BIOCH 590: Biomacromolecules Spring 2010

Optical Spectroscopy

Quantum Mechanics;
UV/IR Absorption, CD, Fluorescence

Key Reference: van Holde Chapter 8 (theory heavy; weak in application)
Tinoco Chapter 10 (more application oriented)

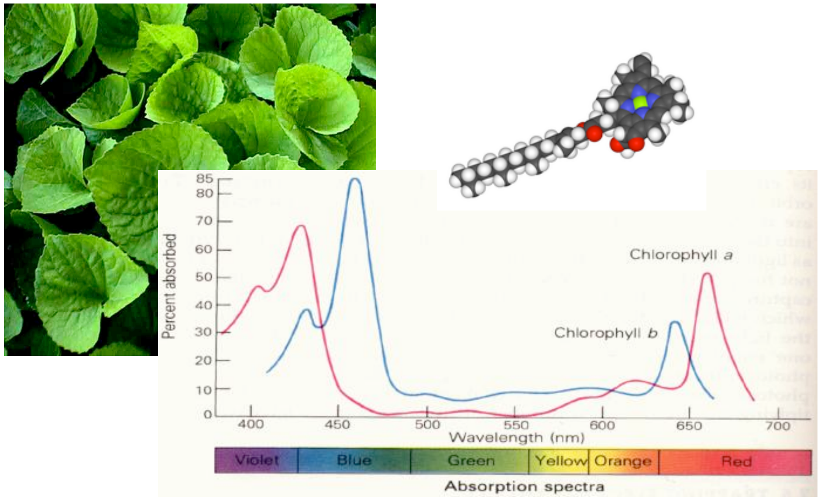
Overview

- Overview of spectroscopy
  - Types
  - Light
- Brief introduction to quantum mechanics
  - Postulates of quantum mechanics
  - Energy states and transitions
- Absorption spectroscopy
  - UV
  - IR
  - CD
- Emission spectroscopy
  - Fluorescence and FRET: molecular ruler

Spectroscopy

- Study of the interaction of electromagnetic radiation with matter: "observation of quantum mechanics"
  - Light absorbed or emitted by a molecular undergoing a transition between discrete energy levels.
  - Energy levels predicted by quantum mechanics: key to interpretation
- Types of spectroscopy
  - Absorption: transition to a high-energy level
    - UV and visible (UV): electronic states
    - Infrared (IR): vibration states
    - Circular dichroism (CD): differential absorption of polarized lights
  - Emission: transition to a low-energy level
    - Fluorescence: re-emission with ns
    - Phosphorescence: > ms (and up to weeks)
- Information derived: identification, quantification, monitoring

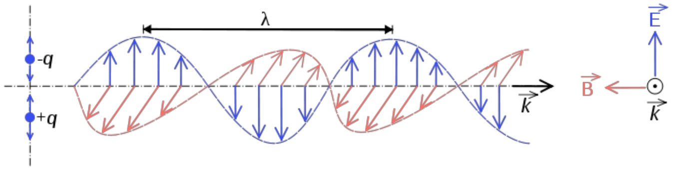
Color and Absorption Spectrum



Light

- Classically: electromagnetic radiation

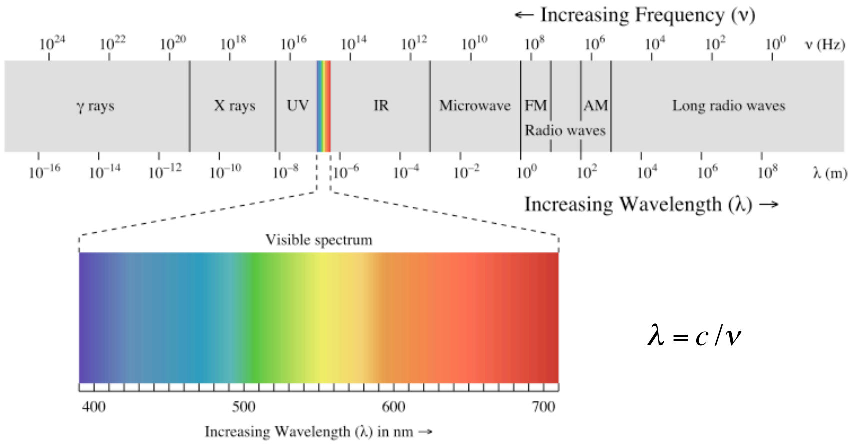
$$E = E_0 \cos(2\pi\nu t - x_0)$$
$$B = B_0 \cos(2\pi\nu t - x_0)$$



- Quantum mechanically: photon particles

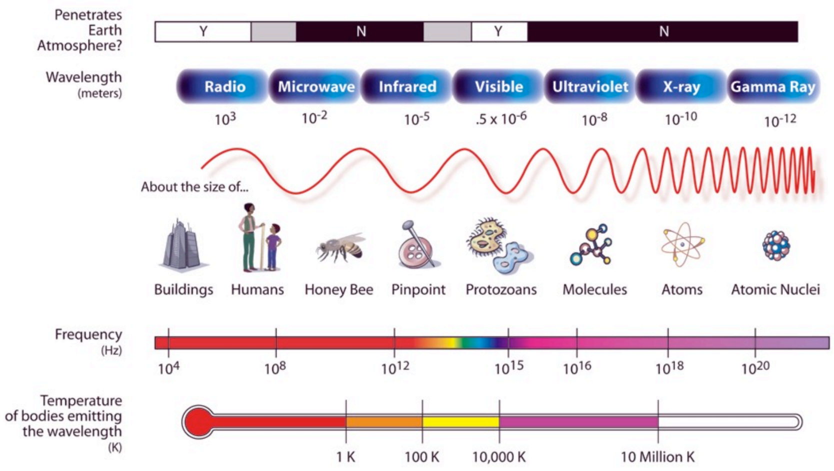
$$E = h\nu = hc/\lambda$$

Electromagnetic Spectrum



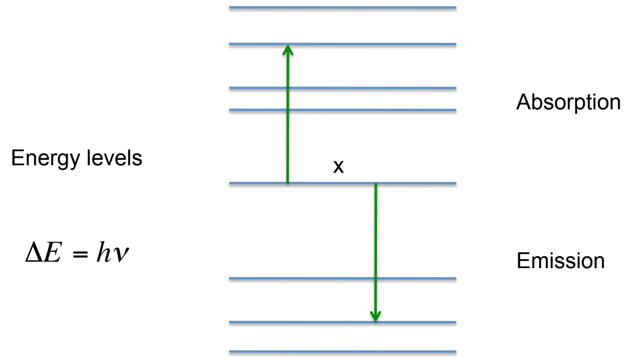
$$\lambda = c/\nu$$

THE ELECTROMAGNETIC SPECTRUM



Transitions of Energy Levels

- Light absorbed or emitted only for photons with an energy that matches the energy difference between levels
  - Frequency, intensity, direction

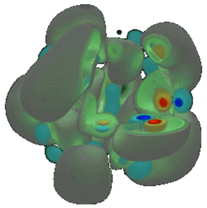


$$\Delta E = h\nu$$

Postulates of Quantum Mechanics

- Six postulates serve as the foundation of QM
- Postulate I: state of system described by wavefunction

$$\Psi(q_1,\ldots,q_{3n},t)$$



http://www.psc.edu/Packages/VFleet_Home/

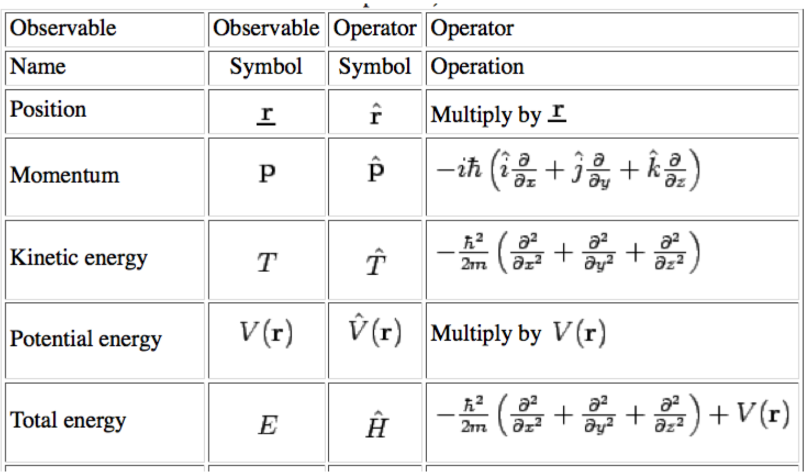
- Postulate II: probability of finding the system is given by

$$\Psi^* \, \Psi \, d\tau$$

$$\int_{-\infty}^{\infty} \Psi^*(\mathbf{r},t)\Psi(\mathbf{r},t)d\tau = 1$$

Postulates of Quantum Mechanics

- Postulate III & V: physical observables as Hermitian operators



Observable Name	Observable Symbol	Operator Symbol	Operator Operation
Position	$\underline{\mathbf{r}}$	$\hat{\mathbf{r}}$	Multiply by $\underline{\mathbf{r}}$
Momentum	$\mathbf{p}$	$\hat{\mathbf{p}}$	$-i\hbar\left(\hat{i}\frac{\partial}{\partial x}+\hat{j}\frac{\partial}{\partial y}+\hat{k}\frac{\partial}{\partial z}\right)$
Kinetic energy	T	$\hat{T}$	$-\frac{\hbar^2}{2m}\left(\frac{\partial^2}{\partial x^2}+\frac{\partial^2}{\partial y^2}+\frac{\partial^2}{\partial z^2}\right)$
Potential energy	$V(\mathbf{r})$	$\hat{V}(\mathbf{r})$	Multiply by $V(\mathbf{r})$
Total energy	E	$\hat{H}$	$-\frac{\hbar^2}{2m}\left(\frac{\partial^2}{\partial x^2}+\frac{\partial^2}{\partial y^2}+\frac{\partial^2}{\partial z^2}\right)+V(\mathbf{r})$

Postulates of Quantum Mechanics

- Postulate IV: average value of observable Â given by:

$$< A >= \int_{-\infty}^{\infty} \Psi^* \hat{A} \Psi d\tau$$

  - Only eigenvalues (a_i) will be observed:

$$\hat{A}\Psi_i = a_i\Psi_i \qquad \int \Psi_i^*\Psi_{j\neq i} = 0$$

$$\Psi = \sum c_i\Psi_i$$

$$\langle A\rangle = \sum c_i^2 a_i$$

a_i: always real for Hermitians

Postulates of Quantum Mechanics

- Postulate VI: (time-dependent) Schrödinger Equation

$$\hat{H}\Psi(\mathbf{r},t) = i\hbar\frac{\partial\Psi}{\partial t}$$

  - Ĥ: Hamiltonian operator <-> Energy (E = T+V)

$$\hat{T} = -\frac{\hbar^2}{2m}\left(\frac{\partial^2}{\partial x^2}+\frac{\partial^2}{\partial y^2}+\frac{\partial^2}{\partial z^2}\right) = -\frac{\hbar^2}{2m}\nabla^2$$

$$\mathbf{H} = -\frac{\hbar^2}{2m}\nabla^2 + V(\mathbf{r},t)$$

Solving Schrödinger Equation

- Central problem of QM
  - Generally not (analytically) solvable except for simplest systems (see examples that follow)
- Time-independent Schrödinger Equation
  - Stationary state: states not change with time are quantized at certain energy values!
  - If V (and thus H) is independent of time:

$$\Psi(r,t) = \Psi(r)e^{-iEt/\hbar}$$

$$\hat{H}\Psi(r) = E\Psi(r)$$

  - Stationary state: what is stationary?
  - Origin of quantized energy levels: confinement (of wavefunction)

