tangent to the protein surface. These angular displacements were measured from the N2, O2 and C14 (Figure 1), with respect to the center of mass of the protein to define the reference 0 angles.

Overall, the data in Figure 2 indicate that in the initial 35 ns the two main rotations associated with the Atto molecule are rotation of ϕ and ψ where Atto 390 “flips” between two configurations where each side of the a ring alternatively faces the surface of the protein.

At ~ 35 ns until the end of the simulation, the mobility of all three rotations increases. Thus, in the latter part of the simulation the stable parallel configuration indicated by ligand scissoring is lost, as the angular displacement repeatedly increased and decreased before plateauing at ~60 ns. After ~ 47 ns ϕ and ψ angles show a sharp increase whereby the ligand reorients and pulls away from the surface of the protein leaning alternately on each side of the coumarin ring. On a longer time scale the spinning motion increases and the scissoring motion decreases until the ligand returns to a more stable conformation where ϕ , θ and ψ fluctuated around 295°, 125° and 160° respectively.

These motions indicate an increasing rotational freedom throughout the simulation trajectory, which is verified through a RMSD analysis of the ligand motion (Figure 2, bottom). It is clear that the RMSD of the ligand follows the dynamics indicated by the angular displacements described above. The first 35 ns demonstrate that the ligand was in motion and except for a sharp rise at 23 ns, the fluctuations generally remained below 5 Å. At times longer than 35 ns the mobility of the ligand increases as corroborated by the noticeable increase in RMSD, e.g. at 50 ns.

The analysis of the origin of the mobility of the fluorescent tag was also undertaken by considering the interaction of Atto 390 with the environment throughout the simulation (e.g., the formation of hydrogen bonds, electrostatic/Van der Waals interactions, etc.). The electronic interactions between the ligand and the protein (Figure 3) show an overall trend of attractive electrostatic and larger repulsive VdW forces. The overall effect is therefore repulsive and results in keeping Atto 390 at a location that maximizes its distance from the protein interior. Throughout the simulation the VdW contribution remains consistently repulsive, whereas the electrostatic force shows a slight increase in the attractive contribution corresponding to the first ~ 35 ns of the simulation, followed by two episodes of larger repulsive contributions corresponding to the “flips” at 50 and 57 ns of Figure 2, whereby the changes in electrostatic (attractive or repulsive) contributions (at ~ 35, 50 and 57 ns) cause large conformational rearrangement of all motion angles of Atto 390 at the same time point. Given the location of the Atto 390 covalent label at the N-terminus, the survey of the structure of HEWL leads us to believe that the most likely charged residues responsible for the electrostatic contributions (Figure 4A) are the acidic Glu7, the Asp residues (at positions 52, 66, and 87), as well as basic residues Lys1 and Arg45, Arg86, and Arg128. An analysis of the formation of hydrogen bonds between Atto 390 and the protein did not show extensive hydrogen bonding formed between the ligand and polar residues (e.g., Asn39, Gln41 or the Ser residues in positions 81, 85, and 86) or hydrophobic residues such as Val2, Thr43, Ala82 and Leu84, indicating that hydrogen bond formation did not substantially contribute to the ligand stability.

When examining the trajectory for evidence of conformational changes at the significant conformational events (Figure 2), the reason for the ligand to exhibit such complex motility becomes clear. A very intriguing interaction between the C-terminus residue Arg128, and the N-terminus residues Glu7 and Lys1 appears to dictate the position and angular displacements of Atto 390. The side chain amine of Lys1 acts as an anchor to the N-terminus of the protein. As shown in Figure 4A the N-terminus of HEWL, to which Atto 390 is attached, is formed by 5 residues leading to the first α -helix. Often such a short

terminal segment of unordered residues would show a large flexibility which would then create substantial mobility of the Atto molecule “in-tow”. In the case of HEWL the N_ε amine of Lys1 is held in place though electrostatic attractions with the negatively charged residue Glu7 (with possible contribution from Asp87), as visualized in Figures 4A and 4B. This interaction effectively anchors the 5 amino acids-long N-terminus to the first α-helix of HEWL and prevents large displacements (not rotations) of the Atto 390 over the surface of the protein. Occasionally, however, the positively charged Arg128 rotates close to Glu7 and produces a perturbation of the electrostatic interactions that cause the separation between the positively charged N_ε amine of Lys1 and the negatively charged Glu7 residue by simultaneously repelling the former and attracting the latter. This causes the temporary loss of the Lys1/Glu7 anchor, which causes the ligand to rotate away from the protein surface and reorient itself such as, for instance, at *t* ~ 50 ns in Figure 2.

