

UV-Visible, Fluorescence and CD Spectroscopy Of Proteins

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http://en.wikipedia.org/wiki/UV-vis_spectroscopy

http://en.wikipedia.org/wiki/Fluorescence_spectroscopy

http://en.wikipedia.org/wiki/Circular_dichroism

The Electromagnetic Spectrum

Examples of Spectroscopic Measurements:

NMR (MHz)
nuclear spin spectroscopy

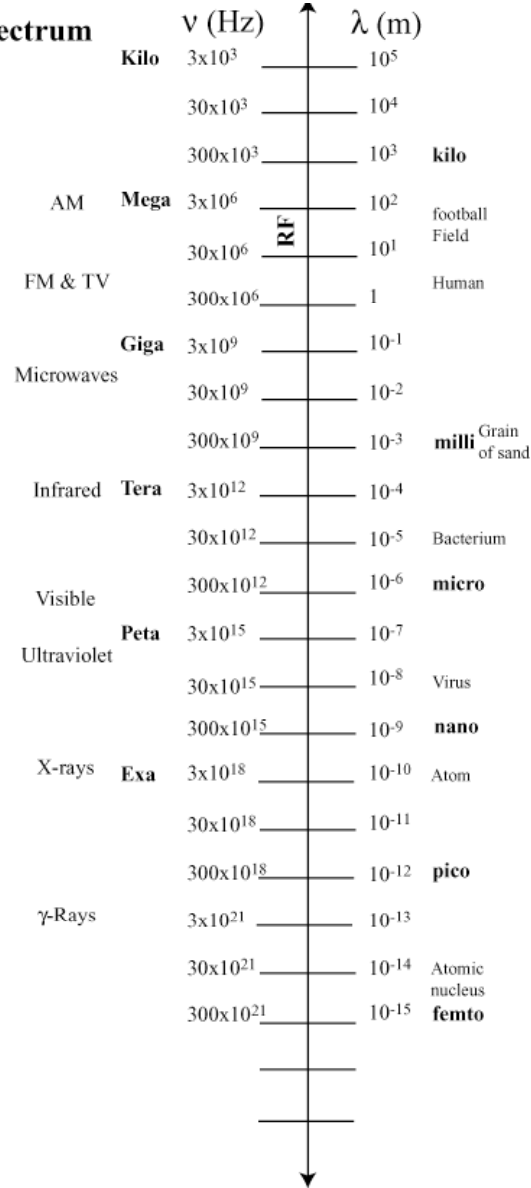
EPR/ESR (GHz)
electron spin

vibrational spectroscopy, IR
molecular bond vibrations
wavenumbers in cm^{-1}

Visible and UV
electronic spectroscopy
electronic structure in nm
Absorbance, Fluorescence, CD, Light Scattering

crystallography and scattering
molecular structure in Å

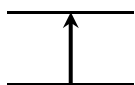
Mössbauer Spectroscopy
Nuclear/Atomic structure



A **photon** of light energy can be absorbed by a molecule causing a quantized jump from one energy level to another.

$$E \text{ (J)} = h\nu = hc/\lambda$$

$$h = \text{Planck's const} = 6.63 \times 10^{-34} \text{ J s}$$



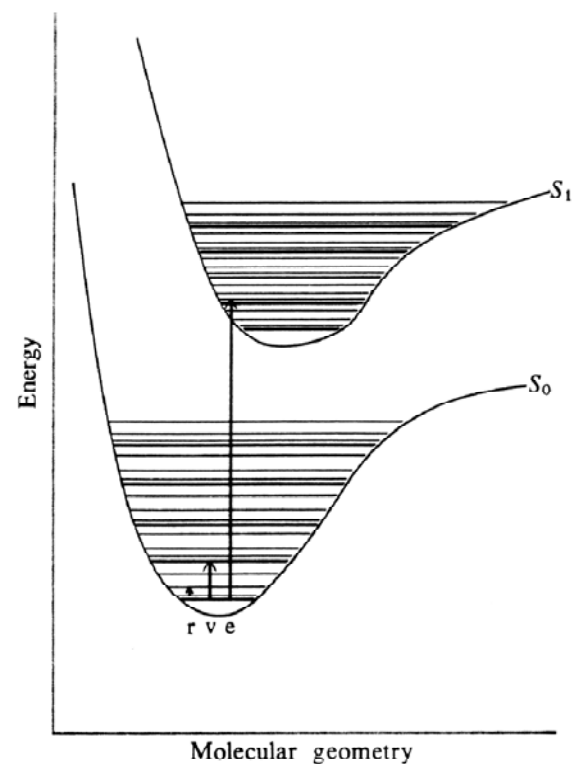
According to **Boltzmann** distribution the population of states can differ significantly depending on the energy difference and the temperature of the system:

$$N_e = N_g e^{(-\Delta E / kT)}$$

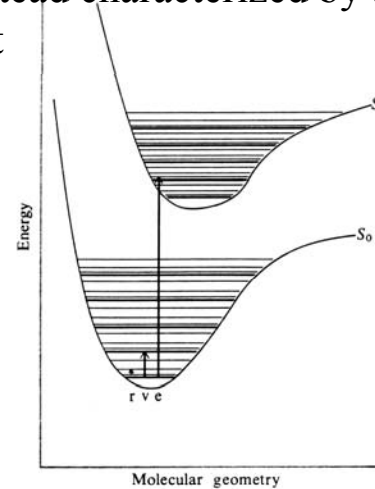
where $k = 1.38 \times 10^{-23} \text{ J/K}$

Figure 7-1

Energy levels of a small molecule. Selected rotational sublevels of the vibrational levels of each of two electronic states are shown. Transitions corresponding to electronic (e), vibrational (v), and rotational (r) spectra are indicated.

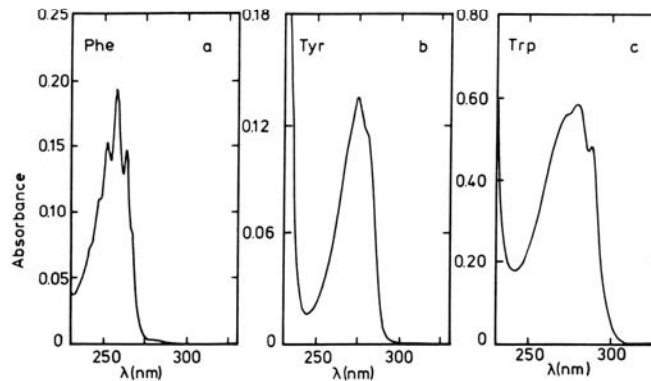


Selected rotational sublevels of the vibrational levels of each of two electronic states are shown. Transitions corresponding to electronic (e) vibrational (v) and rotational (r) spectra are indicated. Visible and UV radiation induce transitions between electronic states while IR induces transitions between vibrational states within a single electronic state. Transitions between vibrational states in a given electronic manifold is the reason why electronic transitions are not characterized by a spectrum containing a narrow absorption line, but are instead characterized by a broad band. Also, the environment surrounding a molecule can affect characteristics of its Absorption Spectrum.



