Spectral Methods of Characterizing Protein Conformation and Conformational Changes

CD and Fluorescence Spectroscopy

Circular Dichroism

- One only gets a CD signal when two conditions are both fulfilled: you are looking
  - at an asymmetric molecule
  - At a \( \lambda \) at which the molecule absorbs light
- The primary use of CD in biochemistry is to monitor regular (and asymmetric) features in biopolymers
Nucleic Acids

• We can use CD to monitor the extent of stacking or helix formation
• One can determine what fraction of the bases are stacked, but not where in a molecule a stacked helix (helices) are located
• CD signal centered around 260 nm

Proteins

• random coils
• α-helices all have characteristic CD spectra
• β-sheets
• In principle, we can tell if β structure is present in a protein and how much β structure is present
• In practice: this is very difficult (virtually impossible) to do because local structure affects CD signal and CD bands overlap considerably
• The theory of CD is not well-developed, and one cannot take a CD spectrum and determine precisely the percentage of protein that is $\alpha$-helix

  – This CANNOT BE DONE !!

  – CD cannot be use to provide an absolute structure. But it is extremely good at determining changes in the structure.

Conformational changes

• The origin of changes in CD signal will be:
  – $\alpha$-helix $\Rightarrow$ less $\alpha$-helix (or vice versa)
  – $\alpha$-helix $\Rightarrow$ random coil “
  – $\beta$-sheet $\Rightarrow$ random coil “
  – stacked NA $\Rightarrow$ unstacked NA “

• But you will not be able (usually) to identify exactly where the change occurred, or what happened
  – CD tells “if”, not “what” or “where”
Circular Dichroism

• Far UV or amide region (170-250 nm)
  – dominated by contributions of peptide bonds
  – used to characterize the secondary structure and changes therein
• Near-UV region (250-300 nm)
  – originate from the aromatics amino acids
  – are observed when in a folded protein, aromatic side chains are immobilized in an asymmetric environment. Represents a highly sensitive criterion for the native state of a protein

\[ \Delta \varepsilon_L - \varepsilon_R \]

\[ [\theta]_\lambda = 3300 \Delta \varepsilon \]
Fluorescence Spectroscopy

Spectrophotometer vs Spectrofluorometer
Why aren’t all molecules that absorb light fluorescent?

- Because there are other ways for an excited electron to lose its energy and get back to ground state
  - Internal conversion
    - by collision with solvent
    - by dissipation through internal vibrational modes
  - Singlet-singlet energy transfer
    - by non-radiative ET to another electron through dipole-dipole coupling
  - Collisional quenching by solute Q
    - by collision with solute molecules that efficiently absorb the excited state energy
  - Intersystem crossing
    - by the electron moving from an excited state into an excited triplet state

The Phenomenon

\[
S_0 \xrightarrow{h\nu_A, 10^{-15} \text{s}} S_1 \xrightarrow{k_F, 10^{-2}-10^{-8} \text{s}} T_1 \xrightarrow{k_{ET}} A_1 \xrightarrow{h\nu_{A0, Acc}} A_0
\]
All these non-radiative (nr) pathways of depopulating the excited state compete with the fluorescence (F) mode

- rate of depopulation = \( k_F + \frac{k_{iC} + k_{ET} + k_{IS} + k_{q}[Q]}{k_{nr}} \)
  = \( k_F + k_{nr} \)

- Efficiency of fluorescence emission = quantum yield (\( \Phi \))
  \( \Phi = \frac{\text{photons emitted}}{\text{photons absorbed}} \)
  \( \Phi = \frac{k_F}{k_F + k_{nr}} \)

Fluorescence lifetime (\( \tau \))
\( \tau = \frac{1}{k_F + k_{nr}} \)

Fluorophores

- The intensity of the fluorescence emission depends upon both
  - the efficiency of absorption (\( \varepsilon \))
  - the efficiency of emission (\( \phi \))

- Natural Fluorophores
  - Intrinsic: Trp and Tyr in proteins

- Other Fluorophores
  - Extrinsic: high \( \varepsilon \) and \( \phi \) values
Relative contribution of the aromatic amino acids to the total fluorescence of a protein