When the Arg128 side chain moves away from Glu7 its interaction with Lys1 and the anchoring effect are re-established and the Atto 390 returns to a more stable configuration.

This process is summarized in Figures 4, where the events that lead to the larger configuration changes at ~ 50 ns are highlighted. Figure 4B and 4C show the large reorientation that occurs at ~ 50 ns. In this case, Arg128 rotates and forces the Lys1 amine to rotate away from the Glu7 side chain. This rotation, in turn, forces the ligand away from the protein and disrupts any hydrogen bonds that the Atto molecule may have formed with other amino acids. After Arg128 rotates back to the previous location, the Lys1/Glu7 interaction is re-established and Atto 390 assumes a new and stable configuration closer to the surface of HEWL (Figure 4D). By comparing Figure 4A with Figure 4D one can appreciate the difference in orientation of Atto 390 between the two conformations that yield the most stable configuration over our time simulations.

The effect of Arg128 motion in this process correlates with the major conformational changes of the Atto 390 through its disruption of the anchoring of the N-terminus by the Lys1 to Glu7 electronic interactions (Figures 2–4). Further evidence is shown in Figure 5 whereby the distance between Arg128 and Glu7 is inversely proportional to the distance between the anchoring amine of Lys1 and the amide of Glu7. Every time the Arg128-Glu7 distance decreases, the Lys1-Glu7 distance increases and vice versa. In turn the behavior of Figure 5 correlates with the rotational changes prompted in Atto 390 (Figure 2 and 4).

Also noticeable (see movie in Supplemental Materials) is the behavior of the first five residues of the N-terminus (Lys1, Val2, Phe3, Gly4 and Arg5) that lead to the first α-helix (Figure 5A) of HEWL. Throughout the simulation their side chains do not undergo substantial rotations and do not affect the rotations of Atto 390. Conversely, it is clear that the carbon backbone linkage (Figure 2) that fixes the Atto molecule to the N-terminus is the pivot around which the Atto molecule rotates. The most active dihedral area surprising was not around the aromatic nitrogen or terminal amine linker in Lys1, but instead between the C3 and C4 carbons and the C2 to C3 dihedral contributions.

Experimental Fluorescence Anisotropy

The simulations discussed above demonstrate that Atto 390 is highly mobile when attached to the N-terminus (Lys1) of HEWL and that its anisotropy is better described by the combination of the fast motion (simulated and described above) and the overall protein diffusional motion. In this model the fluorescent tag rotates fast in a “propeller-like” fashion around the three angles discussed above, however, this motion must be combined with the slow rotational diffusion of the protein to which the dye is attached.

MD Simulation of anisotropy decay (Atto 390 segmental motion)

The demonstrated local mobility justifies the analysis of the fluorescence anisotropy decay in terms of segmental mobility model (Equation 4) when fitting the experimental fluorescence anisotropy decay with the simulated segmental motion of Atto 390 coupled with the overall rotation of the protein. Fitting of the anisotropy decay yields three correlation times: i.) a picosecond dipole relaxation correlation time θ_F , ii.) a nanosecond ligand correlation time θ_L , independent of the overall protein motion, and iii.) a longer correlation time θ_P , corresponding to the overall rotation of HEWL. Since our MD model only simulated the motion of the ligand, the value of θ_P was fixed at 8 ns, which is the rotational correlation time reported for HEWL of $8 \text{ ns} \pm 2 \text{ ns}$ ¹⁴.

