Fluorescence anisotropy of oligomeric proteins

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Abstract. Previous studies of protein oligomerization using time-resolved fluorescence anisotropy assumed a single fixed probe per oligomeric complex and an identical probe orientation in complexes of different stoichiometry. However, an oligomer consisting of “n” singly labeled monomers must necessarily have “n” probes. Moreover, in the expression for the anisotropy decay, the molecular axes from which the probe orientation is defined are different for complexes that differ in stoichiometry. Here, we derive an expression for the decay of the anisotropy for molecules with any number of fixed probes, and show how an explicit understanding of the probe orientation is necessary to properly assess oligomerization.

1. Introduction

One of the mechanisms by which the emission from a fluorophore can become depolarized with time is through the rotational diffusion of the fluorophore [1–3]. For a fluorescent group that is immobile on a macromolecule, depolarization may then be owing to the rotational diffusion of the carrier macromolecule, which is a property that depends on its size [4–6]. A measure of the rate of depolarization, with time-resolved fluorescence anisotropy, can thus be used to determine the size of macromolecules [7–9], and, further, to study their state of oligomerization [10–13].

However, in previous studies of oligomerization, the anisotropy was interpreted based on two assumptions that may not be valid for all investigations of oligomerization. First, the equation that was used to describe the anisotropy had been previously derived for the case of a single probe per rotating molecule [14–16]. Yet oligomers that are composed of singly labeled monomers (as in these earlier studies) will have the same number of probes as their stoichiometry. The rotational diffusion of all of the probes associated with the same oligomer will therefore not be independent, and so, any measure of the rotational diffusion, such as the anisotropy, may be expected to depend on the number of probes per oligomer. Secondly, it was assumed that the orientation of the probe is the same for all complexes. This probe orientation is defined with respect to the set of axes, the principal axes, that diagonalize the diffusion tensor of the particular oligomeric complex with which the probe is associated. Since the principal axes of a complex with one stoichiometry do not coincide with those of a complex of a different stoichiometry (which can be seen by comparing the axes of symmetry of any two complexes that differ in stoichiometry), it is not clear whether the probe orientations could be the same in any of the complexes that differ in stoichiometry.

Here we extend the expression that was previously derived for the case of a singly labeled molecule to that of any number of probes per molecule, and show that the anisotropy depends on the relative orientations of the probes on the same molecule. In addition, we show how the inevitable difference in
the location of the molecular reference system in complexes of different stoichiometry can, alone, change the expected decay of fluorescence anisotropy.

2. Theory

Fluorescence anisotropy is defined as

\[ r(t) = \frac{I_Z - I_Y}{I_Z + 2 \cdot I_Y}, \] (1)

where \( I_Z \) and \( I_Y \) are, respectively, the intensities with polarization along the \( Z \)- and \( Y \)-axis in the laboratory coordinate system, and the initial pulse of light is polarized along the (laboratory) \( Z \)-axis. Consider first the case of a collection of molecules, each with a single fixed probe. Denote the absorption and emission dipole moments of this probe by \( \mu_a \) and \( \mu_e \), respectively, defined with respect to the molecular coordinate system. Assuming that depolarization occurs solely via rotational diffusion, the emitted intensity along the \( Z \)-axis is determined by the probability to first excite the collection of probes, the probability that the ensemble of molecules then changes from one to another orientation during the time of observation, and then the probability that the probe emits along the \( Z \)-axis [16]. Using Euler angles, \( \Omega \), to transform the laboratory coordinate system into the molecular coordinate system and denoting \( W(\Omega_o, \mu_a) \) as the probability that the probe is excited with the \( Z \)-polarized light for all initial molecular orientations, \( \Omega_o \), the evolution of the excited fluorophore population can be expressed as [16]

\[ P(\Omega, t) = \int W(\Omega_o, \mu_a) P(\Omega_o | \Omega, t) \, d\Omega_o, \] (2)

where \( P(\Omega_o | \Omega, t) \, d\Omega \) is the probability that the ensemble of molecules has an orientation between \( \Omega \) and \( \Omega + d\Omega \) at time, \( t \), given the initial molecular orientation \( \Omega_o \). With the magnitude of the intensity of the emitted fluorescence along the \( Z \)-axis at any time proportional to \( (Z \cdot \mu_e)^2 \), the emitted intensity along this axis is

\[ I_Z = \int P(\Omega, t) (Z \cdot \mu_e)^2 \, d\Omega \]
\[ = \int \int W(\Omega_o, \mu_a) P(\Omega_o | \Omega, t) (Z \cdot \mu_e)^2 \, d\Omega_o \, d\Omega. \] (3)