<table>
<thead>
<tr>
<th>Amine</th>
<th>Contribution</th>
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</thead>
<tbody>
<tr>
<td>Trp</td>
<td>78</td>
</tr>
<tr>
<td>Tyr</td>
<td>21</td>
</tr>
<tr>
<td>Phe</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Environment sensitive fluorophores

In polar environment: solvent relaxation
Less energy = higher $\lambda_{em}$
Effect of Trp environment on the emission spectra of proteins

Environment-sensitive probes

Acrylodan
Badan
IANBD
mBBr
NBD is a good reporter group for the presence of water in its environment

Lifetimes of NBD

<table>
<thead>
<tr>
<th>Environment</th>
<th>τ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>~1 ns</td>
</tr>
<tr>
<td>Nonaqueous</td>
<td>~ 8 ns</td>
</tr>
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</table>
Lifetime vs Intensity measurements

<table>
<thead>
<tr>
<th>Intensity change</th>
<th>Lifetime</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% of NBD ↑ 2 times</td>
<td>2 - 1 component</td>
</tr>
<tr>
<td>50% of NBD ↑ 3 times</td>
<td>2 - 2 components - % intensity of each</td>
</tr>
</tbody>
</table>

Extrinsic fluorophores

- Fluorophores added to the sample to provide fluorescence when none exists or to change the spectral properties of the sample.
- Great variety of commercial available fluorophores
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Cy3</td>
<td>4</td>
<td>PBS</td>
<td>540</td>
</tr>
<tr>
<td>Cy5</td>
<td>27</td>
<td>PBS</td>
<td>620</td>
</tr>
<tr>
<td>Cresyl Violet</td>
<td>53</td>
<td>Methanol</td>
<td>580</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>95</td>
<td>0.1 M NaOH, 22°C</td>
<td>496</td>
</tr>
<tr>
<td>POPOP</td>
<td>97</td>
<td>Cyclohexane</td>
<td>300</td>
</tr>
<tr>
<td>Quinine sulfate</td>
<td>58</td>
<td>0.1 M H₂SO₄, 22°C</td>
<td>350</td>
</tr>
<tr>
<td>Rhodamine 101</td>
<td>100</td>
<td>Ethanol</td>
<td>450</td>
</tr>
<tr>
<td>Rhodamine 6G</td>
<td>95</td>
<td>Water</td>
<td>488</td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>31</td>
<td>Water</td>
<td>514</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>13</td>
<td>Water, 20°C</td>
<td>280</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>14</td>
<td>Water</td>
<td>275</td>
</tr>
</tbody>
</table>

Table 1. List of common standards for fluorescence quantum yield measurements.

Some cautions

- Internal conversion is temperature dependent
  - Intensity (Temp)
- Photobleaching
- A protein may contain more than one fluorophore
- Light scattering is frequently a problem
- Inner filter effect
Inner filter effect

- Absorbance of a sample at $\lambda_{ex}$ and $\lambda_{em}$ should be less than 0.03

Collisional quenching ($k_q[Q]$)

- $\phi \propto F$
  
  $\frac{F_0}{F} = \frac{\phi_0}{\phi} = \frac{(k_F + k_{ic} + k_{ET} + k_{IS} + k_q[Q])}{k_F + k_{IC} + k_{ET} + k_{IS}}$

- $\tau_0 = \text{lifetime in the absence of collisional quenching and ET}$
  
  $\tau_0 = \frac{1}{(k_F + k_{IC} + k_{IS})}$

- $F_0/F = (1/\tau_0 + k_q[Q])/(1/\tau_0) = 1 + \tau_0 k_q[Q]$
Collisional quenching measures solvent accessibility

\[ \text{slope} = K_{sv} \]
\[ K_{sv} = \tau_0 k_q \]

If \( \tau_0 \) is the same in both situations:
You can compare \( K_{sv} \),
if not you must compare \( k_q \)

Fluorescence anisotropy

- Photoselection

- \( r = \frac{l_{||} - l_{\perp}}{l_{||} + 2l_{\perp}} \)

\[ r = 0.40 \]
  - in a totally rigid system, if the emission transition dipole is \( \parallel \) to the absorption emission dipole

\[ r = -0.20 \]
  - in a totally rigid system, if the emission transition dipole is \( \perp \) to the absorption emission dipole

\[ 0.40 > r > -0.20 \]