The resultant calculated anisotropy curve was fitted to the experimental one using Equation 8 (Figure 6) where the average value on the left-hand side of the equation is calculated by averaging the angles obtained from the MD simulations. As one notices from the data in Figure 6 the experimental points tend to greatly deteriorate the S/N ratio after the first ~ 10–15 ns because of the fewer counts at these longer channels. This is a known limitation of the experimental technique at longer time delays and of finite computational methods of time correlations averaging^{15,16}. This is an additional factor in selecting the length of the simulations. As mentioned earlier our computed trajectory sampled the conformational space over 67 ns, however as shown in Figure 6 only the first ~ 15 ns yield anisotropy data with sufficient S/N ratio, therefore we think that the 67 ns time scale was sufficiently long and sufficiently stable to provide a reliable simulation of the motion of the fluorescent tag. The corresponding simulated curve showed good agreement with the experimental data. The overall R^2 value is poor (~ 0.7), however closer inspection reveals that the reason for such a value distant from the optimal value of 1 is mostly due to the small S/N ratio at times longer than 15 ns. For the first 10 ns of the decay the model of Equation 8 calculated using MD simulation of the motion of Atto 390, yield a $R^2 > 0.85$, which validates the segmental model. The results, shown in Table 1, yield a very fast 90 ps correlation time, along with a slower (1.8 ns) intermediate and the longer $\theta_P = 8 \text{ ns}$ rotational times. The ultra-short component to the anisotropy decay is well below the time-resolution limit of our instrument (estimated at ~ 300 ps) and causes depolarization of the emitted fluorescence even before the first decay data point is collected. Therefore, this component lowers the detectable limiting anisotropy from the theoretical maximum value of $r_0 = 0.4$, to an effective value $r_{0,eff}$ which for our system was expected to be 0.35 (Table 1). In the simulations, $r(0)$ was used because the value found through steady-state measurement was $0.39 (\pm 0.04)$ and because in the decay measurements anisotropy decay is fitted from the time point corresponding to the maximum counts (peak count) for the two positions of the emission polarizer. The position

of the peak count depends on jittering of the light source and other instrumental factors. Since this can account for > 1 detection channel, it is likely that very short component (< 100 ps) to the depolarization occur within the uncertainty of the peak position and therefore be “missed” by the experimentally recorded decay. Thus, the very first channel of the decay would be recorded after depolarization in < 100 ps has already occurred. In turn, this would lower the value of $r_{0,eff}$ even further compared to the simulated value as shown by Table I. We also cannot completely rule out the “instantaneous” rotation between absorption and emission transition dipoles, and further calculations (e.g., density functional theory) may be needed. The simulated curve obtained from Equation 8 yields a positive r_{∞} which remains slightly above the experimental data ($r_{\infty} = 0.003$) that can be attributed to the relatively short fitting range of 7 ns and deviations shown in Figure 6A. This indicates that the MD simulations restrict the motion of the chromophore on the longer time scale. Longer simulations most likely would yield more a more in-depth picture of this issue.

Fitting with Atto 390 segmental motion

In order to validate the model just presented, we compared it with the anisotropy fitting provided by the DAS6.6 software using three decay components in line with the θ_F , θ_L and θ_P parameters used to fit the experimental data with MD simulations. In addition, we carried out time-resolved fluorescence anisotropy measurements of free Atto 390 at room temperature in buffer and in a highly viscous (70% sucrose) solution (Figure 6B). The resultant anisotropy decay of the dye alone showed rotational correlation times below the limit of time-resolution of the instrument. The fast depolarization was expected, due to high rotational diffusion rates resulting from the low solution viscosity and the small molecular volume of the free dye. The anisotropy decays of the tagged lysozyme showed no evidence of the hindered rotation observed in the viscous sucrose environment (Figure 6). The fitting of the experimental decays with DAS6.6 shows good agreement with the ones obtained through MD simulations. The overall correlation time θ_P attributed to the protein tumbling was found to be 6.85 ± 0.34 ns which is relatively close ($\sim 15\%$) to the value of 8 ns assumed for the MD fitting and larger than the values of ~ 4 ns obtained in other studies (see below). This value of θ_P is therefore much closer to the actual expected value for a globular protein of the size of HEWL. The ligand correlation time θ_L also showed good agreement with the value retrieved through MD simulations (Table 1). The fitting with DAS6.6, however, was unable to resolve the fast (< 300 ps) component.