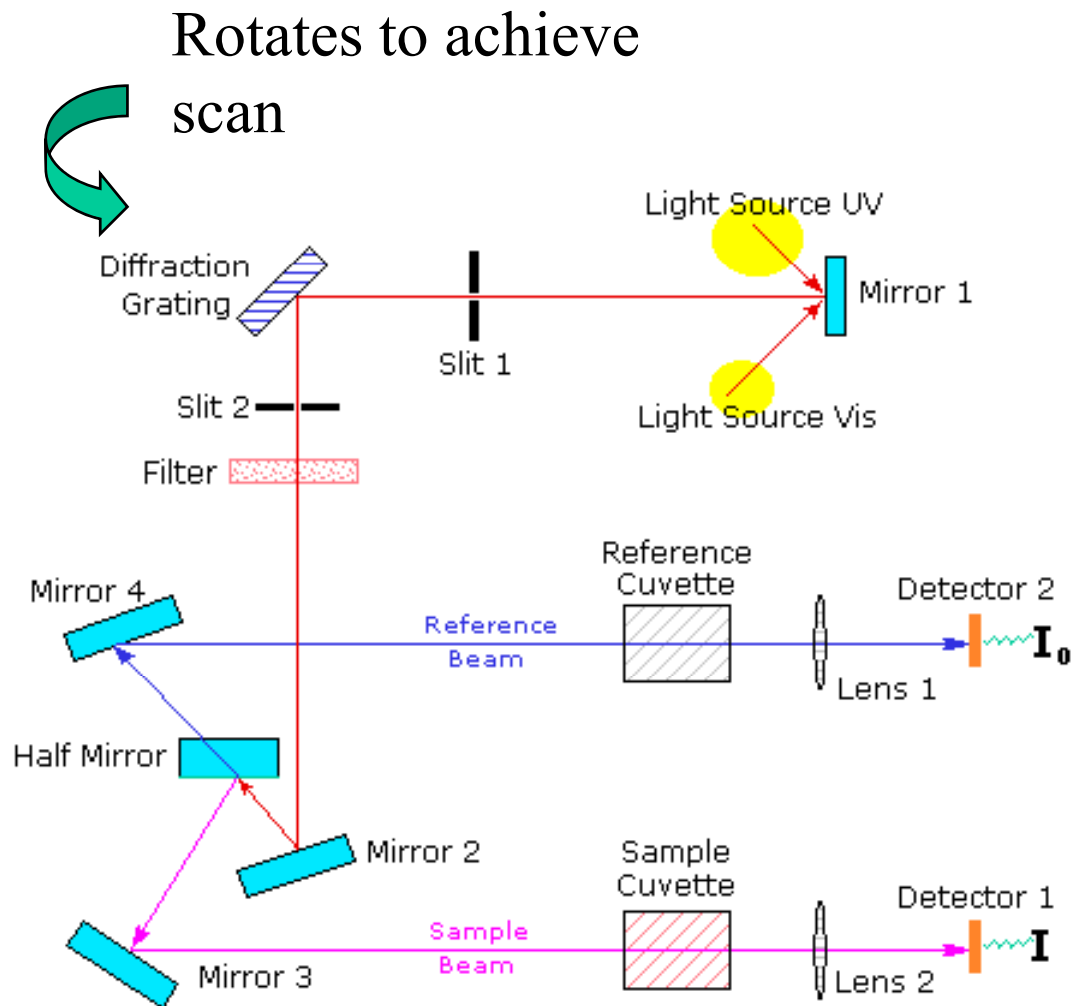
Transitions between vibrational states in a given electronic manifold is the reason why electronic transitions are not characterized by a spectrum containing a narrow absorption line, but are instead characterized by a broad band.

The environment surrounding a molecule can affect characteristics of its Absorption Spectrum.



Ultraviolet absorbance spectra of the aromatic amino acids in a 1 cm cell in 0.01 M potassium phosphate buffer pH 7.0 25°C. (a) 1 mM phenylalanine; (b) 0.1 mM

Scanning UV spectrometers



Matched quartz cuvettes

Sample in solution at ca. 10^{-5} M.

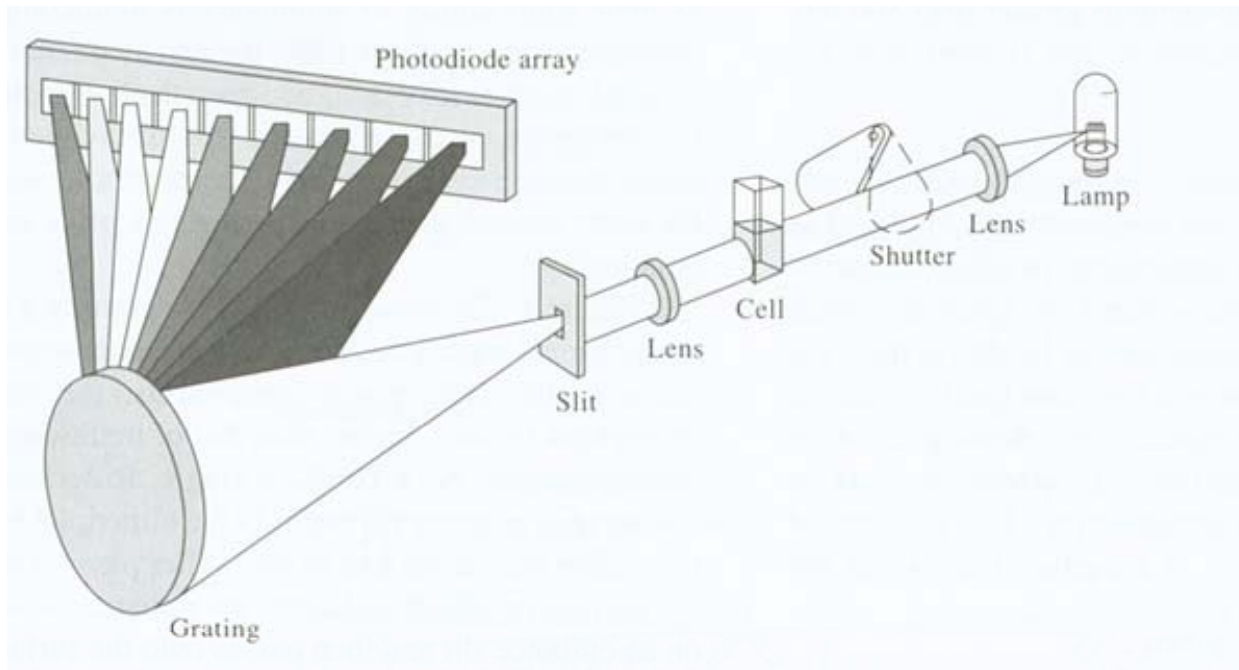
System protects PM tube from stray light

D2 lamp-UV

Tungsten lamp-Vis

Double Beam makes it a difference technique

Diode Array Detector UV Spectrophotometer



Diode array alternative puts grating, array of photosensitive Semiconductors after the light goes through the sample.