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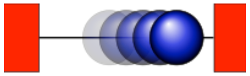
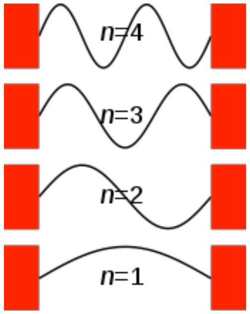
1. Particle in a Box



$V = 0 \quad 0 < x \leq L$

$= \infty \quad otherwise$



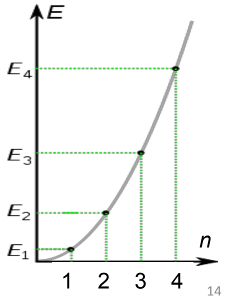
$$\mathbf{H} = -\frac{\hbar^2}{2m}\nabla^2 + V(\mathbf{r},t)$$

$$-\frac{\hbar^2}{2m}\frac{d^2}{dx^2}\Psi(x) = E\Psi(x)$$

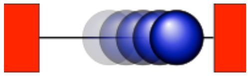
$$-\frac{\hbar^2}{2m}\frac{d^2}{dx^2}\Psi(x) + \infty\Psi(x) = E\Psi(x)$$

$$\psi_n = \sqrt{\frac{2}{L}}\sin\left(\frac{n\pi x}{L}\right)$$

$$E_n = \frac{n^2h^2}{8mL^2}$$



Particle in a Box



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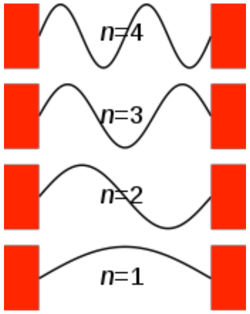

$$\psi_n(x,t) = \begin{cases} A\sin(k_n x)e^{-i\omega_n t}, & 0 < x < L, \\ 0, & \text{otherwise}, \end{cases}$$

$$k_n = \frac{n\pi}{L}, \quad \text{where} \quad n = \{1, 2, 3, 4, \ldots\},$$

$$P_n(x) = \begin{cases} \frac{2}{L}\sin^2\left(\frac{n\pi x}{L}\right); & 0 < x < L \\ 0; & \text{otherwise.} \end{cases}$$

$$\langle x \rangle = \int_{-\infty}^{\infty} \psi^*(x)x\psi(x)\,\mathrm{d}x.$$ = L/2 for all levels!

<p> = 0 (home work)

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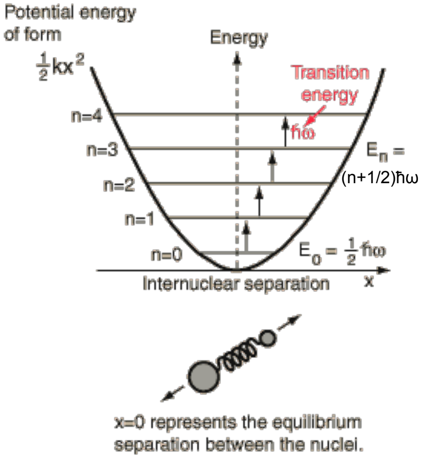
2. Harmonic Oscillator



x=0 represents the equilibrium separation between the nuclei.

$$V(x) = \frac{1}{2}m\omega^2x^2,$$

$$\hat{H} = \frac{\hat{p}^2}{2m} + \frac{1}{2}m\omega^2\hat{x}^2,$$

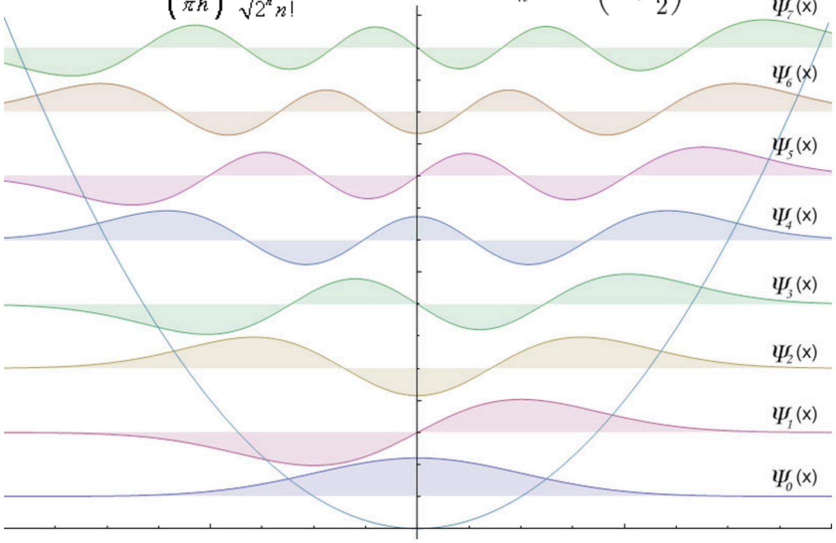
$$\psi_n(x) = \left(\frac{m\omega}{\pi\hbar}\right)^{\frac{1}{4}}\frac{1}{\sqrt{2^n n!}}H_n(\xi)e^{-\xi^2/2}$$

$$\xi = \sqrt{m\omega/\hbar}\,x$$

$$H_n(x) = (-1)^n e^{x^2}\frac{d^n}{dx^n}\left(e^{-x^2}\right)$$

$$E_n = \hbar\omega\left(n + \frac{1}{2}\right)$$

$$\psi_n(x) = \left(\frac{m\omega}{\pi\hbar}\right)^{\frac{1}{4}} \frac{1}{\sqrt{2^n n!}} H_n(\xi) e^{-\xi^2/2} \qquad E_n = \hbar\omega\left(n + \frac{1}{2}\right)$$



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3. Hydrogen-like Atom

- Atoms with one electron (H, He$^+$…)
  - ε_0: vacuum permittivity
  - Z: atom number
  - e: electron charge
  - r: electron-nucleus. Distance
  - See van Holde Table 8.2 for atomic unit (au)

$$V(r) = -\frac{1}{4\pi\varepsilon_0}\frac{Ze^2}{r} = \frac{Z}{r}$$

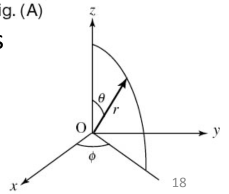
- Nucleus much heavier: only electronic Hamiltonian considered

$$\hat{H} = -\frac{\hbar^2}{2(m_e+m_n)}\nabla_R^2 - \frac{\hbar^2}{2\mu}\nabla_r^2 + V(r) \approx -\frac{\hbar^2}{2m_e}\nabla_r^2 + V(r)$$

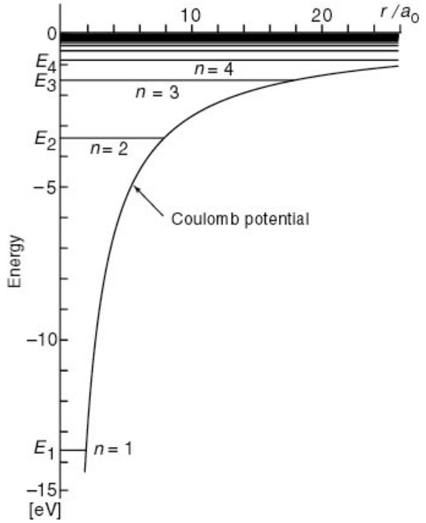
- Separation of radial and angular wavefunctions

$$\psi(\bar{r}) = R_l(r)Y_{lm}(\theta,\phi)$$

Fig. (A)



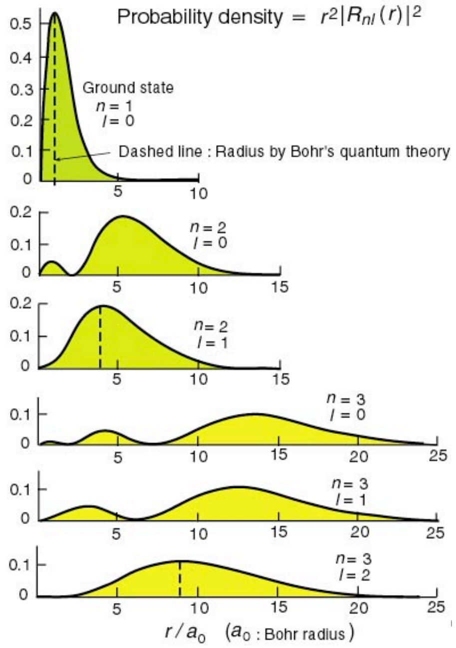
Hydrogen-like Atom



$$E = -\frac{Z^2e^2}{2an^2} = \frac{Z^2}{2n^2} \quad \text{(in a.u.)}$$

$$\psi_{nlm} = R_{nl}(r)\,Y_{lm}(\theta,\phi)$$

$$n = 1, 2, 3, 4, \ldots$$
$$l = 0, 1, 2, \ldots, n-1$$
$$m = -l, -l+1, \ldots, 0, \ldots, l-1, l$$

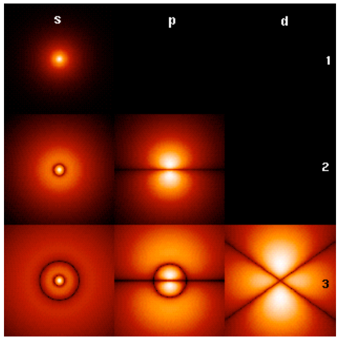


1s $\frac{1}{\sqrt{\pi}}\left(\frac{1}{a_0}\right)^{3/2} e^{-r/a_0}$

2s $\frac{1}{4\sqrt{2\pi}}\left(\frac{1}{a_0}\right)^{3/2}\left(2 - \frac{r}{a_0}\right) e^{-r/2a_0}$

2p$_z$ $\frac{1}{4\sqrt{2\pi}}\left(\frac{1}{a_0}\right)^{3/2} \frac{r}{a_0} e^{-r/2a_0}\cos\theta$

2p$_x$
2p$_y$ $\frac{1}{8}\sqrt{\frac{1}{\pi}}\left(\frac{1}{a_0}\right)^{3/2} \frac{r}{a_0} e^{-r/2a_0}\sin\theta e^{\pm i\phi}$