As expected the fundamental anisotropy, r_0 , of Atto 390 yielded by the DAS6.6 software is lower (Table 1) than the expected value of 0.4. Moreover, while the MD simulations produce a positive value of r_{∞} , the DAS software is able to represent the fact that the decay returns to basically the baseline value. This is probably due to the fact that the overall motion of the protein is not described to the detail necessary to reproduce the anisotropy decay on longer time-scales. In other words this model, at this stage needs to be improved to better account for the slower overall motion of the protein which contributes to the depolarization of the dye but is not reproduced well by the current approaches to MD simulations.

Fitting with rigid rotor

The fitting was also carried out using the rigid rotor model (Equations 3) provided by the DAS6.6 software. The comparison between the results of the rigid rotor and the segmental motion model (either through MD simulations or DAS6.6 software) in Table 1 shows some interesting results. A difference in correlational times attributed to the protein motion was found between the segmental mobility and rigid rotor models. The segmental mobility model θ_p value of 6.85 ± 0.34 ns was almost twice the value of the rigid rotor θ_R value of 3.64 ± 0.26 ns. The latter is instead in the range of those reported by others using the same model^{17–19}. Considering the rotational correlation expression assuming the protein as a sphere

$$d=2\left(\frac{3\theta RT}{4\pi\eta}\right)^{1/3} \quad (9)$$

with a solvent viscosity $\eta = 1.4$ cP, gas constant R and temperature $T = 310$ K, the spherical diameters of HEWL from the two models were calculated from θ_p and θ_R . The results showed that the relative diameters found were $d_{SM} = 40.4 \pm 15.7$ Å and $d_{RR} = 32.7 \pm 8.9$ Å. Measuring the average maximum diameter of the protein from the simulation, with 10 Å solvation water shell¹ the diameter was found to be 44.9 ± 15.1 Å. It is clear that the rigid rotor results underestimate the size of the protein in aqueous solvents by ~ 16%, while the segmental mobility model that we propose better approximates (within 10%) the diameter of the protein. The rigid rotor model was not the right model to use in this instance, due to the high mobility of the attached Atto 390. However if one considers that segmental mobility may hide within the rotor fitting results, the use of the harmonic mean correlation time θ_H (Equation 5) shows a value of 4.02 ± 0.23 ns (Table 1), which is similar to the rigid rotor time 3.64 ± 0.26 ns and the times reported in other studies^{17–19}.

Discussion

Atto 390, a UV fluorescent dye with high quantum yield, was conjugated to the N-terminal Lys1 residue of HEWL and its dynamics were characterized through fluorescence anisotropy decay. Our results stress the importance of knowing the local mobility of the fluorescent tag, consisting in this case of the three-ring aromatic group, as the degree of coupling between its local motion and the overall rotation of the protein affects the more appropriate model to be used to analyze fluorescence anisotropy decay. A 67 ns MD simulation was conducted with a parameterized Atto 390 connected to a crystal structure of HEWL in order to establish the local mobility of the ligand with respect to the fixed protein frame. A simulated fluorescence anisotropy decay was then computed using the second order Legendre, time-averaged inner products of successive absorption transition dipoles, which was compared to the experimental decay in terms of two molecular rotational diffusion models: segmental mobility and restricted rigid rotors.