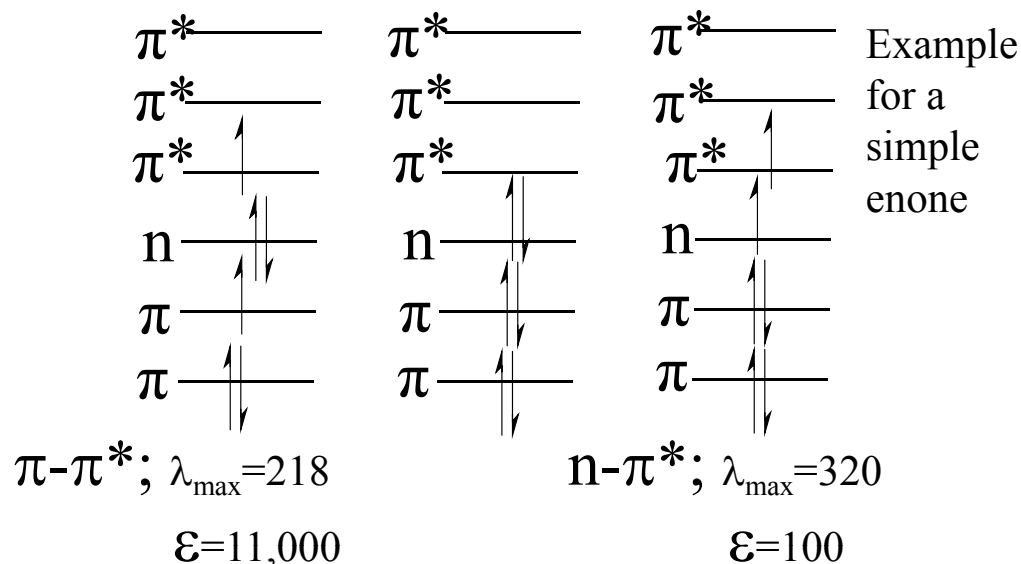
Advantage: speed and sensitivity

Disadvantage: light is used in full range

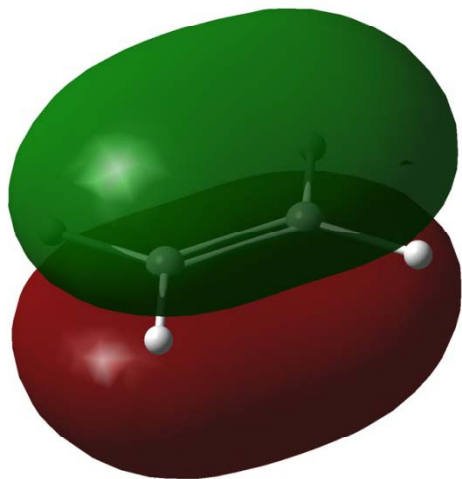
The UV Absorption process

- $\sigma \rightarrow \sigma^*$ and $\sigma \rightarrow \pi^*$ transitions: high-energy, accessible in vacuum UV ($\lambda_{\max} < 150$ nm). Not usually observed in molecular UV-Vis.
- $n \rightarrow \sigma^*$ and $\pi \rightarrow \sigma^*$ transitions: non-bonding electrons (lone pairs), wavelength (λ_{\max}) in the 150-250 nm region.
- $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions: most common transitions observed in organic molecular UV-Vis, observed in compounds with lone pairs and multiple bonds with $\lambda_{\max} = 200-600$ nm.
- Any of these require that incoming photons match in energy the gap corresponding to a transition from ground to excited state.
- Energies correspond to a 1-photon of 300 nm light are ca. 95 kcal/mol

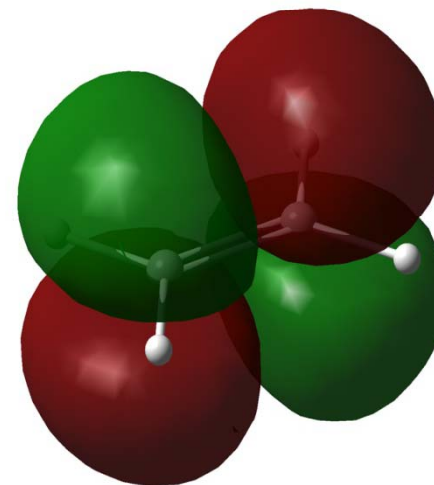
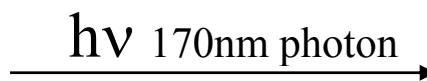
What are the nature of these absorptions?



Example: $\pi \rightarrow \pi^*$ transitions responsible for ethylene UV absorption at ~ 170 nm calculated with ZINDO semi-empirical excited-states methods (Gaussian 03W):



HOMO π_u bonding molecular orbital



LUMO π_g antibonding molecular orbital

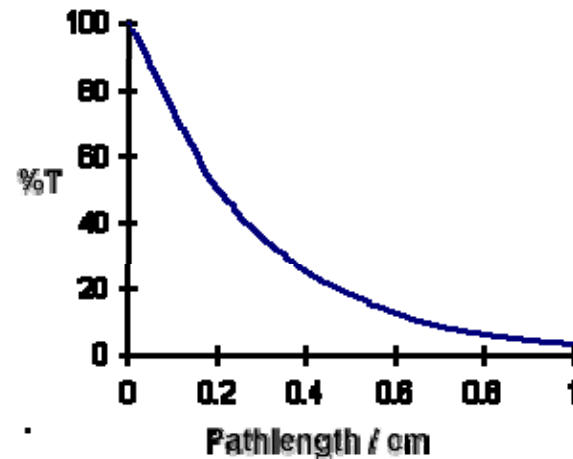
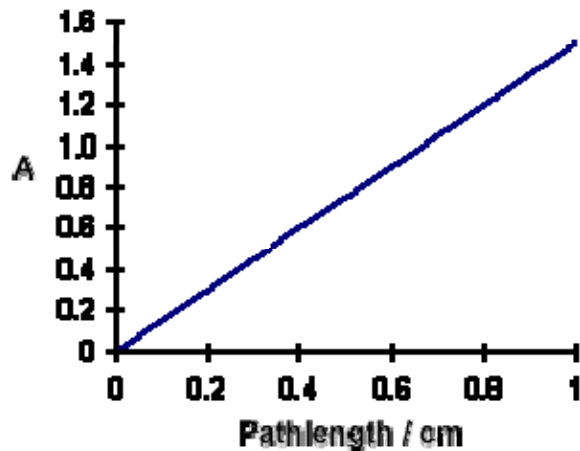
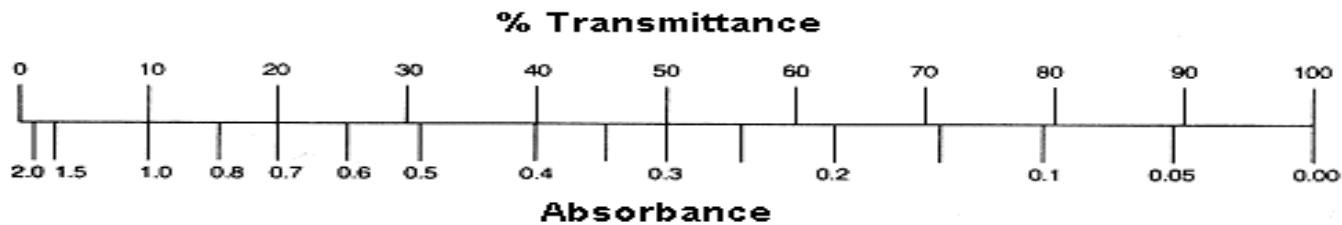
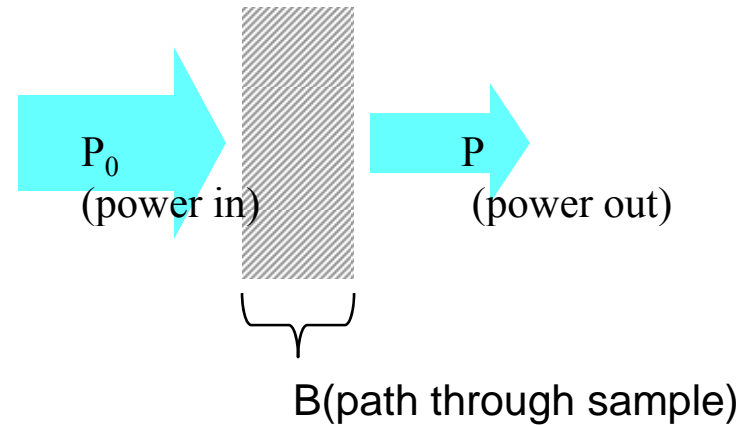
The Quantitative Picture

- Transmittance:

$$T = P/P_0$$

- Absorbance:

$$A = -\log_{10} T = \log_{10} P_0/P$$



- The Beer-Lambert Law (a.k.a. Beer's Law):

$$A = \epsilon bc$$

Where the absorbance A has no units, since $A = \log_{10} P_0 / P$

ϵ is the molar absorptivity with units of $L \text{ mol}^{-1} \text{ cm}^{-1}$

b is the path length of the sample in cm

c is the concentration of the compound in solution, expressed in mol L^{-1} (or M, molarity)

