Chemistry 431

Lecture 22

Fluorescence anisotropy
Amide vibrations and conformation
Time-resolved polarization
Circular dichroism
Fluorescence anisotropy
Fluorescence occurs after vibrational relaxation

1. Excitation
2. Vibrational relaxation
3. Fluorescence
4. Ground state vibrational relaxation
Fluorescence and Competing Processes

This is known as a Jablonski diagram. Different vibrational states with each electronic state are shown as parallel lines.
Fluorescence lifetime and quantum yield

The intrinsic lifetime for a single state is given by a single exponential with time constant $\tau_{\text{obs}}$:

$$N(t) = N(0)e^{-t/\tau_0}$$

The quantum yield is the ratio of the molecules that decay by the fluorescent pathway to the total:

$$\phi = \frac{k_f}{k_f + k_{\text{other}}} = \frac{\tau}{\tau_0}$$
Polarization anisotropy in emission

If a molecule is excited using polarized light the polarization of the emission will change in a time-dependent fashion as the molecule rotates.

The transition moment rotates with the molecule

NOTE: The molecule can rotate by any angle prior to emission.
Fluorescence anisotropy

The fluorescence anisotropy is defined as:

\[ r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \]

We consider the case of high viscosity where molecular rotation does not occur, \( r_0 \). The angle between the absorbing transition dipole and the emitting transition dipole is \( \gamma \).

\[ r_0 = \frac{1}{5} \left(3\cos^2 \gamma - 1\right) \]
The role of amide bands of the peptide as conformational markers
Background

• FTIR and Raman studies of amide band vibrations can be used to monitor the change in conformation of the peptide backbone.

• X-ray requires a protein that can be crystallized.

• NMR is limited to molecular weights less than 35 kD.

• Both require much more data analysis than FTIR spectra.
Description of the amide normal mode vibrations

• The peptide group, the structural repeat unit of the protein backbone gives up to nine characteristic bands.

• Amide A (3500 cm$^{-1}$) and B (3100 cm$^{-1}$) are N-H stretches.

• Amide I is predominantly C=O stretch.

• Amide II is N-H bending and C-N stretching.

• Amide III and IV are complex in-plane modes.

• Amide V-VII are out-of-plane vibrations.

IR spectrum of the amide vibrations
Correlations are observed between frequency and structure

Once the correlation of a set of frequencies of band shape with $\alpha$-helix, $\beta$-sheet, and random coil structures has been established, the complex line shape of a protein that contains these structures can be fit using Lorentzian and Gaussian line shapes.

Susi & Byler
Methods Enzymol 1986;130:290
Native Lysozyme

The structure is predominantly $\alpha$-helix in the core with some surface exposed $\beta$-sheet regions. The ribbon follows the peptide backbone of the protein.
FTIR of amide I of native lysozyme

The secondary structure prediction based on the amide I band is in agreement with the lysozyme structure.

Lysozyme

\( \beta \)-sheet  \( \alpha \)-helix  random  \( \beta \)-sheet
Denatured lysozyme

It would be very difficult to determine the crystal structure of a denatured protein. the FTIR technique allows one to predict the composition of various secondary structural components in various folding states.
Structure of a β-sheet
β-helix model

Experiment

Calculation
Transitions of Elastin-like Protein
Infrared spectroscopy as a marker for myoglobin dynamics

Time-resolved spectroscopy
Polarization anisotropy
Myoglobin Structure

Globular α-helical protein

Takano, JMB, 1977, 110, 569
Ligand recombination in myoglobin

\[ \text{His-FeP}^* \text{-CO} \]

\[ \text{His-FeP} \text{::CO} \]

\[ \text{His-FeP} + \text{CO} \]

\[ \text{His-FeP-CO} \]

The electronic absorption spectrum of Mb changes when CO is photolyzed.
The iron in heme is the binding site for oxygen and other diatomics.

Heme is iron protoporphyrin IX. Fe is found in Fe$^{2+}$ and Fe$^{3+}$ oxidation states. Diatomics bind to Fe$^{2+}$. Examples, CO, NO, O$_2$. O$_2$ is the physiologically relevant ligand, but it can oxidize iron and it is difficult to study directly.
The structure of the globin changes upon deligation of carbon monoxide

Deoxy Mb
MbCO

The peptide backbone shifts and the heme changes structure when CO is photolyzed. The structures shown are at equilibrium.

X-ray data
Kuriyan
Takano
CO recombination can be monitored by transient absorption spectroscopy.

The spectrum changes with time.