Tricks for Approaching "Complex" Systems

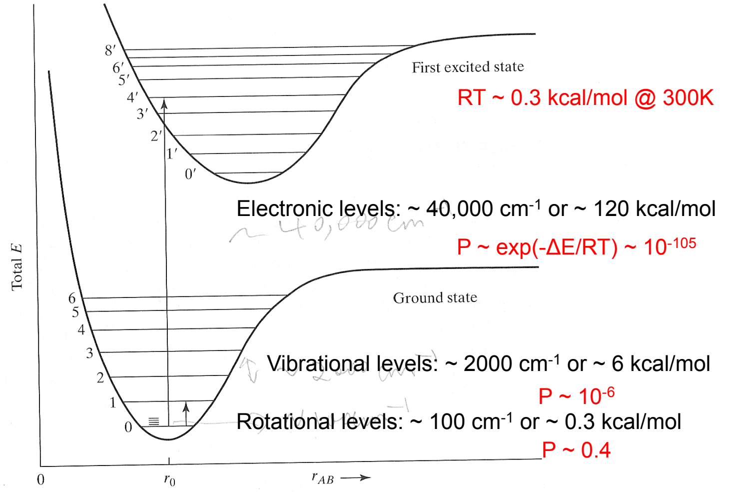
- Approximate solutions for chemical systems
- Separability of Hamiltonians
- Perturbation theory
- Variation method

- Linear combination of atomic orbitals (LCAO)

- Born-Oppenheimer approximation: separation of nuclear vibration and electronic states

- Density Fuctional Theory (DFT)

Electronic, vibrational and rotational states



RT ~ 0.3 kcal/mol @ 300K

Electronic levels: ~ 40,000 cm^{-1} or ~ 120 kcal/mol

P ~ exp(-ΔE/RT) ~ 10^{-105}

Vibrational levels: ~ 2000 cm^{-1} or ~ 6 kcal/mol

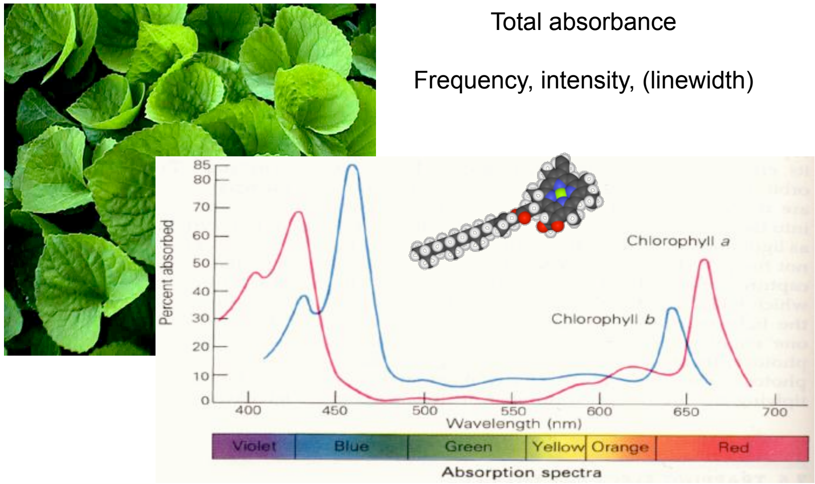
P ~ 10^{-6}

Rotational levels: ~ 100 cm^{-1} or ~ 0.3 kcal/mol

P ~ 0.4

Figure 8.11 The total energy for the electronic ground state and electronic first-excited state of a hydrogen molecule that result from the approximate quantum mechanical treatment described in the text. Some vibrational energy levels and a few representative rotational energy levels are also shown.

Features of Absorption Spectra



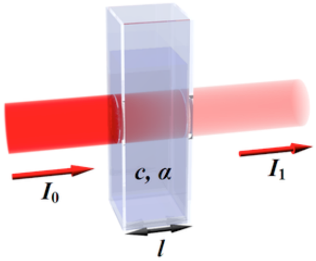
Total absorbance

Frequency, intensity, (linewidth)

Transition Intensities

- Beer-Lambert Law

$$T = \frac{I}{I_0} = 10^{-\alpha\ell} = 10^{-\varepsilon\ell c}$$



  - T: transmission
  - α: absorption coefficient
  - ℓ: absorption length
  - ε: molar absorptivity (or extinction coefficient): $\varepsilon = \varepsilon(\nu)$
  - c: concentration

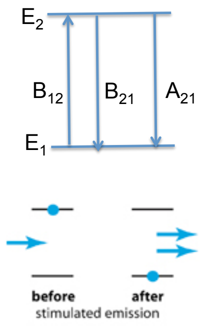
$$T = \frac{I}{I_0} = e^{-\alpha' l} = e^{-\sigma\ell N}$$

  - σ: absorption cross section
- A powerful way for quantification: $c = -\frac{1}{\xi l}\log T$

Induced vs Spontaneous Emission

Consider a system at equilibrium: (black-body radiation) induced transition and spontaneous transition

$h\upsilon = E_2 - E_1$



$$\frac{dN_{12}}{dt} = B_{12}N_1\rho(\nu)$$

$$\frac{dN_{21}}{dt} = B_{21}N_2\rho(\nu) + A_{21}N_2$$

$$\rho(\nu) = \frac{A_{21}N_2}{B_{12}N_1 - B_{21}N_2} = \frac{8\pi h\nu^3}{c^3(e^{h\nu/k_BT} - 1)}$$

$$N_2/N_1 = e^{-h\nu/k_BT}$$

$$\boxed{A(\nu) = \frac{8\pi h\nu^3}{c^3}B(\nu)}$$

- Electronic and vibrational spectroscopy: *B(υ)*
- Fluorescence: *A(υ)*

Quantum Mechanical Intensity

- Perturbation theory allows one to calculate the transition probability between any two states:
  - Transition dipole: for the transition from *m* to *n* state

$$\vec{u}_{fi} = \int \psi_f^* \hat{u}\, \psi_i = \langle \psi_f^* \hat{u}\, \psi_i \rangle$$

  - QM intensity:

$$C_f(t) = \frac{1}{2}\vec{E}_0 \bullet \vec{u}_{fj}\left[\frac{e^{i(E_f - E_i + h\nu)t/\hbar} - 1}{E_f - E_i + h\nu} + \frac{e^{i(E_f - E_i - h\nu)t/\hbar} - 1}{E_f - E_i - h\nu}\right]$$

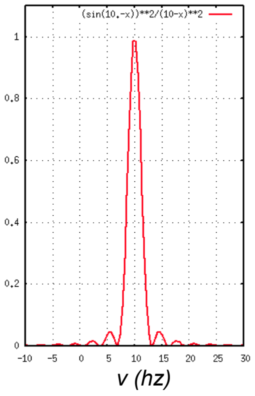
  - Absorption: peak @ h*ν* ~ ΔE

$$C_f^*(t)C_f(t) = E_0^2 D_{fi}\cos^2\theta\left[\frac{\sin^2\frac{1}{2}(E_f - E_i - h\nu)t/\hbar}{(E_f - E_i - h\nu)^2}\right]$$

  - Transition dipole strength:

$$D \sim \int_{band} \xi(\nu)/\nu\, d\nu$$

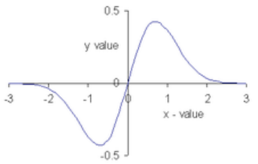
(E_f - E_i)/h = 10 Hz



Transition Dipole and Selection Rules

- The basis of spectroscopic selection rules: transition only probable for transitions with non-vanishing transition dipoles

$$\vec{u}_{fi} = \int \psi_f^* \hat{u}\, \psi_i = \langle \psi_f^* \hat{u}\, \psi_i \rangle$$



- What the hell is the dipole operator?

Transition type	**μ transforms as**	**note**
Electric dipole	x, y, z (or simply r)	Optical spectra
Electric quadrupole	x^2, y^2, z^2, xy, xz, yz	Constraint x2 + y2 + z2 = 0
Electric polarizability	x^2, y^2, z^2, xy, xz, yz	Raman spectra
Magnetic dipole	R_x, R_y, R_z	Optical spectra (weak)

- In practice: sufficient to consider only the symmetry (even/odd parity) to identify "forbidden" transitions
  - ΔS=0: singlet to triplet forbidden (Spin Rule)
  - Δl = ±1: requires polarity change in centrosymmetric systems; s-s, p-p etc not allowed; (Orbital Rule)
  - Δ*n* = ±1 for vibrations, or Δ*J* = ±1 for rotations

Additional reading: http://en.wikipedia.org/wiki/Selection_rule

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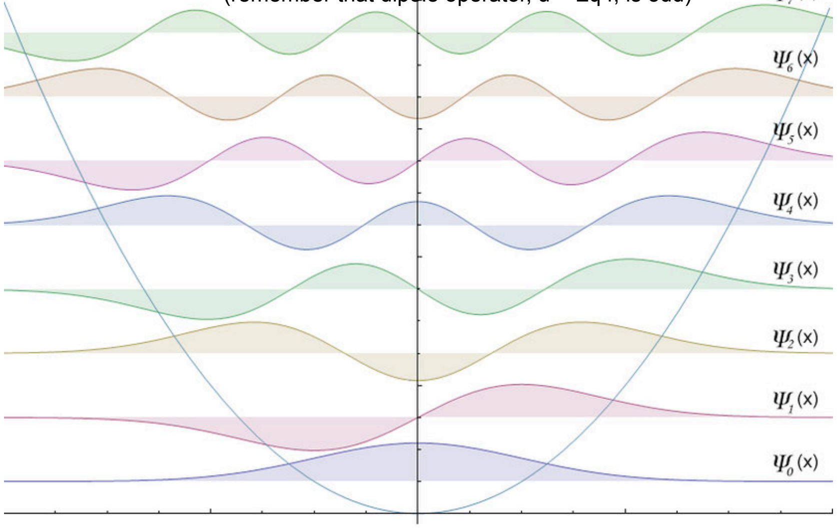
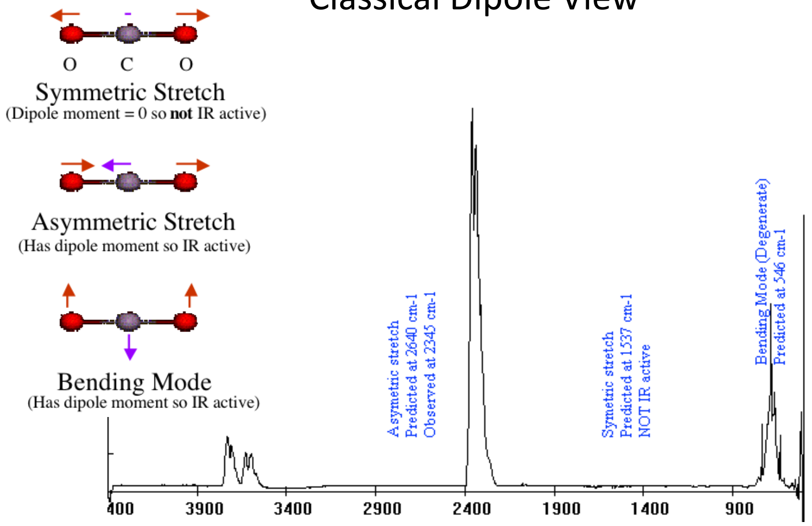
$\vec{u}_{fi} = \int \psi_f^* \hat{u}\, \psi_i = \langle \psi_f^* \hat{u}\, \psi_i \rangle$ Δ*υ* = ±1 for vibrations: Can you see why?