With a similar expression for \( I_Y \), the denominator in Eq. (1), corresponding to the total fluorescence intensity, is

\[ \int \int W(\Omega_o, \mu_a) P(\Omega_o | \Omega, t) \left[ (Z \cdot \mu_e)^2 + 2 \cdot (Y \cdot \mu_e)^2 \right] \, d\Omega_o \, d\Omega, \] (4)

which, when evaluated as in [16], yields a (properly normalized) value of 1, as expected. Evaluation of the numerator in Eq. (1)

\[ \int \int W(\Omega_o, \mu_a) P(\Omega_o | \Omega, t) \left[ (Z \cdot \mu_e)^2 - (Y \cdot \mu_e)^2 \right] \, d\Omega_o \, d\Omega, \] (5)
yields
\[
\sum_{\tau=-2}^{2} C_{\tau} \exp(-E_{\tau} \cdot t),
\]
with
\[
C_{2} = \frac{3}{10} M^{2} \{ \left( \frac{a^{2}}{3} \right) (3z_{a}^{2} - 1)(3z_{e}^{2} - 1) + \left( \frac{b^{2}}{3} \right)(x_{a}^{2} - y_{a}^{2})(x_{e}^{2} - y_{e}^{2}) \\
+ \left( \frac{ab}{3} \right)(x_{a}^{2} - y_{a}^{2})(3z_{e}^{2} - 1) + (x_{a}^{2} - y_{a}^{2})(3z_{a}^{2} - 1) \},
\]
\[
C_{1} = \frac{6}{5} y_{a} z_{a} y_{e} z_{e},
\]
\[
C_{0} = \frac{3}{10} M^{2} \{ \left( \frac{b^{2}}{3} \right)(3z_{a}^{2} - 1)(3z_{e}^{2} - 1) + \left( \frac{a^{2}}{3} \right)(x_{a}^{2} - y_{a}^{2})(x_{e}^{2} - y_{e}^{2}) \\
+ \left( \frac{ab}{3} \right)(x_{a}^{2} - y_{a}^{2})(3z_{e}^{2} - 1) + (x_{a}^{2} - y_{a}^{2})(3z_{a}^{2} - 1) \},
\]
\[
C_{-1} = \frac{6}{5} x_{a} z_{a} x_{e} z_{e},
\]
\[
C_{-2} = \frac{6}{5} x_{a} y_{a} x_{e} y_{e},
\]
\[
D_{L} = (D_{1}^{2} + D_{2}^{2} + D_{3}^{2} - D_{1} D_{2} - D_{1} D_{3} - D_{2} D_{3})^{1/2},
\]
\[
a = 3^{1/2}(D_{1} - D_{2}), \quad b = 2D_{3} - D_{1} - D_{2} + 2D_{L}, \quad M = 2(D_{L}b)^{1/2},
\]
\[
D_{s} = (1/3)(D_{1} + D_{2} + D_{3}),
\]
\[
E_{2} = 6D_{s} + 2D_{L}, \quad E_{1} = 3(D_{s} + D_{1}), \quad E_{0} = 6D_{s} - 2D_{L}, \quad E_{-1} = 3(D_{s} + D_{2}),
\]
where \(D_{1}, D_{2}, D_{3}\) are the diffusion constants in the molecular coordinate system that diagonalizes the diffusion tensor, and the adsorption and emission dipole moments in this molecular coordinate system are, respectively,
\[
\mu_{a} = (x_{a}, y_{a}, z_{a}), \quad \mu_{e} = (x_{e}, y_{e}, z_{e}).
\]

This molecular coordinate system is defined by the principal axes of the molecule (since these axes diagonalize the rotational diffusion tensor), and is used to describe the basis set for the corresponding eigenvalue problem [17]. Transforming to spherical coordinates
\[
\mu_{a} = (\cos \phi_{a} \sin \theta_{a}, \sin \phi_{a} \sin \theta_{a}, \cos \theta_{a}), \quad \mu_{e} = (\cos \phi_{e} \sin \theta_{e}, \sin \phi_{e} \sin \theta_{e}, \cos \theta_{e}),
\]
and considering the symmetric case \((D^1_1 = D^2_2)\) for these monomers, the anisotropy is given by

\[
n(t) = \sum_{\tau=1}^{3} A^m_\tau \exp\left(-E^m_\tau \cdot t\right),
\]