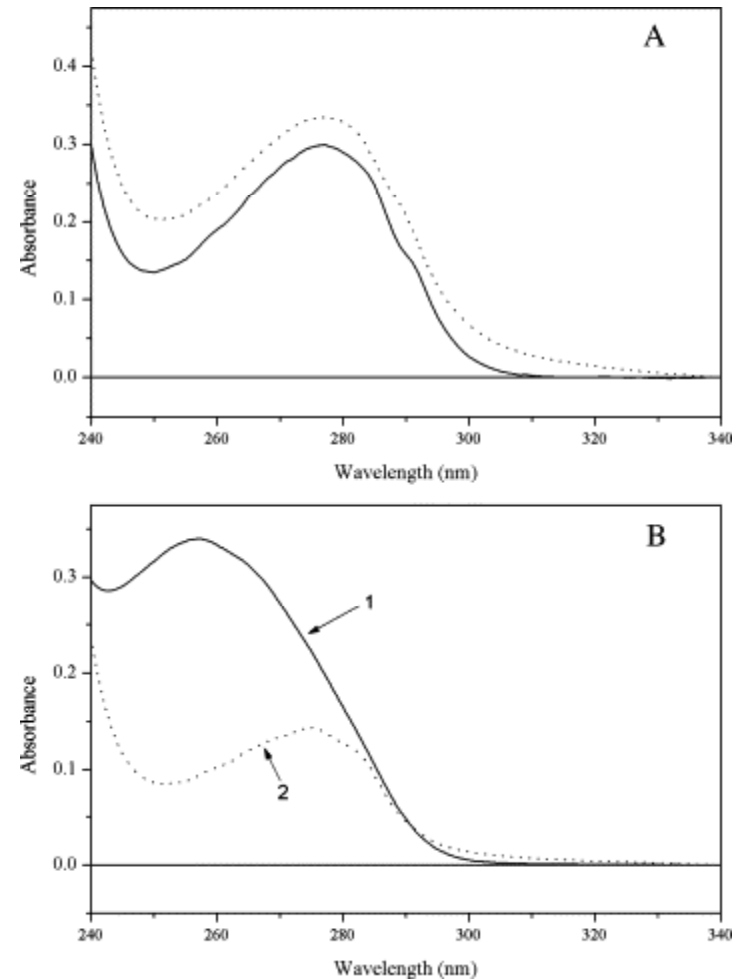
Limitations of the Beer-Lambert law

The linearity of the Beer-Lambert law is limited by chemical and instrumental factors.

Causes of nonlinearity include:

- deviations in absorptivity coefficients at high concentrations due to electrostatic interactions between molecules in close proximity
- scattering of light due to particulates in the sample
- fluorescence or phosphorescence of the sample
- changes in refractive index at high analyte concentration
- shifts in chemical equilibria as a function of concentration
- non-monochromatic radiation

Problems encountered in measuring the absorbance of protein solutions. Panel A shows the absorption spectrum of a solution of the actin-bundling protein *scp1p* from *Saccharomyces cerevisiae* before and after centrifugation to remove aggregated protein. Panel B shows the absorption spectrum of the SNARE protein syntaxin 4 from *S. cerevisiae* expressed as a recombinant His-tagged protein and purified by immobilised metal-ion chromatography. Spectrum 1 (solid line) shows that there is considerable contamination by nucleic acids, since the peak of absorption is close to 260 nm. Spectrum 2 (dotted line) shows the absorption spectrum of syntaxin 4 where the cell extract has been incubated with DNase before chromatography.



Calculated and experimental A_{280} values for lysozyme, chymotrypsinogen and insulin

Note that in each protein all the cysteine side chains form disulphide bonds, no disulphide means no nC term.

Gill & von Hippel $\epsilon_{280} [\text{M}^{-1} \text{cm}^{-1}] = \# \text{ of Trp} \times 5690 + \# \text{ of Tyr} \times 1280 + \# \text{ of Cys} \times 60$

Pace $\epsilon_{280} [\text{M}^{-1} \text{cm}^{-1}] = \# \text{ of Trp} \times 5500 + \# \text{ of Tyr} \times 1490 + \# \text{ of Cys} \times 62.5$

Protein	Mass (Da)	n_W	n_Y	n_C	A_{280} (1 mg/ml: 1 cm)		
					Calculated ^a	Calculated ^b	Experimental ^c
Lysozyme	14,314	6	3	8	2.69	2.65	2.65
Chymotrypsinogen	25,666	8	4	10	2.00	1.97	1.98
Insulin	5734	0	4	6	0.96	1.10	0.97

Spectroscopic properties of proteins containing prosthetic groups

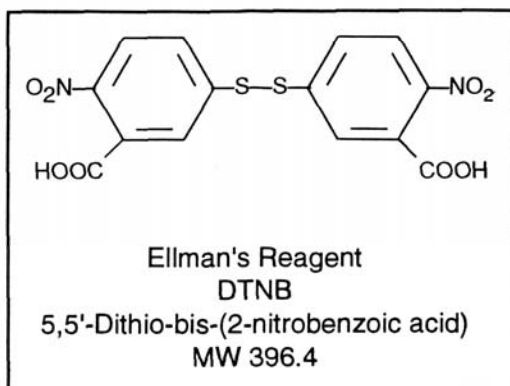
Protein	Prosthetic group	Longest-wavelength absorption band		Second-longest absorption band	
		λ_{\max} (nm)	ϵ_{\max} ($\times 10^{-4}$)	λ_{\max} (nm)	ϵ_{\max} ($\times 10^{-4}$)
Amino acid oxidase, rat kidney	FMN	455	1.27	358	1.07
Azurin, <i>P. fluorescens</i>	Cu ^{II}	781	0.32	625	0.35
Ceruloplasmin, human	8 Coppers (3 distinct classes)	794	2.2	610	1.13
Cytochrome <i>c</i> , reduced, human	Fe ^{II} -heme	550	2.77	—	—
Ferredoxin, <i>Scenedesmus</i>	(2 Fe ^{III} , 2 sulfide) cluster	421	0.98	330	1.33
Flavodoxin, <i>C. pasteurianum</i>	FMN	443	0.91	372	0.79
Monoamine oxidase, bovine kidney	Flavins plus Cu	455	4.7	—	—
Pyruvic dehydrogenase, <i>E. coli</i>	FAD	460	1.27	438	1.46
Rhodopsin, bovine	Retinal-Lys	498	4.2	350	1.1
Reubredoxin, <i>M. aerogenes</i>	(Fe ^{III} , 4 Cys) tetrahedron	570	0.35	490	0.76
Threonine deaminase, <i>E. coli</i>	4 Pyridoxal phosphates	415	2.6	—	—
Xanthine oxidase	Fe, Mo	550	2.2	—	—

Measurement of the number of moles of free sulfhydryl groups in proteins

A water-soluble compound (Ellman's Reagent) can be used to quantitate free sulfhydryl groups in solution. DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB), which gives color in the visible range. The target of DTNB is the conjugate base (R-S-) of a free sulfhydryl group. Therefore the rate of this reaction is dependent on:

- 1) the reaction pH,
- 2) The pKa of the sulfhydryl
- 3) steric and electrostatic effects.

Molar extinction coefficients of Ellman's Reagent in various solvents	
Solvent	epsilon 412
2% SDS	12,500
0.1 M phosphate, pH 7.27, 1 mM EDTA	14,150
Buffered 6 M guanidine hydrochloride	13,700
6 M guanidine hydrochloride	13,880
8 M urea	14,290



Major attributes of fluorescence:

specificity, sensitivity, spectral information, temporal resolution,
and spatial resolution (if combined with microscopy)

Fluorescence Lifetimes:

measure molecular dynamics

Fluorescence Polarization and Anisotropy:

measure orientation and molecular dynamics

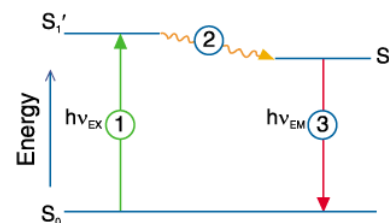
Fluorescence Resonance Energy Transfer:

measure molecular distances

Further Reading:

<http://probes.invitrogen.com/handbook/>

Fluorescence is the result of a three-stage process involving poly aromatic hydrocarbons or heterocycles called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific stimulus. The process responsible for the fluorescence is shown with a simple electronic-state diagram (Jablonski diagram).