(remember that dipole operator, u = Σq r, is odd)



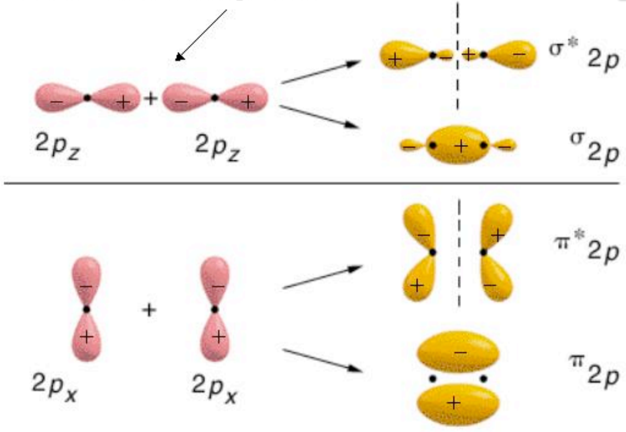
Classical Dipole View



Molecular Orbitals

Are these transitions allowed?

2*p* atomic orbitals give rise to both σ and π type molecular orbitals. The strongest overlap occurs between the $2p_z$ orbitals.



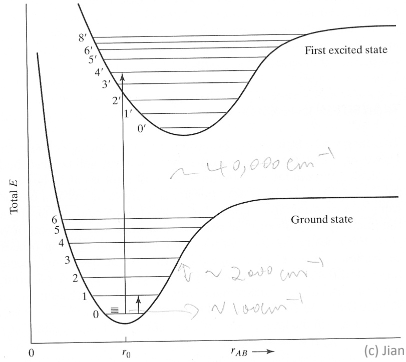
Expected intensities of electronic transitions

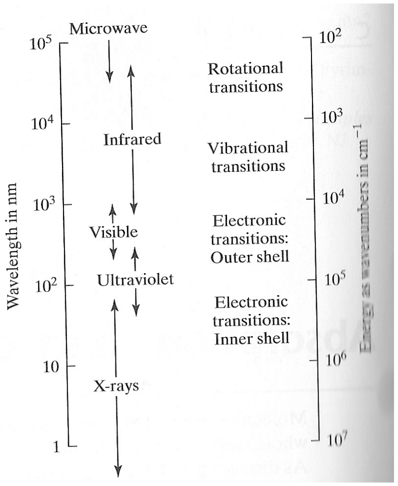
Transition type	**Example**	**Typical value of ε**
Spin forbidden, Laporte forbidden	$[Mn(H2O)_6]^{2+}$	0.1 m^2 mol^{-1}
Spin allowed (octahedral complex) Laporte forbidden	$[Ti(H2O)_6]^{3+}$	1 m^2 mol^{-1}
Spin allowed (tetrahedral complex), Laporte partially allowed by d-p mixing	$[CoCl_4]2-$	50 m^2 mol^{-1}
Spin allowed, Laporte allowed e.g. charge transfer bands	$[TiCl_6]^{2-}$ or MnO^{4-}	1000 m^2 mol^{-1}

Forbidden is only true to the first order: in reality, it only means lower probability!
(The molecular/atomic orbitals are only approximate after all: couplings)

Absorption Spectroscopy

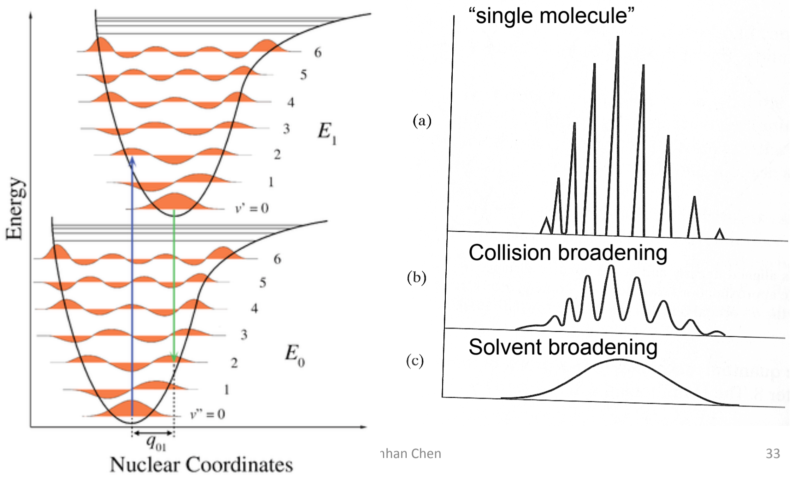
- Types of absorption: relation to energy levels
- Key information from spectra:
  - Band: energy levels
  - Intensity: transition dipoles
  - Line width: life times
  - Absorbance: concentration





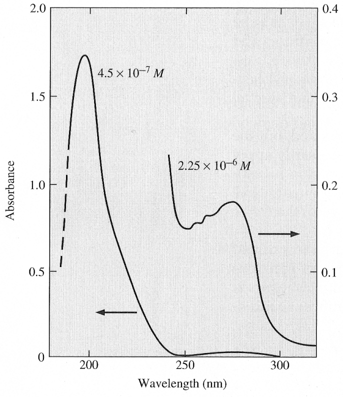
Frank-Condon Principle

- Electronic transitions that conserve the nuclear displacement have the highest probability: various broadening effects lead to absorption bands



UV Absorption Spectra of Proteins

- Dominated by absorption from backbone amides: ~195 nm and 220 nm
- A few sidechains also visible (aromatics, Cys, Met, S-S): ~280 nm



▲ FIGURE 10.10

UV-absorption spectrum of bovine serum albumin. Solution in 10^{-3} *M* phosphate buffer pH 7.0, 1.0-cm path. The wave-

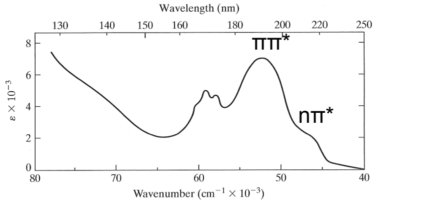
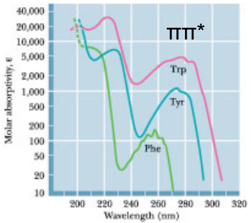


Figure 9.12 The electronic absorption spectrum of N,N-dimethylacetamide, which consists primarily of the electronic transitions for the amide chromophore. [Redrawn from data in Basch et al. (1968), *J. Chem Phys.*



See van Holde pg 430-433 for a detailed analysis of amide chromophore.

Secondary Structure Dependence

Interactions among ππ* transitions of oriented amides and hypochromism: low-energy transitions lose intensity compared to isolated monomer.

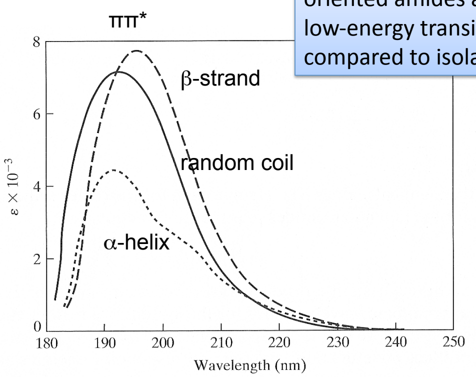
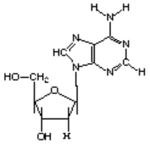
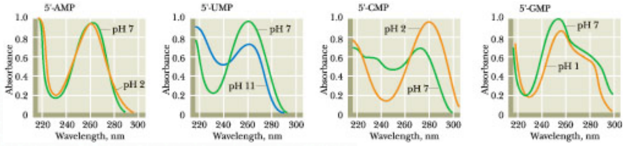
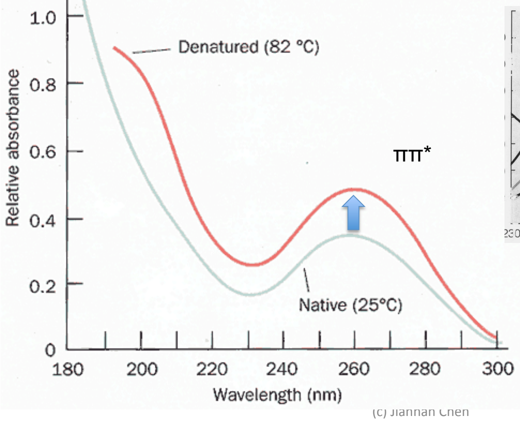


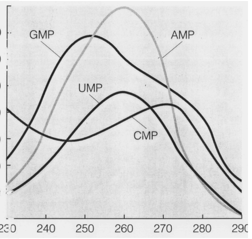
Figure 9.16 The electronic absorption spectra for poly-L-lysine hydrochloride in aqueous solution as a random coil at pH 6.0, 25°C (—); *α* helix at pH 10.8, 25°C (---); *β* strand at pH 10.8, 52°C (–·–). [Adapted from K. Rosenheck and P. Doty (1961), *Proc. Natl. Acad. Sci. USA* **47**, 1775–1785.]

UV Spectra of Nucleic Acids





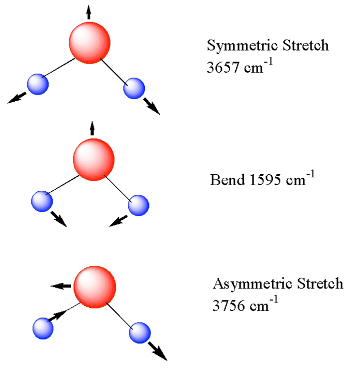


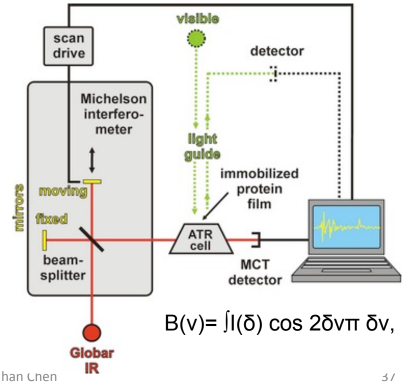


Hypochromism: degree of orientation of bases (or degree of melting)

Vibrational Absorption: FT-IR

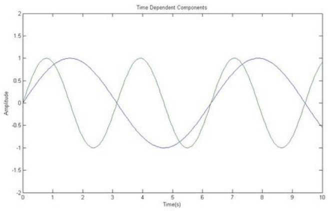
- Normal modes of nuclei vibration: n=0 -> n=1
  - Number of vibrational degrees of freedom: 3N – 6 (or 3N – 5 for linear molecules)
- Modern IR spectra acquired with an interferometer: Fourier transform (FT) used to obtain intensity as a function of frequency in less a second!