(10)

with

\[
A^m_1 = \left(\frac{3}{10}\right) \sin^2 \theta_a \sin^2 \theta_e (\cos^2 \Phi - \sin^2 \Phi),
\]

\[
A^m_2 = \left(\frac{6}{5}\right) \sin \theta_a \cos \theta_a \sin \theta_e \cos \theta_e \cos \Phi,
\]

\[
A^m_3 = \left(\frac{1}{10}\right) (3 \cos^2 \theta_a - 1) (3 \cos^2 \theta_e - 1),
\]

(11)

\[
E^m_1 = 2D^m_1 + 4D^m_3, \quad E^m_2 = 5D^m_1 + D^m_3, \quad E^m_3 = 6D^m_1,
\]

where \(\Phi = \phi_e - \phi_a\). Notice that this expression does not depend on the actual magnitude of the azimuthal angle, \(\phi\), but only on the difference, \(\phi_e - \phi_a\).

Now consider the case with \("n"\) probes per molecule, which corresponds to the case of a collection of identical oligomers of stoichiometry \("n"\), with a single fixed probe per monomer. The emitted fluorescence along the \(Z\)-axis is now given by the sum of the fluorescence emitted by all of the probes on each molecule, integrated over all possible orientations of the collection of molecules:

\[
I_Z = \int \int \left\{ \left[ W(\Omega_o, \mu_{a1})(Z \cdot \mu_{e1})^2 \right] + \left[ W(\Omega_o, \mu_{a2})(Z \cdot \mu_{e2})^2 \right] + \cdots \right. \\
\left. + \left[ W(\Omega_o, \mu_{an})(Z \cdot \mu_{en})^2 \right] \right\} \cdot P(\Omega_o|\Omega, t) \ d\Omega_o \ d\Omega,
\]

(12)

with a similar expression for \(I_Y\). Here, \(P(\Omega_o|\Omega, t) \ d\Omega\) has the same form as in the single probe case, describing the evolution of the collection of molecules from their initial orientation, \(\Omega_o\), to their final orientation, \(\Omega\). Resolving each term separately, the denominator of Eq. (1) will be a sum of \(n\) terms, each of the form

\[
\int \int W(\Omega_o, \mu_{ei})P(\Omega_o|\Omega, t) [(Z \cdot \mu_{ei})^2 + 2 \cdot (Y \cdot \mu_{ei})^2] \ d\Omega_o \ d\Omega,
\]

(14)

which resembles Eq. (4) for the single probe case. Since the value of each of these is 1 (as the total fluorescence per probe will not change because of the rotation of the molecule), the denominator is simply \(n\), the number of probes per molecule.

The numerator of Eq. (1) is also a sum of \(n\) terms, but each of these is of the form

\[
\int \int W(\Omega_o, \mu_{ei})P(\Omega_o|\Omega, t) [(Z \cdot \mu_{ei})^2 - (Y \cdot \mu_{ei})^2] \ d\Omega_o \ d\Omega,
\]

(15)

which resembles Eq. (5) for the single probe case and thus yields

\[
\sum_{\tau=-2}^{2} C^\tau_i \exp(-E^\tau \cdot t),
\]

(16)
where each of the pre-factors, $C^i_i$, has the same form as in Eq. (7).

Thus, taken together, the anisotropy for a molecule with $n$ fixed probes is given by

$$r_n(t) = \frac{1}{n} \sum_{i=1}^{n} \sum_{\tau=-2}^{2} C^i_i \exp(-E_{\tau} \cdot t),$$

(17)

with $C^i_i$ given by Eq. (7) for each probe $i$. In words, in the case of an oligomer of “$n$” singly labeled monomers, the anisotropy is the sum of five exponentials, where the pre-factor of each exponential is the average of the corresponding pre-factors of the monomers, with the probe orientation defined with respect to the principal axes of the oligomer. That is, defining this average as $C^i_n(\equiv (1/n)[C^1_i + C^2_i + \ldots + C^n_i])$, in the oligomeric coordinate system,

$$r_n(t) = \sum_{\tau=-2}^{2} C^i_n \exp(-E_{\tau} \cdot t)$$

for a molecule with “$n” probes. For example, for two probes per molecule in the symmetric case ($D^d_1 = D^d_2$), the anisotropy is given by

$$r^d(t) = \sum_{\tau=1}^{3} A^d_\tau \exp(-E^d_{\tau} \cdot t),$$

(18)