Stage 1: Excitation

A photon of energy $h\nu_{EX}$ is supplied by a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

Stage 2: Excited-State Lifetime

The excited state exists for a finite time (typically $1-10 \times 10^{-9}$ seconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (Stage 1) return to the ground state (S_0) by fluorescence emission. Other processes such as collisional quenching, fluorescence energy transfer and intersystem crossing (see below) may also depopulate S_1 . The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these processes occur.

Stage 3: Fluorescence Emission

A photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state S_0 . Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon $h\nu_{EX}$. The difference in energy or wavelength represented by $(h\nu_{EX} - h\nu_{EM})$ is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

Fluorescence Spectra

The fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching), the same fluorophore can be repeatedly excited and detected. For polyatomic molecules in solution, the discrete electronic transitions are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum.

The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected. Typically, the fluorescence excitation spectrum is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime.

The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength

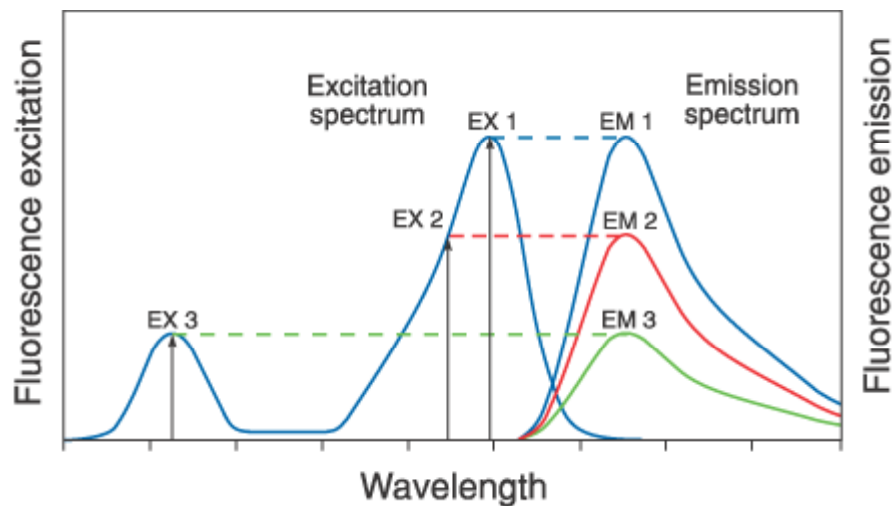


Table Absorbance and fluorescence properties of the aromatic amino acids^a

Amino acid	Absorbance		Fluorescence		Sensitivity
	λ_{\max} (nm)	ϵ_{\max} ($\text{M}^{-1}\text{cm}^{-1}$)	λ_{\max} (nm)	φ_{F}^b	$\epsilon_{\max} \times \varphi_{\text{F}}^b$ ($\text{M}^{-1}\text{cm}^{-1}$)
Tryptophan	280	5600	355	0.13	730
Tyrosine	275	1400	304	0.14	200
Phenylalanine	258	200	282	0.02	4

^a In water at neutral pH; data are from ref. 10.

^b φ_{F} , Fluorescence quantum yield.

CD Spectroscopy of Proteins

Reference:

How to study proteins by circular dichroism

Sharon M. Kelly, Thomas J. Jess and Nicholas C. Price (2005)

[Biochimica et Biophysica Acta \(BBA\) - Proteins & Proteomics](#)

[Volume 1751](#), 119-139.

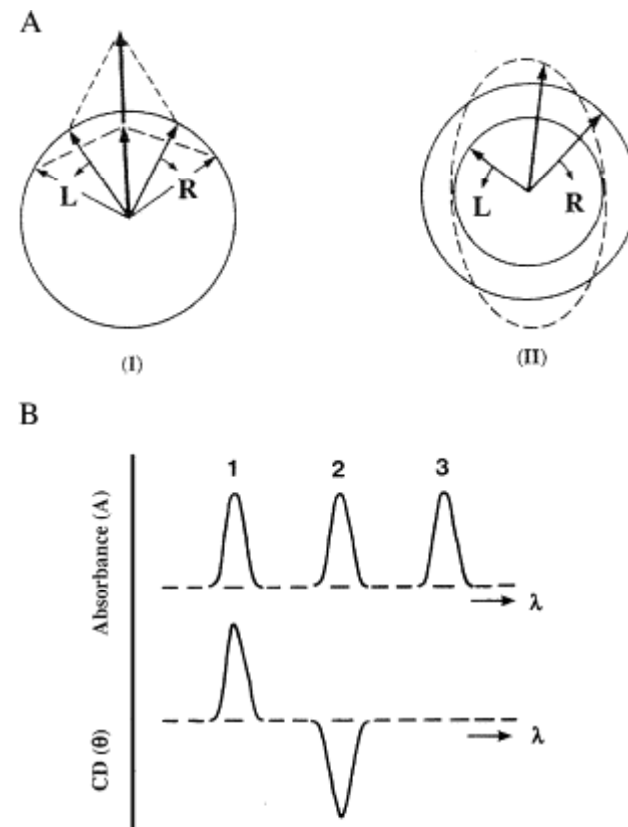
URL

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16027053

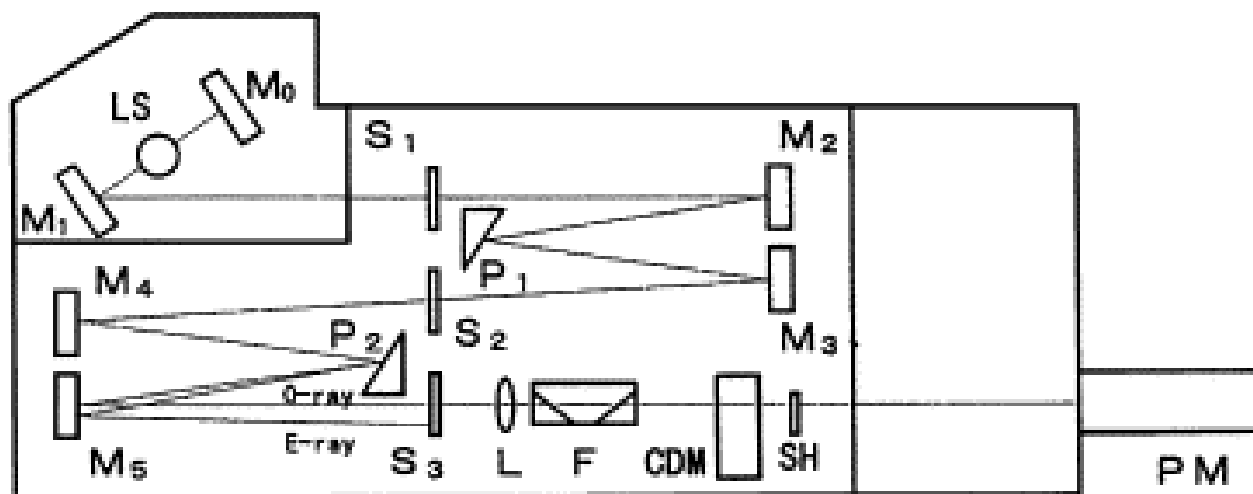
Circular dichroism (CD) is recognized as a valuable technique for examining the structure of proteins in solution. Plane polarized light can be viewed as being made up of 2 circularly polarized components of equal magnitude, one rotating counter-clockwise (left handed, L) and the other clockwise (right handed, R). Circular dichroism (CD) refers to the differential absorption of these 2 components. If, after passage through the sample being examined, the L and R components are not absorbed or are absorbed to equal extents, the recombination of L and R would regenerate radiation polarised in the original plane (I). However, if L and R are absorbed to different extents, the resulting radiation would be said to possess elliptical polarization (II).