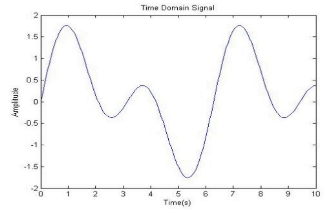




B(v)= ∫I(δ) cos 2δvπ δv,

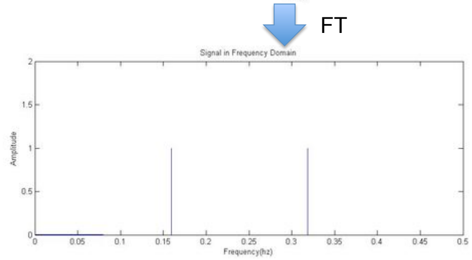
Fourier Transform (FT)



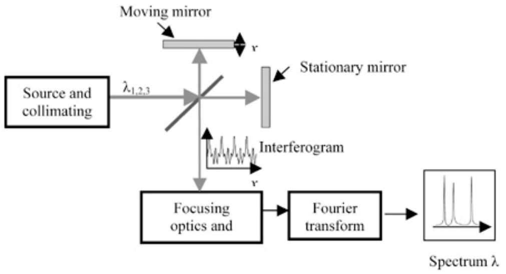


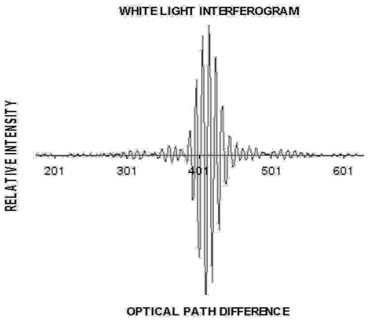
$$F(v) = \int_{-\infty}^{\infty} f(t) e^{-2i\pi vt} dt$$

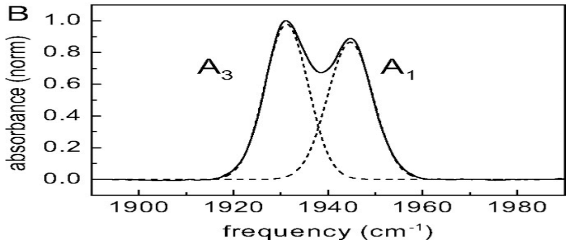
$$f(t) = \int_{-\infty}^{\infty} F(v) e^{-2i\pi vt} dv$$



Interferogram vs. Spectrum

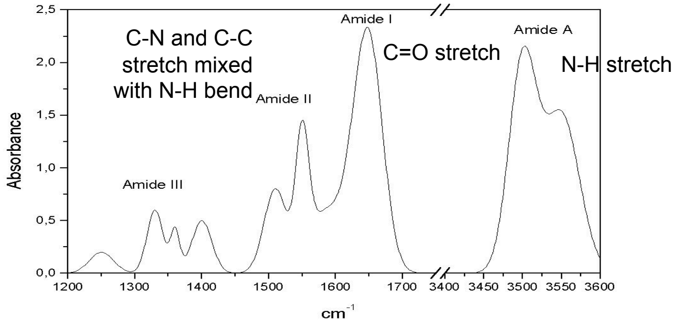






IR of Protein Backbone

- Hydrogen bonding yields characteristic shifts in vibrational bands
- Amide I vibrational band (~1650 cm^{-1}) most often followed

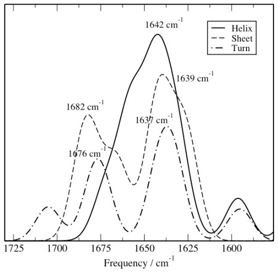


IR Amide I Frequencies

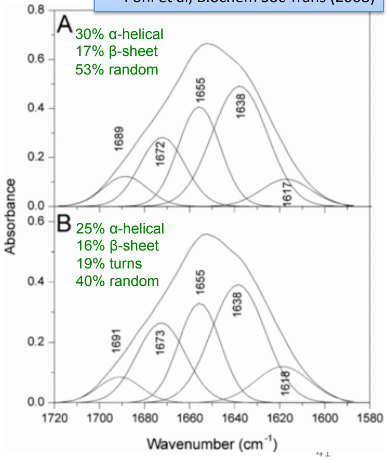
Structure	Frequency (cm^{-1})
α-helix	1648-1655
Random coil	1655-1675
β-sheet	1620-1635,1675-1690

Protein Secondary Structure from IR

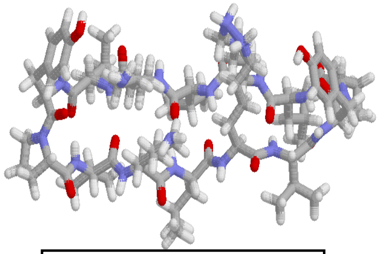
- Needs to establish a "basis set" of IR spectra of Amide I band
- Fourier self-decovlution coupled with iterative curve fitting



IR absorbance spectra of the amide I band of the soluble NADH dehydrogenase before (A) and after reduction with NADH (B)
Pohl et al, Biochem Soc Trans (2008)

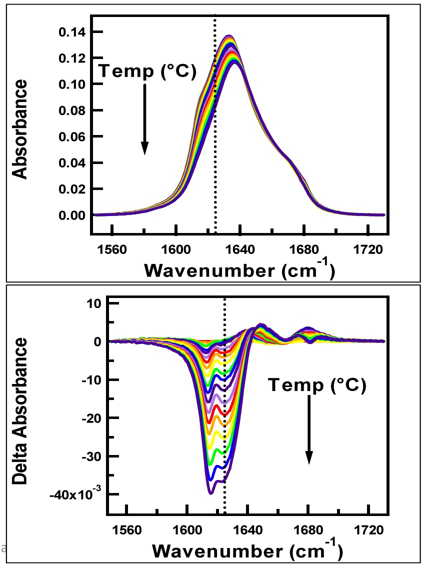


FT-IR Monitoring of Protein Folding

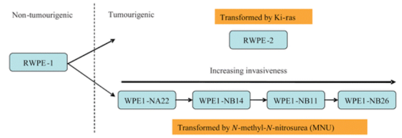


VKLKVYPLKVKLYP

Original slide from: chsfpc5.chem.ncsu.edu/~franzen/Poland/Poznan07/lec4_appl_spec.ppt



FTIR for Cancer Diagnosis



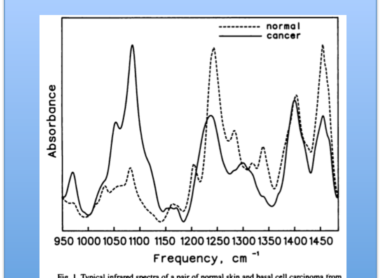
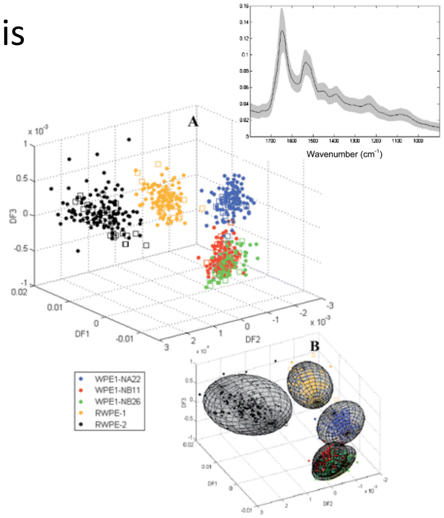


Fig. 1. Typical infrared spectra of a pair of normal skin and basal cell carcinoma from the same patient.

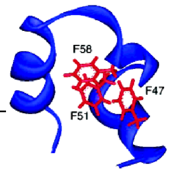
Wong et al, "Distinct Infrared Spectroscopic Patterns of Human Basal Cell Carcinoma of the Skin", Cancer Research (1993).



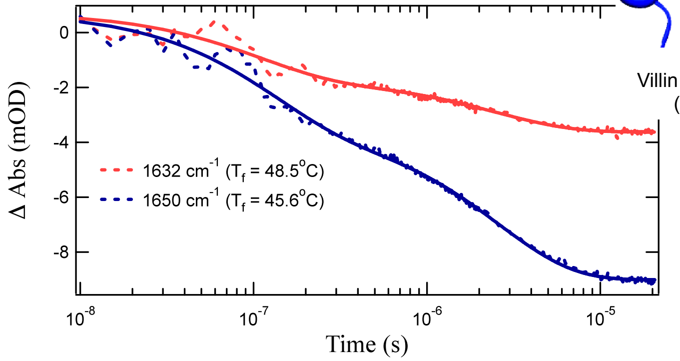
Baker et al., "An investigation of the RWPE prostate derived family of cell lines using FTIR spectroscopy", Analyst (2010)

Time-Resolved IR Spectroscopy

- Two-pulse (pump-probe): provide ps-ns resolution
- Might be limited by spectral overlaps



Villin headpiece (HP35)



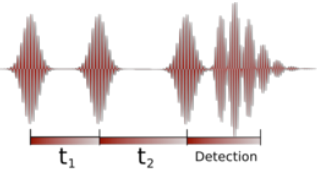
1632 cm^{-1} : 9.63x10^{6} s^{-1}, 3.96x10^{5} s^{-1} (104 ns, 2.53 μs)

1650 cm^{-1} : 8.19x10^{6} s^{-1}, 3.87x10^{5} s^{-1} (122 ns, 2.58 μs)

Original slide from: chsfpc5.chem.ncsu.edu/~franzen/Poland/Poznan07/lec4_appl_spec.ppt

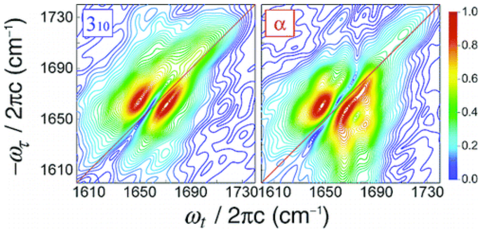
Two-Dimensional FT-IR

- Requires femtosecond (fs) IR pulses: superior time resolution (~ps)!
- Detects coupling between vibrational modes: structural signatures
- State-of-the-art: challenging experiments; lack sequential resolution; interpretation of spectral signature for complex systems



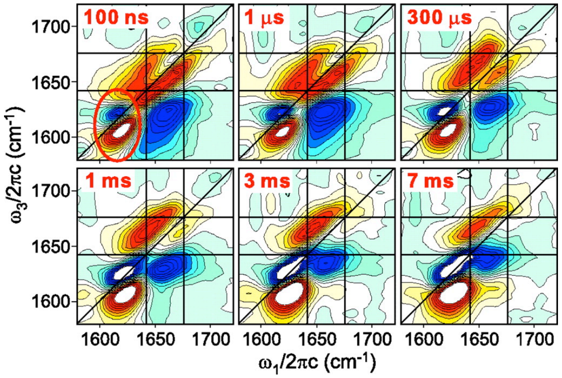
t_2 ~ ps

(Analogous to NMR)



Maekawa et al., "Different Spectral Signatures of Octapeptide 3_{10}- and α-Helices Revealed by Two-Dimensional Infrared Spectroscopy", J. Phys. Chem B (2006).