The simulation found that the three Euler angles, (ϕ , θ and ψ (Figure 1)) describe the protein-uncoupled motion of the chromophore. θ , the angle holding the rings tangential to the protein surface, was found to be the least affected motion of the three. Several important

conformational events were indicated by angular changes at ~ 35, ~ 47 and ~ 57 ns corresponding to changes in electrostatic interactions among the Lys1, Glu7 and Arg128 residues. The electrostatic interaction between Lys1 and Glu7 that stabilizes the motion of Atto 390, by limiting the motion of the 5 residues N-terminus (Figure 4), is disrupted by the rotation of the side chain of Arg128 which brings the positively charged Lys and Arg residues close enough to force (by Coulombic repulsion) Lys1 to temporarily break contact with Glu7 and, in the process, allow Atto 390 to drastically change its angular motions. We also show that the angular displacements of the chromophore are mostly due to rotations of the dihedral angles formed by C1 through C4 (Figure 1).

The simulated anisotropy obtained from MD simulations of the local motion of Atto 390 shows two correlation times: a fast picosecond time and a longer rotational time attributed to the motion of the ligand independent of the rotational motion of the protein. Assuming an eight nanosecond protein correlation time, the resultant decay was compared to the experimental decay, where good agreement was found (Figure 6). We also demonstrated that the fitting derived through MD simulations can resolve contributions that remain undetected by regular multi-exponential fitting of the experimental data (Table 1). It was found that while the rigid rotor model seemed to be the better choice statistically, the segmental mobility model was the appropriate one based on computational results. In fact in the literature one finds fairly consistently reported a correlation time for the rigid rotation of lysozyme in the range of 3.5–4.5 ns^{17–19} which is much shorter than the one expected for a protein the size of HEWL¹. With our approach the protein correlation time better matches the protein diameter. The conflict between correlation times as reported by each model was resolved by noting that the harmonic mean correlation time of the segmental mobility model was close to the rigid rotor model results. This implies that the segmental mobility was hidden inside the rotor model results and underscores the importance of knowing the details of the motion of the ligand relative to the protein in order to extract more precise rotational times and hydrodynamic radii.

We believe that the results presented here have potential practical applications for studies of fluorescence anisotropy decay using fluorescent tag. The MD simulations of a dye attached to a biomolecule can be carried out in a relatively short computer usage provided that one has the correctly parameterized PDB version of the biopolymer and the fluorescent tag. The covalent bond between the tag and the biomolecule can also be created and parameterized through appropriate scripts. The length of time probed (i.e., for how many nanosecond should the trajectories of the dye and the biomolecules be probed) by the simulation should be decided based on the fluorescence lifetime of the tag and the rotational diffusion time of the biomolecule under investigation, estimated from its molecular weight. Through this approach one can then obtained a simulated anisotropy decay that can be compared to the one experimentally obtained. The advantage is that the computational model can greatly improve the interpretation of the experimental anisotropy decay data. With additional computational power and/or smaller systems (i.e., fewer atoms) MD simulations could be run to probe longer time scales. Coarse graining simulations could also increase the time scale that one probes or allow much larger complexes to be investigated, however this approach would, for now, limit the detailed resolution of the motion of the biomolecule.

A final remark should be dedicated to the role of water in the simulations. The water model we employed, TIP3P is the one used by CHARMM force field which was used because is widely accepted as the most accurate for protein simulations. The TIP3P is a classical model that ignores quantum mechanical parameters and adopts a parametrization which yields a certain polarization of the solvent molecule. For this reason future studies will be directed at understanding the role of water in the mobility of the fluorescent tag.

Materials and Methods

Materials

All materials were obtained from Sigma-Aldrich (St. Louis, MO). HEWL (L6876) was labeled with Atto 390 at the N-terminus ⁷ by combining a 5:1 molar excess of Atto-390 NHS ester in anhydrous N-N-dimethylformamide (DMF) with 3 mg/ml HEWL in pH 7.4 PBS at 8 °C. After the reaction was complete, the excess free dye and DMF were removed by extensive dialysis against PBS buffer with 6–8 kDa Pur-A-Lyzer dialysis kit (Sigma-Aldrich). The degree of labeling (DOL) was established through the Equation 1 ²⁰

$$DOL = \frac{\varepsilon_{dye}/A_{dye}}{\varepsilon_{protein}/A_{protein}} \quad (1)$$

using a dual beam Cary 300 spectrophotometer (Agilent Technologies, Santa Clara, CA) assuming molar extinction coefficients of $\varepsilon_{278\text{ nm}} = 37932\text{ M}^{-1}\text{cm}^{-1}$ ²¹ and $\varepsilon_{390\text{ nm}} = 2.4 \times 10^4\text{ M}^{-1}\text{cm}^{-1}$ (provided by the manufacturer) for HEWL and Atto 390 respectively.