A CD signal will be observed when a chromophore is chiral (optically active) for one of the following reasons: (a) it is intrinsically chiral because of its structure, for example, a C atom with 4 different substituents, or the disulphide bond which is chiral because of the dihedral angles of the C–S–S–C chain of atoms, (b) it is covalently linked to a chiral centre in the molecule, or (c) it is placed in an asymmetric environment by virtue of the 3-dimensional structure adopted by the molecule.

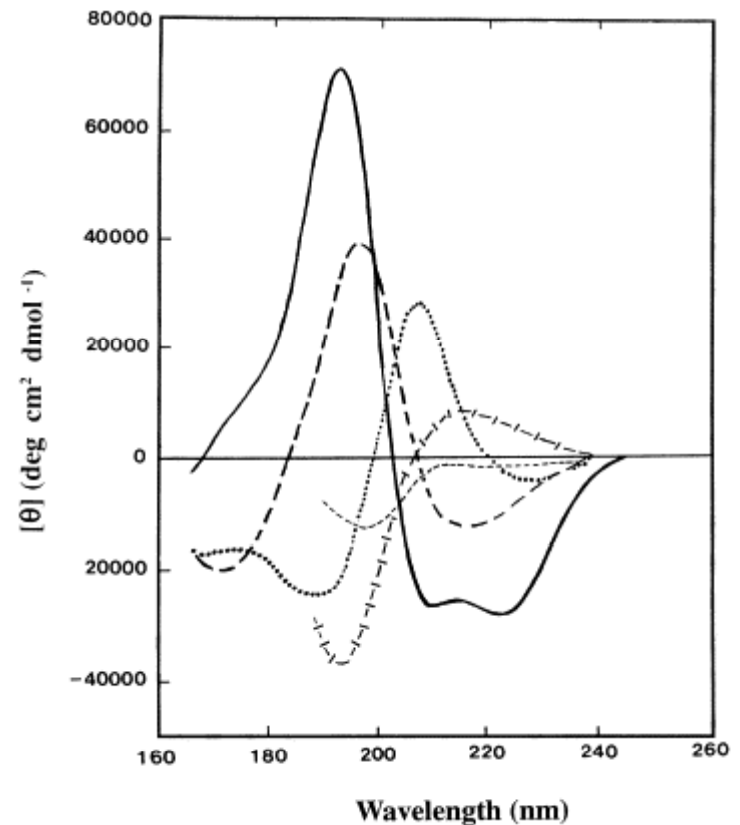
The relationship between absorption and CD spectra. Band 1 has a positive CD spectrum with L absorbed more than R; band 2 has a negative CD spectrum with R absorbed more than L; band 3 is due to an achiral chromophore.



CD instruments (known as spectropolarimeters) measure the difference in absorbance between the L and R circularly polarized components ($\Delta A = A_L - A_R$), but will generally report this in terms of the ellipticity (θ) in degrees. It should be noted that $\theta = \tan^{-1} (b/a)$ where b and a are the minor and major axes of the resulting ellipse. There is a simple numerical relationship between ΔA and ellipticity (in degrees), namely $\theta = 32.98 \Delta A$. The CD spectrum is obtained when the dichroism is measured as a function of wavelength.



Absorption in this region (240 nm and below) is due principally to the peptide bond; there is a weak but broad $n \rightarrow \pi^*$ transition centered around 220 nm and a more intense $\pi \rightarrow \pi^*$ transition around 190 nm. In certain cases aromatic amino acid side chains can also contribute significantly in this spectral range. The different types of regular secondary structure found in proteins give rise to characteristic CD spectra in the far UV. A number of algorithms exist which use the data from far UV CD spectra to provide an estimation of the secondary structure composition of proteins. Most procedures employ basis datasets comprising the CD spectra of proteins of various fold types whose structures have been solved by X-ray crystallography. An online server DICHROWEB has been developed, hosted at Birkbeck College, University of London, U.K. which allows data to be entered in a number of formats including those from the major CD instrument manufacturers, and to be analysed by the various algorithms with a choice of databases.



Far UV CD spectra associated with various types of secondary structure. Solid line, α -helix; long dashed line, anti-parallel β -sheet; dotted line, type I β -turn; cross dashed line, extended 3_1 -helix or poly (Pro) II helix; short dashed line, irregular structure.

CD data are presented in terms of ellipticity $[\theta]$ (degrees). The data are normalized by scaling to molar concentrations of either the whole molecule or the repeating unit of a polymer.

For far UV CD of proteins, the repeating unit is the peptide bond. The Mean Residue Weight (MRW) for the peptide bond is calculated from $MRW = M/(N - 1)$, where M is the molecular mass of the polypeptide chain (in Da), and N is the number of amino acids in the chain; the number of peptide bonds is $N - 1$. For most proteins the MRW is 110 ± 5 Da.

The mean residue ellipticity at wavelength λ ($[\theta]_{mrw,\lambda}$) is given by:

$$[\theta]_{mrw,\lambda} = MRW \times \theta_{\lambda} / 10 \times d \times c$$

where θ_{λ} is the observed ellipticity (degrees) at wavelength λ , d is the pathlength (cm), and c is the concentration (g/ml).

If we know the molar concentration (m) of a solute, the molar ellipticity at wavelength λ ($[\theta]_{molar,\lambda}$) is given by:

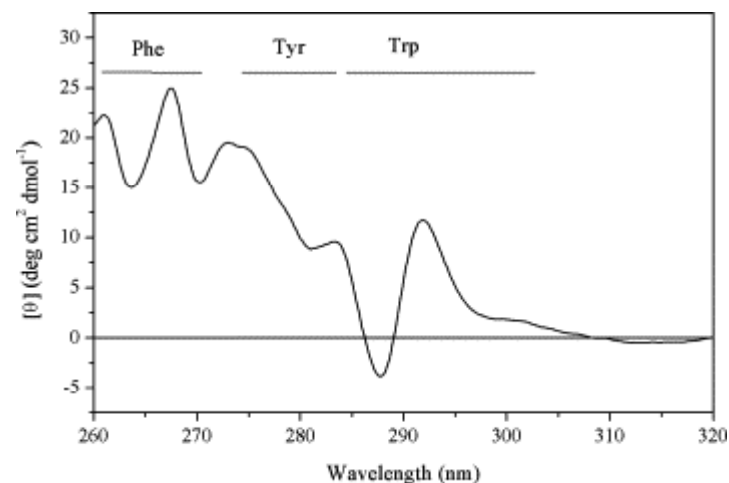
$$[\theta]_{molar,\lambda} = 100 \times \theta_{\lambda} / m \times d$$

where θ_{λ} and d have the same meaning as above.