"The burst phase unfolding of ubiquitin is characterized by a loss of the coupling between vibrations of the β-sheet, …"



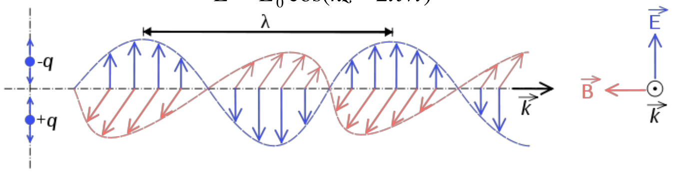
Typically requires molecular dynamics for interpretation!

Chung et al., "Transient 2D IR spectroscopy of ubiquitin unfolding dynamics", PNAS (2007).

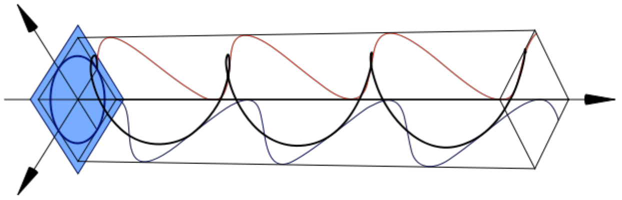
Linear and Circular Dichroism

- Dichroism: different absorption for different directions of polarization
  - Linear or circular dichroism: linearly or circularly polarized light

$$\vec{E} = \vec{E}_0 \cos(kz - 2\pi\nu t)$$

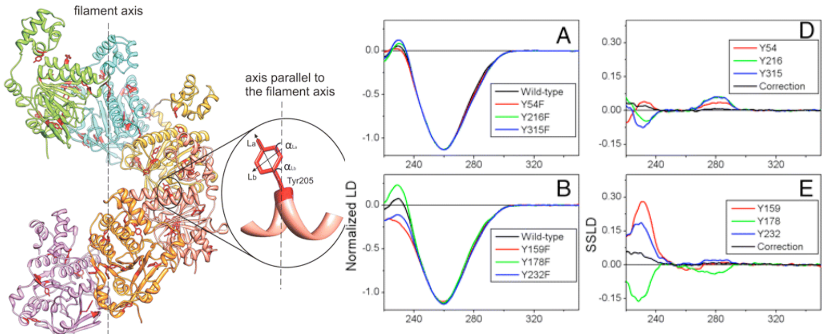


- Circularly polarized light: $\vec{E} = \left|\vec{E}_0\right|[\cos(kz - 2\pi\nu t)\hat{i} + \sin(kz - 2\pi\nu t)\hat{j}]$



Linear Dichroism (LD) $LD(\lambda) = A_{\parallel}(\lambda) - A_{\perp}(\lambda)$

- Oriented materials: such as achieved by stretching polymers
- Absorption depends on the relative direction of electric field of linearly polarized lights and transition dipole
  - Might help to resolve overlapping bands; Contains additional structural information



Sample oriented in a Couette flow device

Reymer et al, Structure of human Rad51 protein filament from molecular modeling and site-specific linear dichroism spectroscopy, PNAS (2009).

Circular Dichroism (CD)

- All biopolymers are optically active with chiral centers.
- CD does not require oriented sample: convenience
- A “standard” technique for quantifying 2nd structural (helical) content

$$CD(\lambda) = A_L(\lambda) - A_R(\lambda)$$

A = -log T = εlc

- Often reported in “ellipticity”

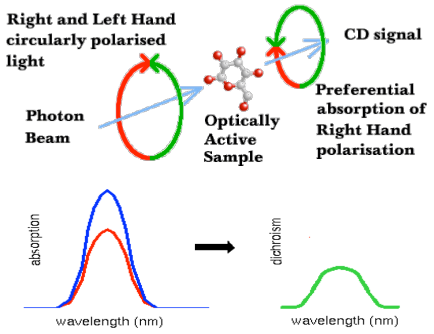
$$\theta(\text{rad cm}^{-1}) = \frac{2.303(A_L - A_R)}{4l}$$

or in “molar circular dichroism”

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \frac{A_L(\lambda) - A_R(\lambda)}{lc}$$

among others parameters

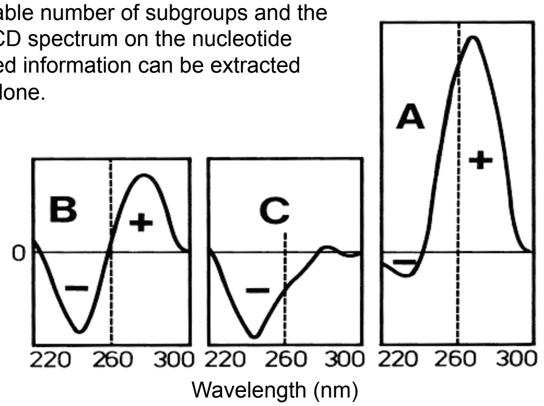
(see Table 10.6 of Tinoco)



CD of Nuclei Acids

On the basis of the measured CD curve, an assignment to one of the structure groups can be made.

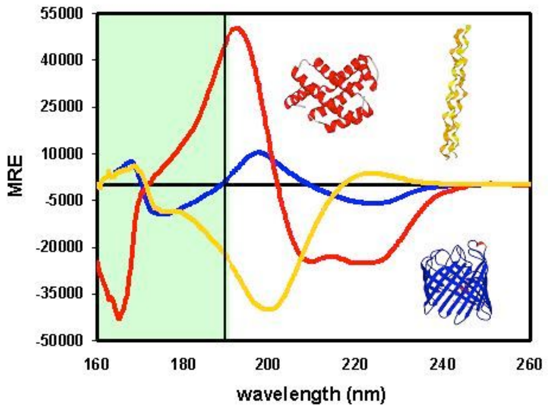
Due to the considerable number of subgroups and the dependence of the CD spectrum on the nucleotide sequence, no detailed information can be extracted from the CD curve alone.



CD of Proteins

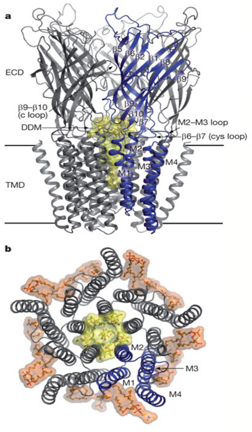
Requires small amount of materials (<100 μg)
Acquired in <30 minutes;
Applicable in almost any solution conditions!

Extremely powerful tool for monitoring structural changes!



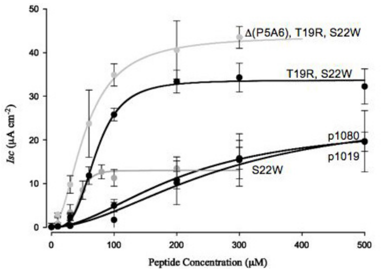
Synchrotron Radiation | UV Range

M2GlyR-Derived Synthetic Channels



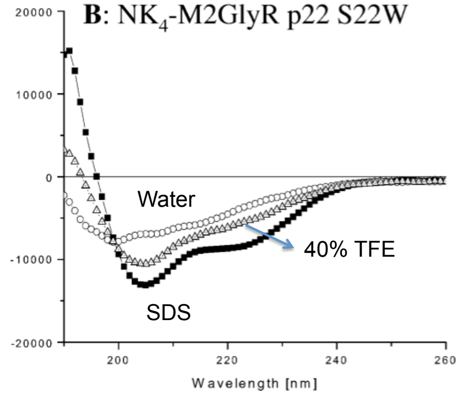
2.9 Å resolution x-ray structure of the GLIC channel protein

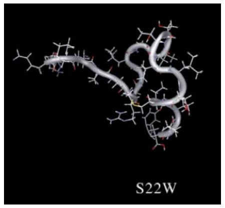
p1080 KKKKPARVGLGITTVLTMTTQS
p1096 KKKKPARVGLGITTVLTMTTQ**W**
p1130 KKKKPARVGLGITTVLTM**R**TQ**W**
p1146 KKKKRVGLGITTVLTM**R**TQ**W**

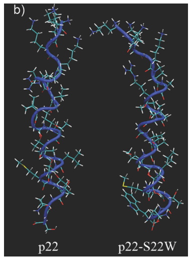


Concentration dependence of I_{sc} induced by modified M2GlyR peptides on epithelial monolayers.

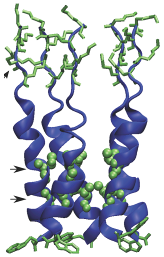
Structures of M2GlyR-Derived Peptides







Right-handed



Herrera et al. PROTEINS (in press).

Peptide Glues

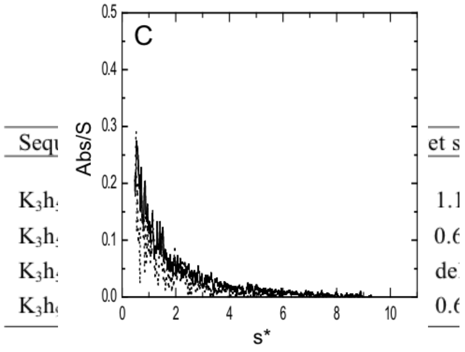
$K_3h_{5ml}K_3$	KKK-FLIVI-KKK
$K_3h_{5n}K_3$	KKK-IVIGS-KKK

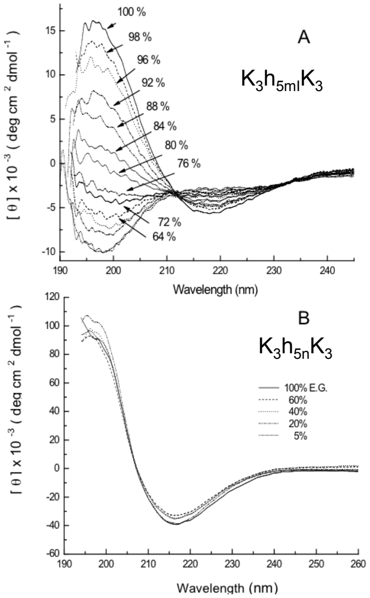
Sequence	dry strength (MPa)	wet strength (MPa)	% residual strength
$K_3h_{5n}K_3$	3.27 ± 0.19	1.13 ± 0.25	34.6
$K_3h_{5c}K_3$	3.24 ± 0.13	0.66 ± 0.20	20.4
$K_3h_{5ml}K_3$	3.44 ± 0.23	delaminated	nil
$K_3h_9K_3^*$	3.05 ± 0.23	0.67 ± 0.08	22.0

Mo et al. Biophys J (2007).

Peptide Glues

$K_3h_{5ml}K_3$	KKK-FLIVI-KKK
$K_3h_{5n}K_3$	KKK-IVIGS-KKK

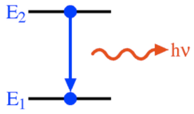


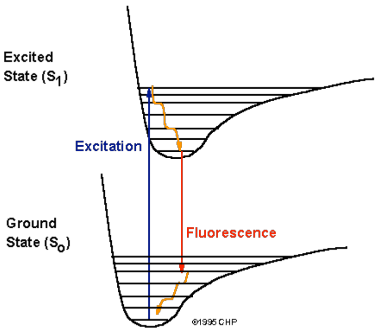


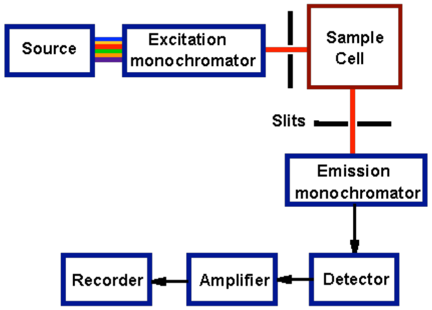
Mo et al. Biophys J (2007).