Excitation 532 nm
Probe 432 nm

Pumpl Probe

Pumped YAG Laser

Xe lamp

MbCO Sample

Silicon detector
Ligand recombination is a sum of single exponential processes

\[ S(t) = \Phi_{ge}(t)e^{-(k_{gem} + k_{esc})t} + \Phi_{bi}(t)e^{-k_{bi}t} \]

Difference spectrum from nanosecond transient absorption spectroscopy
Myoglobin structure changes upon Fe-CO photolysis can be monitored in the amide I band.
Polarization anisotropy in absorption

Photoselection using polarized light means that absorptive transitions of the molecule can be used to probe molecule motions.

The transition moments of all electronic and vibrational transitions rotate with the molecule.

Incident Electromagnetic Wave (PUMP)  Molecular Rotation  Incident Electromagnetic Wave (PROBE)

transition moment 1
transition moment 2
Polarization ratio

The polarization ratio is defined as:

\[ R = \frac{A_\perp}{A_\parallel} \]

For the carbonyl group of heme:

\[ R = \frac{4 - \langle \sin^2 \alpha \rangle}{2 + 2 \langle \sin^2 \alpha \rangle} \]

where \( \alpha \) is the angle of C=O with respect to the heme plane.
Time-resolved infrared difference spectra of photolyzed MbCO

500 ns transient obtained by step-scan in 75% glycerol/buffer solution

Amide I analysis of $\Delta A$ is needed!

Franzen and Dyer, Unpublished
The residues in the turn regions of Mb are most affected by the conformational changes.
It is the change in the projection of the amide C=O dipoles on the heme plane that results in change in anisotropy.

The major changes occur in the turns!
Ultrafast mid-infrared shows CO trapping inside the distal pocket of Mb

Two CO orientations observed in infrared bands $B_1$ and $B_2$

Spectral bands correspond to rotamers of CO trapped in Mb

Nature structural Biology
Lim, Jackson, Anfinrud, 1997, 4, 209
Hemoglobin: the role of the iron as the trigger for the R - T switch
The cooperative R - T switch

Hemoglobin is composed of two $\alpha$ and two $\beta$ subunits whose structures resemble myoglobin.

The frequency of the iron-histidine vibration shows strain in T state

The comparison of photolyzed HbCO in the R state and the equilibrium T state.
Hb*CO at 10 ns
Fe-His = 230 cm\(^{-1}\)
Deoxy Hb
Fe-His = 216 cm\(^{-1}\)
The lower frequency indicates weaker bonding interaction and coupling to bending modes.
The motion of the F-helix tugs on the proximal histidine and introduces strain.

The frequency lowering in the T state arises from weaker Fe-His ligation and from anharmonic coupling introduced by the bent conformation of the proximal histidine.
Time-resolved resonance Raman can follow the R - T structure change

Strain is introduced in stages as intersubunit contacts are made. Based on the x-ray data it was proposed that the iron displacement from the heme plane is a trigger for the conformational changes.

Scott and Friedman JACS 1984, 106, 5877
Ultrafast resonance Raman spectroscopy shows that heme doming occurs in <1 ps.

Equilibrium HbCO

Difference spectra obtained by subtraction of the red spectrum from spectra obtained at the time delays shown. The ultrafast iron doming indicates that the heme iron is the trigger.

Infrared and fluorescence spectroscopic studies of protein folding
Protein folding studied by infrared temperature jump transient absorption

Excitation 2 μ
Probe 1680 cm⁻¹

IR laser
Sample

Pulsed YAG Laser

MCT detector

2 μ excitation excites the bending overtone of H₂O.
Cyclic $\beta$-sheet forming peptides show very rapid (un)folding
Difference FTIR spectra of cyclic hexamer as a function of temperature

This difference spectrum shows the unfolding of the peptide.

Probe at 1620 cm$^{-1}$
Formation of hydrogen bonds in cyclic β-sheet hexamer is rapid.

The kinetics of unfolding of the hexamer is almost identical in 50% glycerol as in buffer.

Probe at $\gtrsim 1620 \text{ cm}^{-1}$.
The time scale for $\alpha$-helix folding can be followed by a T-jump fluorescence experiment.

$\beta$-sheet folding observed by T-jump Tryptophan fluorescence

Dansyl fluorescence

GB1 $\beta$-sheet folding occurs in 3.5 $\mu$s

Tryptophan fluorescence is higher in the hydrophobic folded state and decreases when exposed to water.