The units of mean residue ellipticity and molar ellipticity are $\text{deg cm}^2 \text{ dmol}^{-1}$

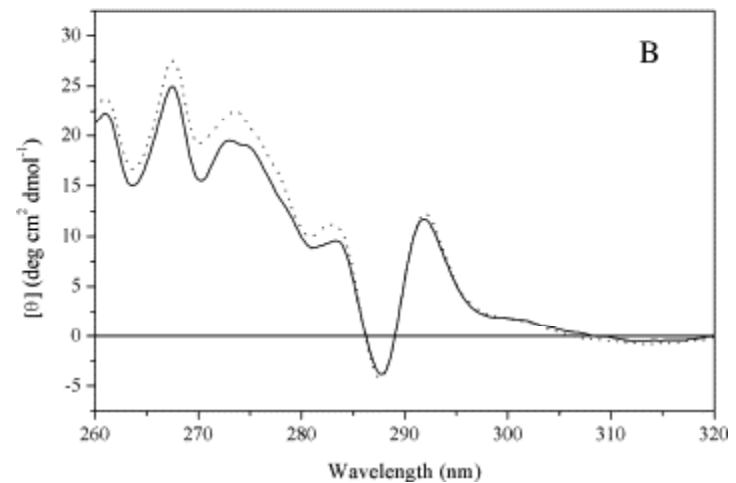
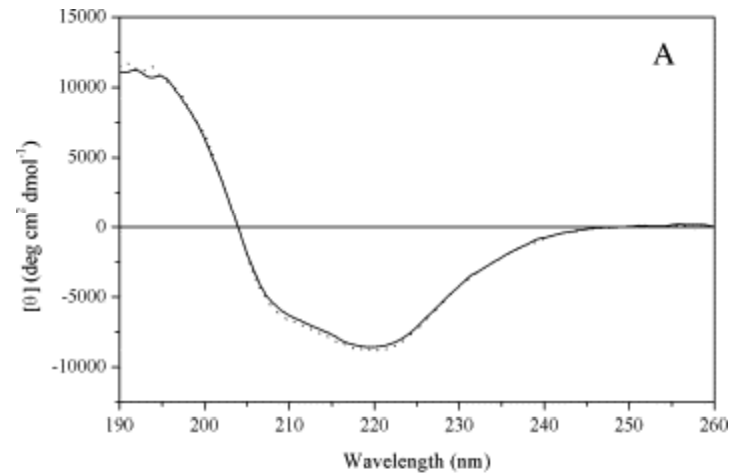
The spectra in the region 260–320 nm arise from the aromatic amino acids. Each of the amino acids tends to have a characteristic wavelength profile. Trp shows a peak close to 290 nm with fine structure between 290 and 305 nm; Tyr a peak between 275 and 282 nm, with a shoulder at longer wavelengths often obscured by bands due to Trp; Phe shows weaker but sharper bands with fine structure between 255 and 270 nm. The fine structure in these bands arises from vibronic transitions in which different vibrational levels of the excited state are involved.

The actual shape and magnitude of the near UV CD spectrum of a protein will depend on the number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups and polarizability). The theoretical treatment of near UV CD spectra is not sufficiently advanced to yield significant structural insights. The near UV CD spectrum of a protein provides a valuable fingerprint of the tertiary structure of that protein, which can be used to compare, for example, wild type and mutant forms of proteins. It can also provide important evidence for the existence of molten globule states in proteins, which are characterized by very weak near UV CD signals, reflecting the high mobility of aromatic side chains.

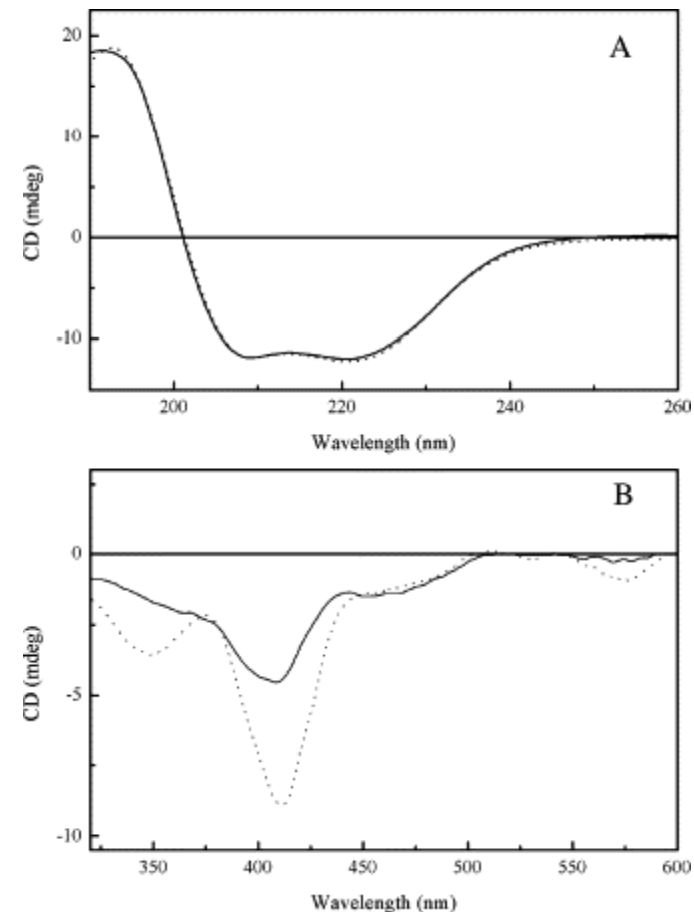


spectrum for type II dehydroquinase. It should be emphasized that there can be considerable overlap between the Tyr and Trp signals.

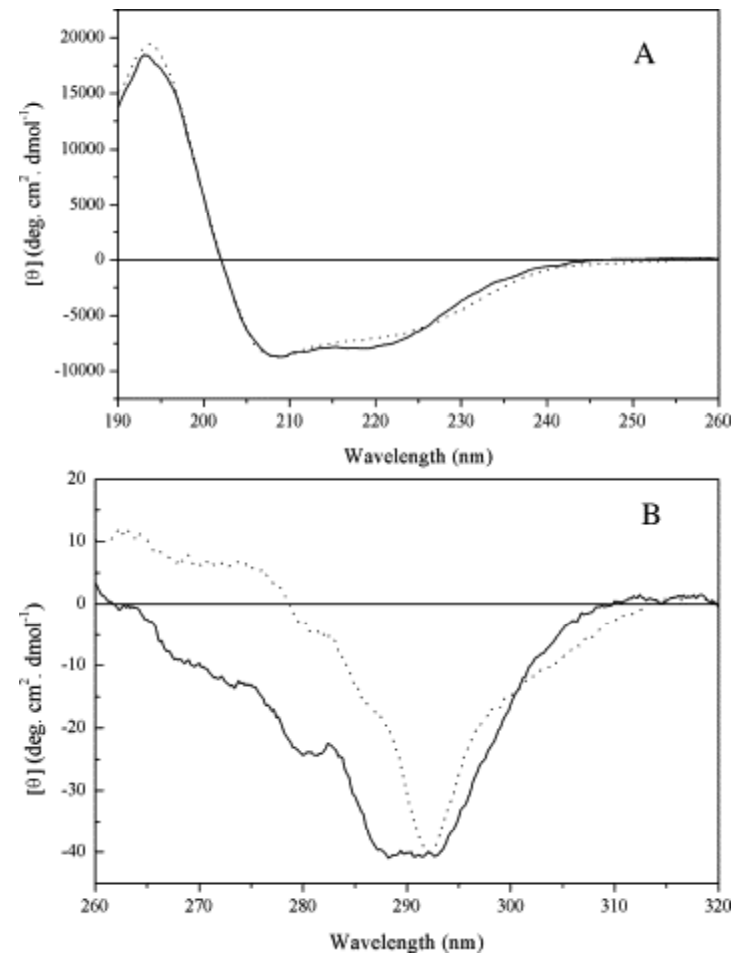
CD spectra of wild type and mutant (R23Q) type II dehydroquinase. The far UV spectrum (panel A) and near UV spectrum (panel B) show that the wild-type (solid line) and mutant (dotted line) enzymes have very similar secondary and tertiary structures. The loss of catalytic activity in the mutant cannot therefore be due to an inability to acquire the correct folded structure.