Emission Spectroscopy

- Emission: transition to a low-energy level
  - Fluorescence: re-emission with ns
  - Phosphorescence: > ms (and up to weeks); weak







Singlet vs Triplet

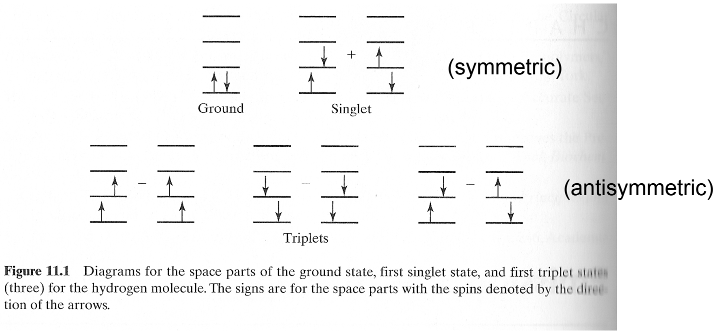
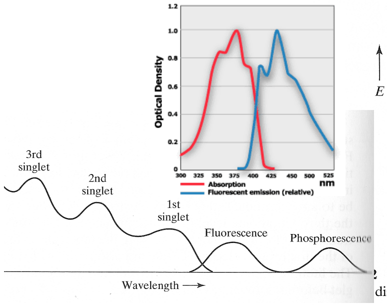


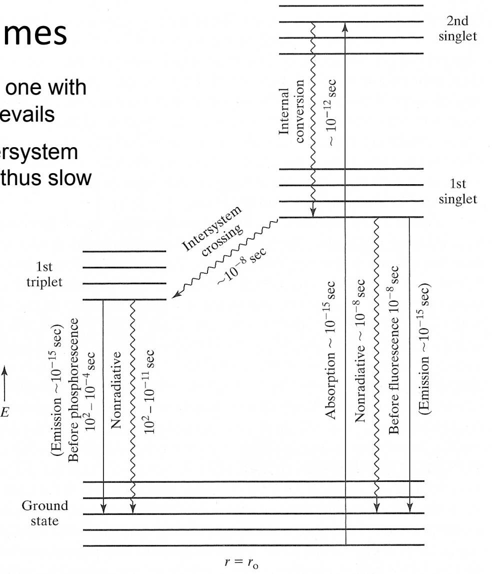
Figure 11.1 Diagrams for the space parts of the ground state, first singlet state, and first triplet states (three) for the hydrogen molecule. The signs are for the space parts with the spins denoted by the direction of the arrows.

- Electron spin: *S* = 1/2
- Degeneracy of the electron spin state: 2 *S* + 1
- Ground state of molecules: singlet
- Transition between different spin states in principle "prohibited" (i.e., very improbable)

Transitions and Lifetimes

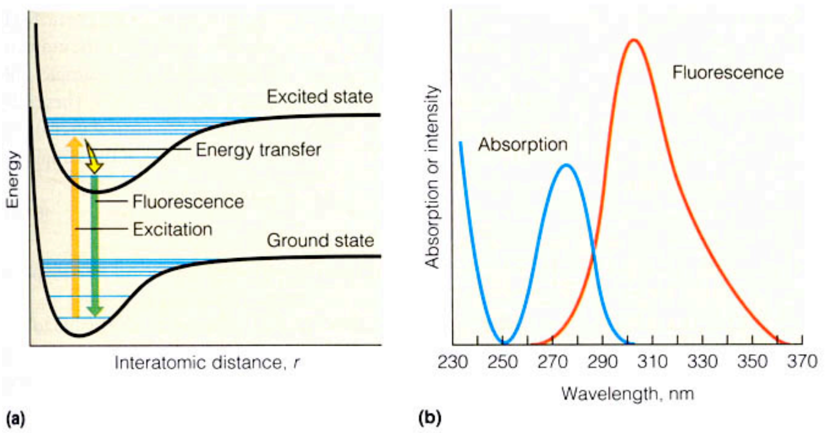
1. All processes compete and the one with the shortest lifetime typically prevails
2. Phosphorescence involves intersystem crossing to triplet states and is thus slow & weak.
3. Phosphorescence is of longer wavelength!
4. Any excitation mechanism





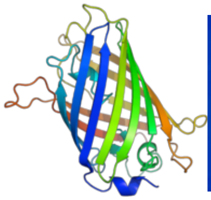
Emission lifetimes of absorption, fluorescence, and phosphorescence at the equili distance of the ground state.

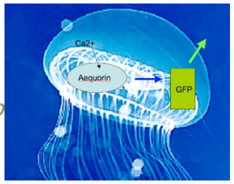
Excitation vs Fluorescence

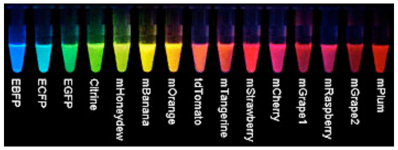


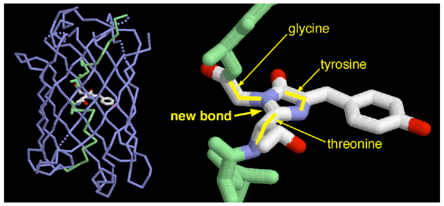
Fluorescence as Molecular Spy

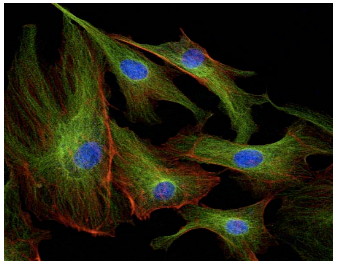
- Green fluorescence protein: GFP (2008 Nobel Prize)
  - Green fluorescence when exposed to blue lights





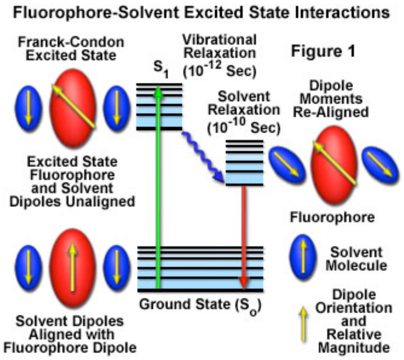


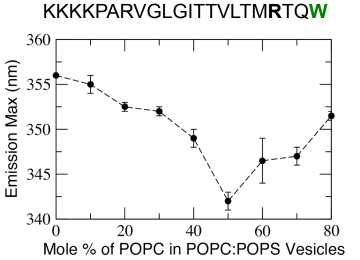




General Solvent Effects

- Polarizability of solvent: solvent relaxation reduces the energy gap and shift fluorescence to longer wavelengths
  - The higher dielectric constant is, the bigger the shift is!
  - Provide a probe for solvent exposure: such as during folding/unfolding



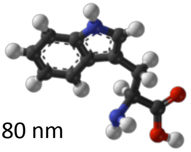


Dependence of average blue shift (nm) of peptide p22-T19R/S22W on the POPC concentration in liposomes

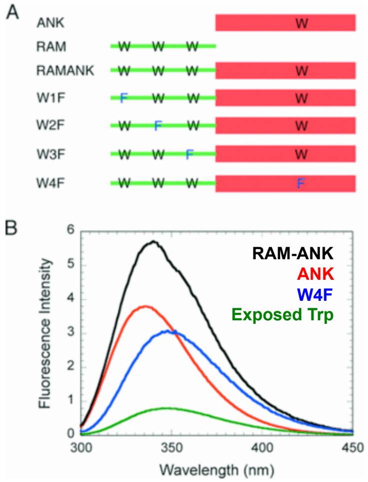
(Tomich, unpublished data)

Intrinsic Fluorescence of Trp

Most of the intrinsic fluorescence emissions of a folded protein are due to excitation of tryptophan residues, with some emissions due to tyrosine and phenylalanine.



- Absorption ~ 280 nm
- Emission @ 300-350 nm: depending on the polarity of the local environment (can be used to infer conformations!)
- Quenched by Asp/Glu
- Excitation transfer (FRET: distance & dynamics info)
- A key internal probe



Bertagna et al, Biochem Soc Trans (2008)

Quenching

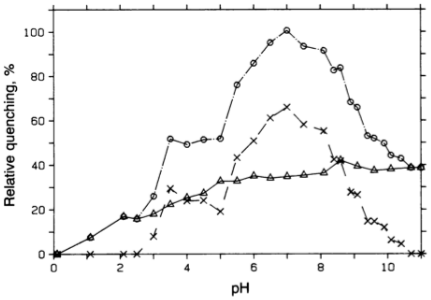
- Multiple processes might decrease fluorescence intensity
  - Static quenching: (ground state) complex-formation
  - Dynamic quenching: excited state (triplet) reactions, collisional quenching, energy transfer etc
  - Common (small and soluble) quenchers for proteins: <u>Iodide</u> (charged, efficiently quenches surface residues). <u>Oxygen</u>: Small and can penetrate the protein to some degree. <u>Acrylamide, nitroxides</u> etc: Neutral (probing accessibility).

Stern-volmer relationship

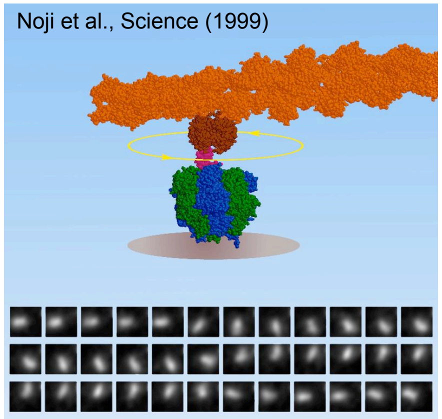
$$A^* + Q \rightarrow A + Q^*$$

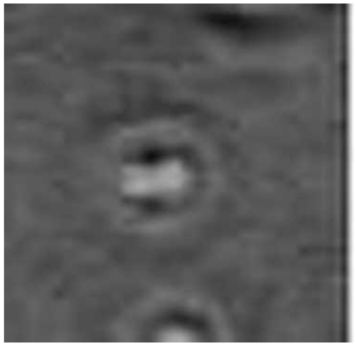
$$\frac{I_f^0}{I_f} = 1 + k_q \tau_0 \cdot [Q]$$

Jang and el-Sayed, "Tryptophan fluorescence quenching as a monitor for the protein conformation changes occurring during the photocycle of bacteriorhodopsin under different perturbations." PNAS (1989).