with

$$A^d_1 = (1/2)(A^1_1 + A^2_1)$$

$$= (3/20)[\sin^2 \theta_{a_1} \sin^2 \theta_{e_1} (\cos^2 \Phi_1 - \sin^2 \Phi_1) + \sin^2 \theta_{a_2} \sin^2 \theta_{e_2} (\cos^2 \Phi_2 - \sin^2 \Phi_2)],$$

$$A^d_2 = (3/5)[\sin \theta_{a_1} \cos \theta_{a_1} \sin \theta_{e_1} \cos \Phi_1 + \sin \theta_{a_2} \cos \theta_{e_2} \cos \theta_{e_2} \cos \Phi_1],$$

$$A^d_3 = (1/20)[(3 \cos^2 \theta_{a_1} - 1)(3 \cos^2 \theta_{e_1} - 1) + (3 \cos^2 \theta_{a_2} - 1)(3 \cos^2 \theta_{e_2} - 1)],$$

$$E^d_1 = 2D^d_1 + 4D^d_3, \quad E^d_2 = 5D^d_1 + D^d_3, \quad E^d_3 = 6D^d_1,$$

(19)

where $\Phi_i = \phi_{ei} - \phi_{ai}$. Notice that each term is dependent on the orientation in a different way, and so the form of the anisotropy in the case of two probes per molecule can be significantly different from that of a single probe per molecule.

3. Two examples

To see how the new expression changes the expected decay of the anisotropy in a specific oligomerization reaction, consider the dimerization of two cylindrical molecules as depicted in Fig. 1A. Assume, for simplicity, that the adsorption and emission dipole moments are identically aligned and oriented at
Fig. 1. Dimerization of two singly labeled cylindrical molecules. (A) As a result of the change in the molecular coordinate system and the change in the number of probes per rotating species, the expression for the anisotropy decay for this dimer will be different from that of the monomer. (B) However, there are situations, such as the one depicted in this figure of two cylindrical membrane proteins, in which the form of the expression for the anisotropy for the dimer would be largely the same as that for the monomer, differing only in the diffusion constants.

an angle, $\theta_o$, from the central axis as shown in Fig. 1A. This axis defines the principal axes of the molecule, which is the coordinate system that diagonalizes the diffusion tensor of this molecule. The probe orientation in the monomer is thus given by

$$\left(\phi^m, \theta^m\right) = (0, \theta_o),$$  \hspace{1cm} (20)$$

and the anisotropy is given by Eq. (10) (since $D^m_1 = D^m_2$), with

$$A^m_1 = \left(3/10\right) \sin^4 \theta_o, \quad A^m_2 = \left(3/10\right) \sin^2 2 \theta_o, \quad A^m_3 = \left(1/10\right) \left(3 \cos^2 \theta_o - 1\right)^2,$$

$$E^m_1 = 2D^m_1 + 4D^m_3, \quad E^m_2 = 5D^m_1 + D^m_3, \quad E^m_3 = 6D^m_1.$$  \hspace{1cm} (21)$$

Now consider the dimer shown in Fig. 1A. There are now two axes of symmetry, given by the $x$- and $y$-axes in the figure, and these axes define the dimer coordinate system, which is the only molecular reference system for which Eq. (17) describes the anisotropy decay. The orientations of the two probes in this system are given by

$$\left(\phi^d_1, \theta^d_1\right) = (45^\circ, 90^\circ - \theta_o),$$

$$\left(\phi^d_2, \theta^d_2\right) = (-45^\circ - \theta_o, 90^\circ).$$  \hspace{1cm} (22)$$
Thus, even though the locations of the probes with respect to their corresponding monomers have not changed, their orientations are different in the dimer owing to the difference in the location of the axes from which the orientation is defined in the expression for the anisotropy.

The anisotropy for the dimer is given by Eq. (18) (since the diffusion constants along both axes of symmetry are equal), but the pre-factors are

$$A_d^1 = \frac{3}{20} \left[ \cos^4 \theta_o + 1 \right], \quad A_d^2 = \frac{3}{10} \cos^2 2 \theta_o, \quad A_d^3 = \frac{1}{20} \left[ (3 \sin^2 \theta_o - 1)^2 + 1 \right],$$

$$E_d^1 = 2D_1^d + 4D_3^d, \quad E_d^2 = 5D_1^d + D_3^d, \quad E_d^3 = 6D_1^d,$$

which clearly differ from the monomeric case. For example, if $\theta_o = 0^\circ$, the anisotropy for the monomer is given by a single term,

$$r^m(t) = \frac{2}{5} \exp(-6D_1^m t),$$

whereas that for the dimer is given by

$$r^d(t) = \frac{3}{10} \exp \left[ -(2D_1^d + 4D_3^d)t \right] + \frac{3}{20} \exp \left[ -(5D_1^d + D_3^d)t \right] + \frac{1}{10} \exp(-6D_1^d t).$$