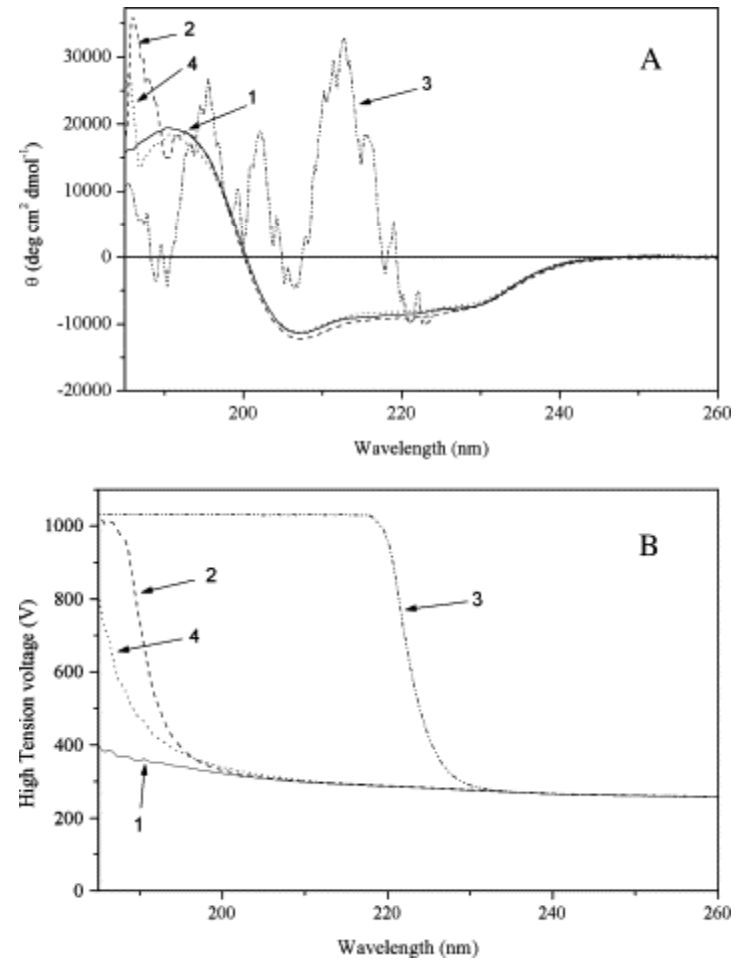
CD spectra of intact cytochrome P450 BM3 from *Bacillus megaterium* and a mixture of its constituent domains expressed as recombinant proteins in *E. coli*. Panels A and B show the far UV and near UV/visible CD spectra respectively for the intact BM3 enzyme (solid line) and an equimolar mixture of the purified P450 and reductase domains (dotted line). The protein solutions are all present at 9.45 μM and the cell pathlengths are 0.02 cm and 0.5 cm for panels A and B respectively. Note that although panel A shows that the secondary structures of the individual domains in the intact enzyme are retained when they are separately expressed, it is clear from panel B that the environment of the haem group (signal at 410 nm) is modified in the intact BM3 enzyme.



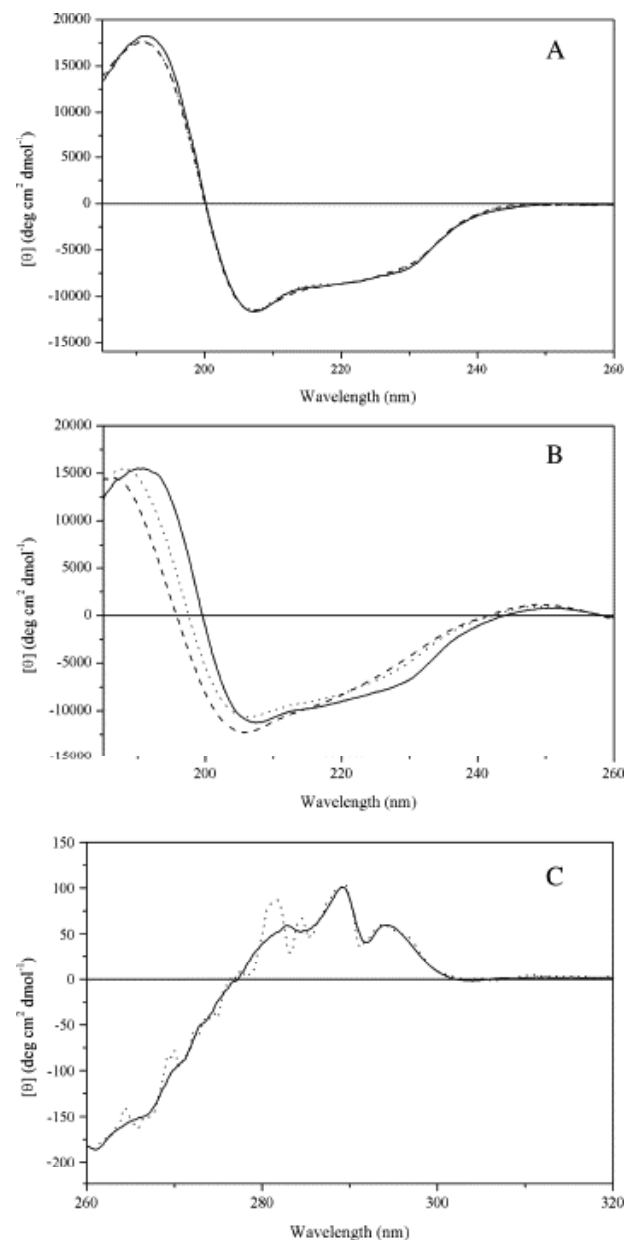
CD spectra of the molybdate-sensing protein ModE from *E. coli* in the absence (solid line) and presence (dotted line) of 1 mM molybdate. Panels A and B show the far UV and near UV CD spectra respectively. The far UV CD spectra show that there is relatively little change in secondary structure on binding ligand. The marked changes in the near UV CD spectrum (signal at 292 nm) on addition of molybdate have been shown to be largely due to changes in the environment of one of the 3 Trp residues (Trp 186) in ModE.



The effects of buffer components on far UV CD spectra. Lysozyme (0.2 mg/ml) was dissolved in 50 mM sodium phosphate buffer, pH 7.5 (spectrum 1, solid line), or sodium phosphate buffer containing either 150 mM NaCl (spectrum 2, dashed line) or 150 mM imidazole (spectrum 3, dash-dot-dot line), or in 50 mM Tris/acetate, pH 7.5 (spectrum 4, dotted line). Spectra were recorded in a 0.02-cm pathlength cell on a Jasco J-810 spectropolarimeter using a scan speed of 50 nm/min, a time constant of 0.5 s and a bandwidth of 1 nm. 8 scans were accumulated. Panel A shows the CD spectra and the panel B the corresponding High Tension voltage traces.



The effects of instrument parameters on CD spectra. The spectra shown are those of lysozyme (0.5 mg/ml) in 50 mM sodium phosphate buffer, pH 7.5. Panels A and B show far UV CD spectra recorded in a cell of 0.02 cm pathlength on Jasco J-810 and J-600 spectropolarimeters, respectively. The solid line, dotted line and dashed line refer to time constants and scan rates of 0.5 s and 50 nm/min, 2 s and 50 nm/min, and 2 s and 100 nm/min, respectively. In each case, the bandwidth was 1 nm and 8 scans were accumulated. Panel C shows the near UV CD spectrum recorded in a cell of 0.5 cm pathlength on a Jasco J-810 spectropolarimeter. The solid and dotted lines refer to accumulations of 20 scans and 1 scan, respectively; the time constant, scan rate and bandwidth were 0.5 s, 50 nm/min, and 1 nm, respectively.



The effects of protein concentration on CD spectra. The spectra shown are those of lysozyme in 50 mM sodium phosphate buffer, pH 7.5 and are recorded on a Jasco J-810 spectropolarimeter using 50 nm/min scan speed, 0.5 s time constant, 1 nm bandwidth and accumulation of 8 scans in each case. The solid, dotted and dashed lines refer to protein concentrations of 0.2 mg/ml, 1.0 mg/ml, and 5.0 mg/ml, respectively. Panel A shows the far UV CD spectra and panel B the corresponding High Tension voltage traces. Panel C shows the near UV CD spectra and panel D the corresponding High Tension voltage traces.