Protein folding studies reveal the time scale for formation of secondary structure

Folding of $\alpha$-helix $\approx 200$ ns
Folding of $\beta$-sheet $\approx 1$-$5$ ms
Folding of cyclic $\beta$-sheet $\approx 50$ ns

Folding consists of a nucleation event followed by chain propagation. The slow step in $\beta$-sheet formation is nucleation.
Vibrational spectroscopy of nucleic acids and DNA
The components of DNA

- The individual nucleobases are:
  - Adenine
  - Guanine
  - Cytosine
  - Thymine

- The nucleoside is formed by a bond with the ribose sugar at the N9 position of A and G and the N6 position of C and T.
The stability of double-helical DNA

The double helical form of DNA is formed by phosphodiester linkages between the 5’ -OH end of one ribose and the 3’-OH of the next. The double helix is stabilized by hydrogen bonding in canonical base pairs and by stacking interactions between the bases.
Infrared spectra of cytosine indicate the correct tautomeric form

Even though the Watson-Crick double helical structure was known in 1953, the hydrogen bond pattern needed to be determined. Infrared spectroscopy played a key role as shown here.

Miles (1961)
DNA conformations

- DNA is a biopolymer with three distinct conformations these are known as B, A, and Z.
- The B form of DNA is most widely observed in fibers at low humidity and solution studies at low salt concentration.
- As the fiber humidity or solution salt concentration is increased the A form and then the Z form are observed.
DNA conformations

A form  B form  Z form
Conformations of the ribose

- B is 2’-endo anti
- A is 3’-endo anti
- Z is 2’-endo syn
The first X-ray crystal structure of d(GCGCGGC) was in the Z-form

• Watson-Crick DNA was determined based on fiber diffraction. This was B form.

• The expectation that crystalline DNA would also be in the B-form was shattered by the structure of the hexamer d(GCGCGGC)

• Raman and infrared spectroscopy have played a key role in comparisons of the various forms of DNA.
Raman spectroscopic markers of A, B, and Z form DNA

Raman Shift (cm$^{-1}$)

protonated
deuterated
Raman difference spectra of poly(dGC) at low and high salt
Infrared spectra of DNA

Poly(dG•dC) fibers

Poly(dA•dT) fibers
Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum, which can contain both positive and negative signals.
Circular dichroism

At a given wavelength,
\[ \Delta A = (A_L - A_R) \]
where \( \Delta A \) is the difference between absorbance of left circularly polarized (LCP) and right circularly polarized (RCP) light. It can also be expressed as:
\[ \Delta A = (\varepsilon_L - \varepsilon_R) C \ell \]
where \( \varepsilon_L \) and \( \varepsilon_R \) are the molar extinction coefficients for RCP and LCP light, where \( C \) is the molar concentration and \( \ell \) is the path length. Then
\[ \Delta \varepsilon = (\varepsilon_L - \varepsilon_R) \]
is the molar circular dichroism.

Although \( \Delta A \) is usually measured, for historical reasons most measurements are reported in degrees of ellipticity. The molar ellipticity is:
\[ [\theta] = 3298 \Delta \varepsilon. \]
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Circularly Polarized Light
Circularly polarised light can be described in terms of electric (e) and magnetic (m) wave components. Linearly and circularly polarized light are contrasted below.
Application to biological molecules
In general, this phenomenon will be exhibited in absorption bands of any optically active molecule. As a consequence, circular dichroism is exhibited by biological molecules, because of the dextrorotary (e.g. some sugars) and levorotary (e.g. some amino acids) molecules they contain. Noteworthy as well is that secondary structure will also impart a distinct CD to their respective molecules. Therefore, the alpha helix of proteins and the double helix of DNA have CD spectral signatures representative of their structures.
The ultraviolet CD spectrum of proteins can predict important characteristics of their secondary structure. CD spectra can be readily used to estimate the fraction of a molecule that is in the alpha-helix conformation, the beta-sheet conformation, the beta-turn conformation, or random conformation. These fractional assignments place important constraints on the possible secondary conformations that the protein can be in.
Circular Dichroism Units

There are several different units of measurement for circular dichroism. Molar ellipticity, mean residue ellipticity and delta epsilons are all mentioned in the literature. Ellipticity is defined as the tangent of the ratio of the minor to major elliptical axes. More modern CD instruments measure the difference in absorption of right and left circularly polarized light as a function of wavelength. In accordance with the Beer–Lambert law, wavelength is equal to the difference in molar extinction coefficients divided by the product of path length and concentration. Mean residue ellipticity is the most common unit (degree cm² dmol⁻¹) and delta epsilons are the new machine unit, often referred to as molar circular dichroism (liter mol⁻¹ cm⁻¹), not to be confused with molar ellipticity (degrees decilitres mol⁻¹ decimeter⁻¹).
Application to Protein Secondary Structure

Precisely as done for infrared spectroscopy, we can correlate certain UV-CD lineshapes with protein structure. This permits one to fit the CD spectrum of a protein of unknown structure to a library of known structures.

Hence, UV-CD is a tool for the determination of secondary structure in proteins.