Fluorescence Experiments

Both steady-state and time-resolved fluorescence anisotropy spectra were collected using a Fluorocube TCSPC lifetime spectrofluorimeter (Horiba Scientific, Edison, NJ), utilizing a 388 nm NanoLED (IBH, Glasgow, UK) with a 1.3 ns pulse-width and a 1 MHz repetition rate. The instrument can be used for lifetime measurements as well as steady-state spectroscopy.

The fundamental anisotropy, r_0 , of Atto 390 was measured in a high viscosity (~4700 cP ²²) 70% w/w aqueous sucrose solution at 8 °C. Light scattering artifacts were reduced by utilizing a long-pass filter (Melles Griot, Rochester, NY) in the collected fluorescence emission path. Emission spectra were recorded in the 450–600 nm range, at 1 nm intervals at a rate of 1 nm/sec, with a 4 nm bandwidth in excitation and emission.

Time-resolved fluorescence decays were collected using the same pulsed excitation source and emission at $500 \pm 8\text{ nm}$ under different polarization of the excitation and emission light. As is explained in the methods below, anisotropy experiments require the acquisition of fluorescence decay under two configurations of the polarizers: 1) excitation and emission polarizers positioned with vertical polarization, $I_{||}$ and 2) the excitation polarizer in vertical configuration and the emission polarizer in horizontal configuration, $I_{\perp}$. The two intensities decays, $I_{||}(t)$ and $I_{\perp}(t)$ were recorded by automatically rotating the emission polarizer every

60 seconds (to ensure equal acquisition times in the two configurations ¹) until the peak count difference between $I_{\parallel}(t)$ and $I_{\perp}(t)$ reached 10,000 photons.

Experiments were carried out at 37 °C and decays were recorded from samples containing either free Atto 390 or Atto 390-labeled HEWL in order to provide a comparison between the free and bound dye. The anisotropy decay was determined using the method described below after collecting fluorescence decays.

Methods

Analysis of the fluorescence emission anisotropy decays was performed using the software DAS6.6 (Horiba, Edison, NJ) for discrete anisotropy fits with no reconvolution. The time-resolved anisotropy decay $r(t)$ can be described by the following equation

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)} \quad (2)$$

where G , $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the instrument factor and polarized emission intensities ¹. The anisotropy $r(t)$ was fitted either to a single-exponential symmetric hindered-rotor model ¹ containing the limiting anisotropy r_{∞} and rotational correlation time θ_R

$$I(t) = (r_0 - r_{\infty}) \exp\left(-\frac{t}{\theta_R}\right) + r_{\infty} \quad (3)$$

or a two-exponential segmental mobility model ¹

$$r(t) = (r_0)_{eff} \left(\alpha_L e^{-t/\theta_L} + (1 - \alpha_L) \right) e^{-t/\theta_P} \quad (4)$$

where θ_P and θ_L are the overall protein correlation time (equivalent to θ_R in the rotor model) and the faster, decoupled segmental motion of the attached ligand, respectively. The two models were compared using the harmonic correlation time θ_H ¹

$$\frac{1}{\theta_H} = \frac{1}{r_0} \sum_i \frac{r_i}{\theta_i} \quad (5)$$

The overall fitting quality was judged by the reduced χ^2 and Durbin-Watson parameter ²³ with optimal values between 1.0 and 1.2, and between 1.8 and 2.0, respectively.