Yet with $\theta_o = 90^\circ$,

$$r^m(t) = \frac{3}{10} \exp \left[ -(2D_1^m + 4D_3^m)t \right] + \frac{1}{10} \exp(-6D_1^m t),$$

and

$$r^d(t) = \frac{3}{20} \exp \left[ -(2D_1^d + 4D_3^d)t \right] + \frac{3}{20} \exp \left[ -(5D_1^d + D_3^d)t \right] + \frac{1}{4} \exp(-6D_1^d t).$$

Thus, the entire form of the expected decay of the anisotropy for the monomer differs from that of the dimer in a manner that depends on the probe orientation. Moreover, it should also be noted that if the monomers can dimerize in two different ways, two expressions, just for the dimers, would be required, which further emphasizes the importance of an explicit knowledge of the relative probe orientation in the interpretation of the anisotropy decay.

Yet, it should also be mentioned that there are cases in which the expression for the anisotropy may, in fact, be largely the same for oligomers of different stoichiometry, differing only in the diffusion constants (owing to their different sizes). For example, consider the dimerization of two cylindrically shaped membrane proteins, depicted in Fig. 1B, and again assume that the absorption and emission dipole moments are identically aligned and oriented by an angle, $\theta_o$, with respect to the central axis. Since these monomers are identical to those in the previous example, the probe orientation is also

$$(\phi^m, \theta^m) = (0, \theta_o),$$

and the anisotropy is given by Eqs (10) and (21), but with $D_1^m = 0$ and $D_3^m = D_2^m$, owing to the confinement of the molecules within the membrane.
Now consider the dimer. The principal axes of the dimer are related to those of the monomer by a translation of the central axis along the plane of the membrane, and so, the polar angles, \( \theta_0 \), of the two probes are identical. The orientations of the probes in this dimer are given by

\[
\begin{align*}
(\phi^d_1, \theta^d_1) &= (0^\circ, \theta_0), \\
(\phi^d_2, \theta^d_2) &= (180^\circ, \theta_0),
\end{align*}
\]

(29)

where, again, the coordinates of at least one of the probes differs from their value in the monomer. However, although such a dimer in solution would not be expected to have similar rotational diffusion constants about the \( x \)- and \( y \)-axes, it is clear that in the present case they may be both taken to be \( \approx 0 \) owing to the confinement of the molecules in the membrane. Hence, in terms of its rotational characteristics, this dimer is effectively symmetric, and so the anisotropy will not depend on the (different) azimuthal angles of the probes, but only on their (identical) polar angles, \( \theta_0 \). The anisotropy for this dimer is therefore also given by the expression for the symmetrical dimer, Eq. (18). Each pre-factor in the dimer, being the average of two identical values, is simply equal to their values in the monomer, \( A^m \), and so the expression of the dimer is

\[
v^d(t) = \sum_{\tau=1}^{3} A^m_{\tau} \exp(-E^d_{\tau} \cdot t),
\]

(30)

with \( D^d_1 = 0 \) and \( D^d_3 = D^d_z (\neq D^m_z) \). That is, in this case (and likewise for any other higher order oligomer of similar membrane proteins), the only difference between the expressions for the anisotropy for differently sized oligomers is in the magnitude of the diffusion constants, and not in the form of the expressions. In this regard, it should be mentioned that an earlier study of the oligomerization of membrane proteins assumed that the form of the expressions for the differently sized oligomers was the same, which enabled a determination of the changes in the relative abundancies of the different oligomers from a measure of the changes in the pre-factors [13]. The results presented in the present report provide a theoretical justification for this assumption.

4. Conclusion

An expression for the anisotropy of multiply labeled molecules is derived here which should find application in studies of the oligomerization of singly labeled molecules. Yet it should also be mentioned that if the state and degree of oligomerization in a particular system are in fact known, this same expression could be used to determine the orientations of the probes in each of the oligomeric complexes that have formed. Thus, in this way, an analysis of the decay of the anisotropy should also be able to provide a means to obtain an understanding of the relative orientation of the monomers within the oligomeric complexes, particularly for those complexes that are not membrane proteins, which has not been heretofore realized.

Acknowledgements

I thank my colleagues in the Shao Laboratory for helpful discussions. This work is supported by grants from US National Institutes of Health (EB002017 and AI39657).
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