Single Molecular Fluorescence


Noji et al., Science (1999)



http://www.youtube.com/watch?v=P0xHuyg9r5k&feature=related

Fröster Resonance Energy Transfer (FRET)

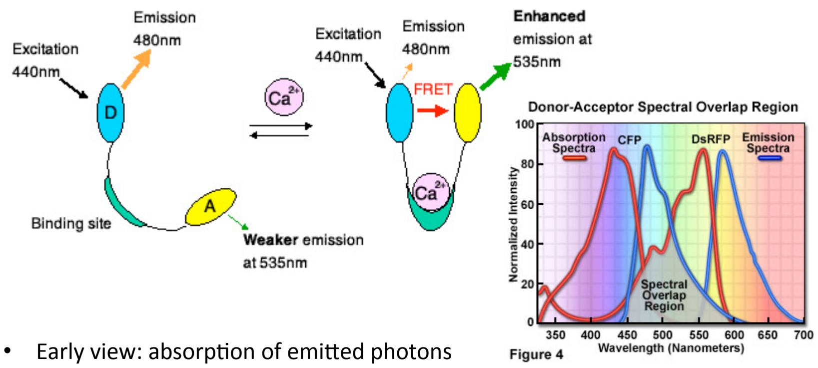


Figure 4

- Early view: absorption of emitted photons
- Fröster argued in 1946 that it is excitation transfer (i.e. direct coupling of quantum mechanical states) and provided the theoretical foundation for all today's FRET analysis
- Transfer efficiency depends on: donor-acceptor distance, spectral overlaps, (transition) dipole orientations

---

FRET as Molecular Ruler

- Definition of efficiency

$$E = \frac{k_{ET}}{k_f + k_{ET} + \sum k_i}$$

$$D \rightarrow D^* \quad + \quad A \xrightarrow{k_{ET}} D + A^* \quad \rightarrow A$$

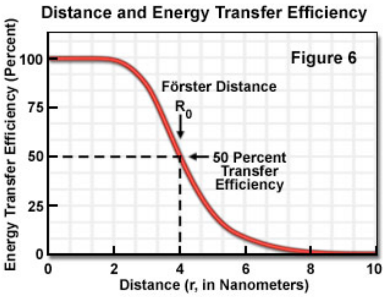
$$k_f \downarrow \qquad k_i$$

D

- Dipole-dipole coupling

$$Eff = \frac{R_o^6}{\left(R_o^6 + r^6\right)}$$

- Calculation of R_0 complicated by anisotropic orientations
- Ensemble averaged distance



Range: ~ 3 to 7 nm

Förster Critical Distance for Common RET Donor-Acceptor Pairs

Donor	Acceptor	Förster Distance (Nanometers)
Tryptophan	Dansyl	2.1
IAEDANS (1)	DDPM (2)	2.5 - 2.9
BFP	DsRFP	3.1 - 3.3
Dansyl	FITC	3.3 - 4.1
Dansyl	Octadecylrhodamine	4.3
CFP	GFP	4.7 - 4.9
CF (3)	Texas Red	5.1
Fluorescein	Tetramethylrhodamine	4.9 - 5.5
Cy3	Cy5	>5.0
GFP	YFP	5.5 - 5.7
BODIPY FL (4)	BODIPY FL (4)	5.7
Rhodamine 6G	Malachite Green	6.1
FITC	Eosin Thiosemicarbazide	6.1 - 6.4
B-Phycoerythrin	Cy5	7.2
Cy5	Cy5.5	>8.0

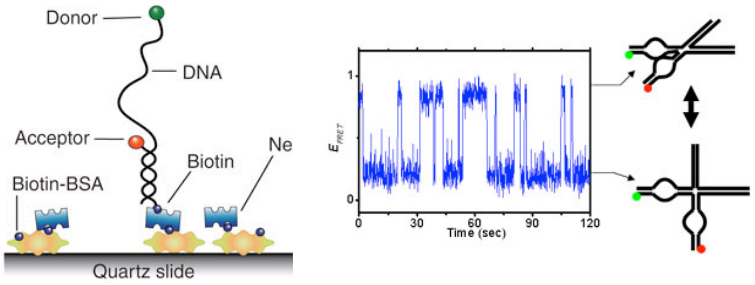
(1) 5-(2-iodoacetylaminoethyl)aminonaphthalene-1-sulfonic acid
(2) N-(4-dimethylamino-3,5-dinitrophenyl)maleimide
(3) carboxyfluorescein succinimidyl ester
(4) 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene

Table 1

http://www.microscopy.fsu.edu/primer/techniques/fluorescence/fret/

Single-Molecular FRET

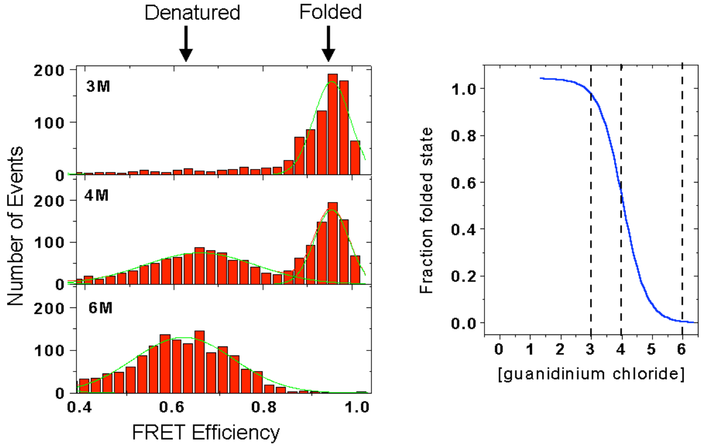
- Time-Resolved FRET on single molecular level: high sensitivity detectors
- Allow direct, time-dependent observation of individual molecular events
- It will/should become one of the standard, but extremely powerful, techniques, like many other single molecular techniques such as optical tweezers, AFM pulling etc.



Also see: Ha et al, A practical guide to single-molecule FRET, Nat Method (2008).

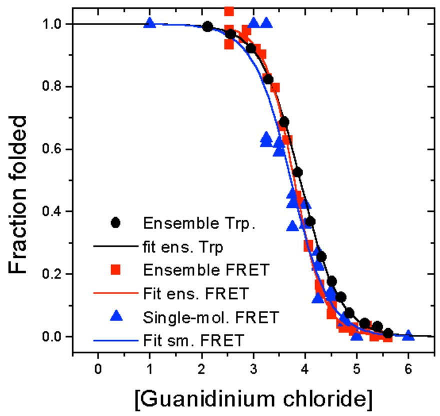
CI2 – folded and denatured subpopulations

Unfolded State ⇌ Folded State



http://www.nigms.nih.gov/news/reports/single_molecules.html

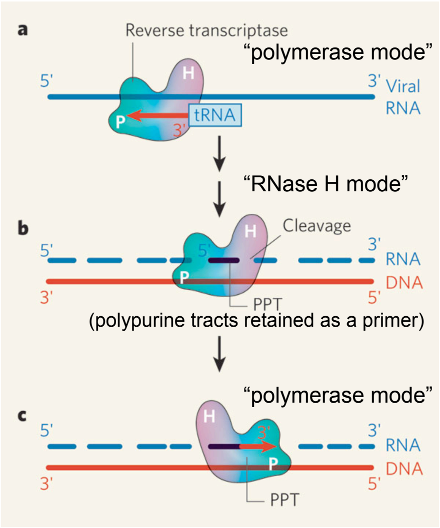
Pwt CI2 denaturation curves: Ensemble vs. single molecule

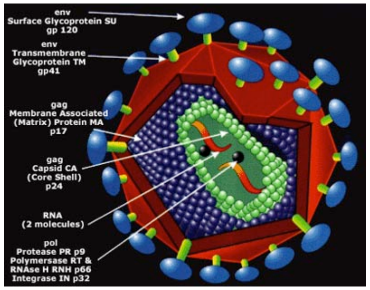


good agreement!

http://www.nigms.nih.gov/news/reports/single_molecules.html

An HIV secret uncovered by single molecule FRET

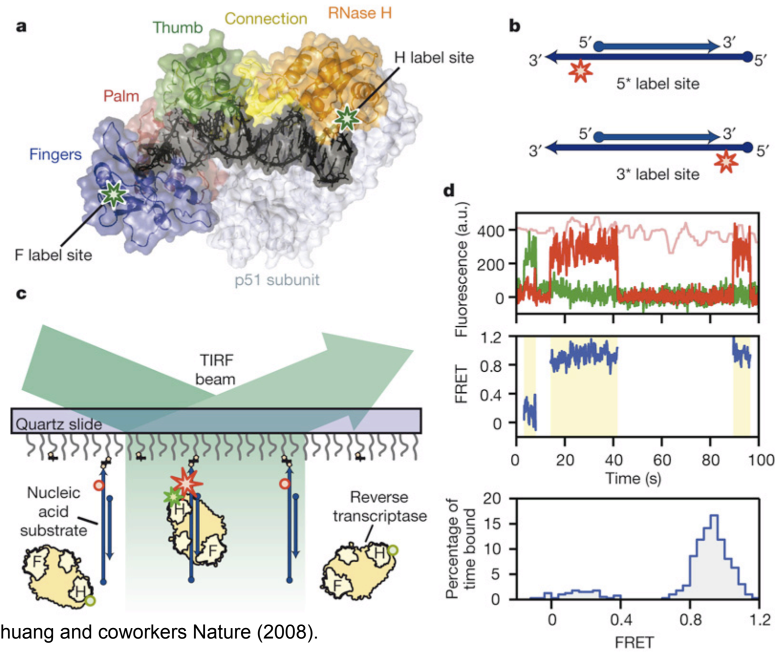




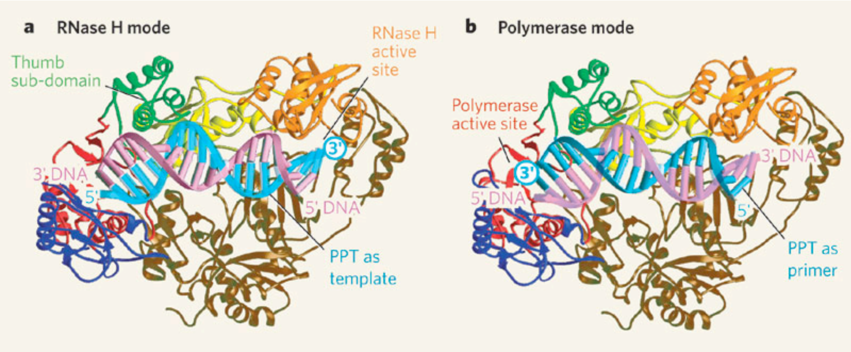
RNA-dependent
DNA polymerase
(PDB: 1HMV)

HIV Reverse
Transcriptase

Arnold & Sarafianos, Nature (2008); Zhuang and coworkers Nature (2008).



Zhuang and coworkers Nature (2008).



a. Before cleavage of the PPT RNA, HIV reverse transcriptase binds to PPT (blue sequence) in an RNase H mode — that is, the RNase H domain of the enzyme is close to the 3 end of PPT (circled), thus blocking the polymerase domain's access to it and preventing premature extension. **b**, When the RNA nucleotides next to the PPT sequence are cleaved, allowing PPT to function as a primer, the enzyme can bind in a polymerase-competent mode (with its polymerase domain close to PPT's 3 end) to initiate DNA synthesis. The HIV reverse transcriptase seems to rapidly switch orientation between these two binding modes.