Molecular dynamics simulations

The motion of Atto 390 attached to HEWL was investigated using MD simulations. The fluorophore was first constructed using Avogadro 1.1.0 ²⁴, followed by parameterization of the molecule using CGenFF based on the CHARMM general force field ²⁵. The ground state of the molecule was then optimized with Gaussian 09 ²⁶ utilizing the B3LYP/6-31G(d) basis

set parameterization. The constructed molecule was connected to the N-terminal of HEWL²⁷ using the all-hydrogen CHARMM36 topology file NTER patch²⁸. The generated dye parameters were added to the CHARMM36 all-hydrogen protein parameter file. Atom type conflicts between the peptide backbone and the CGenFF small-molecule drug design parameter nomenclature were avoided by renaming.

MD simulations were conducted with NAMD 2.9²⁹ based on the edited all-atom CHARMM36 topology and parameter files. The system was explicitly solvated in a water box with 15 Å sides in excess of the size of the protein and neutralized with 150 mM Na⁺ and Cl⁻ ions with 5 Å spacing to eliminate ion clustering. Subsequent simulations conditions were set using Langevin dynamics on an NPT ensemble at 310 K³⁰, with periodic boundary conditions. A Nose-Hoover Langevin piston³¹ with a fixed initial center of mass and 5/ps damping coefficient regulated the pressure set at 1 atm. The long-range interactions were computed using the particle mesh Ewald (PME) method, with cutoff and switching distances of 12 Å and 10 Å and a 1 Å grid spacing. The time-step was 2 fs, with rigid TIP3P waters and fixed bond lengths. The frequency of energy, pressure and trajectory capture was set every 500 steps. After an initial 20 ps system minimization followed by a 1 ns equilibration, the simulation at the target temperature was run for a total of 70 ns. The simulation results were continuously checked in 1 ns intervals with a Maxwell-Boltzmann energy distribution curve to verify the ensemble temperature. These simulations were conducted on a 12-core GPU-enabled node (Tesla M2070) at the Computational Systems Biology Core/Computational Biology Initiative.

From MD simulations to anisotropy simulations

To compute the simulated time-resolved anisotropy of the dye-protein system, first an initial random isotropic ensemble of chromophores¹³ was assumed. The fluorescence anisotropy expression in equation 3 can be re-written in terms of the second order Legendre time correlation function^{1,8,13,32}, containing the normalized absorption and emission transition dipoles $\hat{\mu}_A(t)$ and $\hat{\mu}_E(t)$ and the overall rotational diffusion time, θ_P , of the protein considered as a rigid rotor

$$r(\tau) = r_0 \langle P_2[\hat{\mu}_A(t) \cdot \hat{\mu}_E(t+\tau)] \rangle e^{-t/\theta_P} \quad (6)$$

Extensive derivations shown by Ichiye and Karplus¹³ demonstrated that this equation can be employed when the rotational diffusion of the attached dye is of the same order of the correlation time of the protein (which applies to the Atto 390/HEWL system adopted here). Assuming the absorption and emission transition dipoles of Atto 390 to be collinear due to the high fundamental anisotropy (see Results), the approximation $\hat{\mu}_A \sim \hat{\mu}_E$ was used as substitution for the final form

$$r(t) = r_0 \langle P_2[\hat{\mu}_A(t) \cdot \hat{\mu}_A(t+\tau)] \rangle e^{-t/\theta_P} \quad (7)$$

The correlational motion of the ligand can be separated into a picosecond correlation time, θ_F , which is below the time-resolution of our instrumentation and a longer time θ_L which is due to the composite motion of the dye and the segment of the protein to which it is attached. Assuming $\theta_F \ll \theta_L < \theta_P$, the time-averaged correlation of Equation 7 can be viewed as a product of exponential terms

$$\langle P_2[\hat{\mu}_A(t) \cdot \hat{\mu}_A(t+\tau)] \rangle = \left(\alpha_F e^{-t/\theta_F} + (1-\alpha_F) \right) \left(\alpha_L e^{-t/\theta_L} + (1-\alpha_L) \right) \quad (8)$$

The constant α_L provides the amount of decoupling from the overall protein correlation, as increasing α_L values would indicate higher mobility of the fluorescent ligand^{1,8}. According to Equation 8, for $\theta_F \ll t$, one can estimate the effective fundamental anisotropy $(r_0)_{eff} \approx (1 - \alpha_F)r_0$ which would account for a lower-than-expected value due to the motion of the fluorophore that may occur below the time resolution of the instrument.

The average time-correlations were computed using CPPTRAJ and PTRAJ³³. The direction of the transition state dipole $\hat{\mu}_A$ was calculated using ZINDO-CL^{34,35} in Arguslab 4.0.1³⁶ and as shown in Figure 1, is nearly parallel to the long axis of the three-ring aromatic head group.

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Figure 1.

Representation of lysozyme with Atto 390 (small box) covalently attached the N-terminus. The enhanced Atto 390 (large zoomed box) shows the absorption transition dipole moment (green line), and active angular modes as the Euler angles yaw ψ (blue curved arrow), pitch θ (black curved arrow), and roll ϕ (red arrow). The atoms have been labeled for positional reference.

Figure 2.

Top) Time-dependent Euler angle motions of the Atto 390 ligand shown for pitch (black), roll (red) and yaw (blue). Bottom) RMSD values of hen lysozyme (black solid) and Atto 390 (black dotted) with the initial frame as reference for all values.

Figure 3.

Forces on the Atto 390 ligand due to electrostatic and VDW interactions with HEWL. The red lines represent thermal fluctuation smoothing of the original data (black lines).

Figure 4.

Positional changes of Atto 390 as a result of GLU7-ARG128 interactions. A) The time-zero configuration with type-specific residues colored and labeled as polar (green), hydrophobic (gray), acidic (red) and basic (blue). The polar and hydrophobic residues contribute through hydrogen bonding (not shown). The LYS1:N_ε amine is shown as a blue VDW sphere, docked to GLU7, and connected via yellow to the purple backbone of residues 1 to 5. Atto 390 is shown in orange. B) Atto 390's positional change at ~50 ns, where the amine has undocked (yellow) due to the approach of ARG128 (purple). A better view of the same conformation is seen as a side snapshot in C). In D) the end conformation at 67 ns shows a reversed Atto 390, in a different area than the beginning conformation in A).

Figure 5. Representation of the average separation between Glu7 and Arg128 (black) as well as between Glu7 and Lys1:N_ε amine anchor (red). One can notice that each time the separation between Glu7 and Arg 128 decreases a correlated motion increases the separation between Glu7 and Lys1.

Figure 6. Simulated and measured time-decay anisotropies of Atto 390-labeled lysozyme

A) the simulated decay curve from Equation 8 is shown with the fitted curve for the fast (θ_F) and ligand (θ_L) correlation times, with the approximate contribution of each time to the curve.

B) The simulated anisotropy curve (green line) with the assumed protein correlation time θ_P of 8 ns, as compared to the experimental anisotropy decay of Atto 390-labelled hen lysozyme (red dots) at 37 °C. The experimental decays for free Atto 390 (black dots) and immobilized Atto 390 in 70% sucrose solution (blue dots) are also shown. All dashed black lines represent experimental fitting lines for the decay data.

Table I

Time-resolved anisotropy decay fittings results.

Anisotropy	$\theta_F a, b$	$\theta_L a, b$	$\theta_F a, b$	$\theta_L a, b$	$\theta_R a, b$	$\alpha_F c$	$\alpha_L c$	$r_{0,eff}$	r_∞
Simulated	0.09	1.80	8.0	-	-	73.2	30.7	0.29	0.003
Experimental	-	2.57 ± 0.17	6.85 ± 0.34	4.02 ± 0.23	3.64 ± 0.26	-	38.4 ± 6.3	0.32 ± 0.02	0.00 ± 0.005

a, b in nanoseconds

c in %

The symbols represent correspondingly: the fast (F), ligand (L), protein (P), rigid rotor (R) and harmonic (H) rotational correlational times θ_i effective fundamental anisotropy $r_{0,eff}$ and the limiting anisotropy r_∞ . The pre-exponential factors α_F and α_L are from Eq. 8.