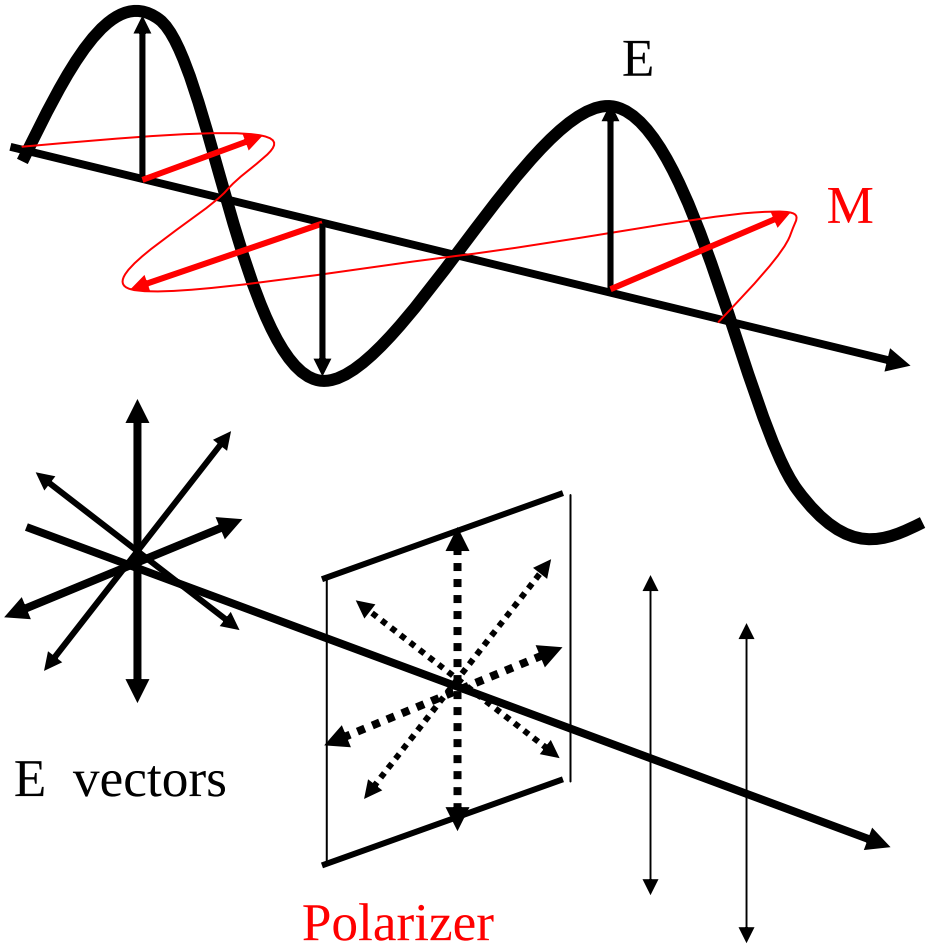
圆二色谱Circular Dichroism（CD)

主要内容

- CD原理
- 蛋白质CD谱
- CD实验要点

CD原理

平面偏振光 (Plane polarized light)



平面偏振光

振动方向保持不变

振幅发生周期性变化

旋光色散(Optical rotatory dispersion)

- 一束平面偏振光通过光学活性分子后，由于左、右圆偏振光的折射率不同，偏振面将旋转一定的角度，这种现象称为旋光(Optical rotation)，偏振面旋转的角度称为旋光度。

- 朝光源看，偏振面按顺时针方向旋转的，称为右旋，用“+”号表示；偏振面按逆时针方向旋转的，称为左旋，用“-”号表示

旋光度

- **$\alpha = [\alpha]lc$**

  [α]是旋光物质的比旋光率，单位是度•厘米^{2} • 10克$^{-1}$

- **对同一物质，[α]值与波长有关**

  旋光率与波长的关系称为旋光色散(Optical rotatory dispersion, ORD)

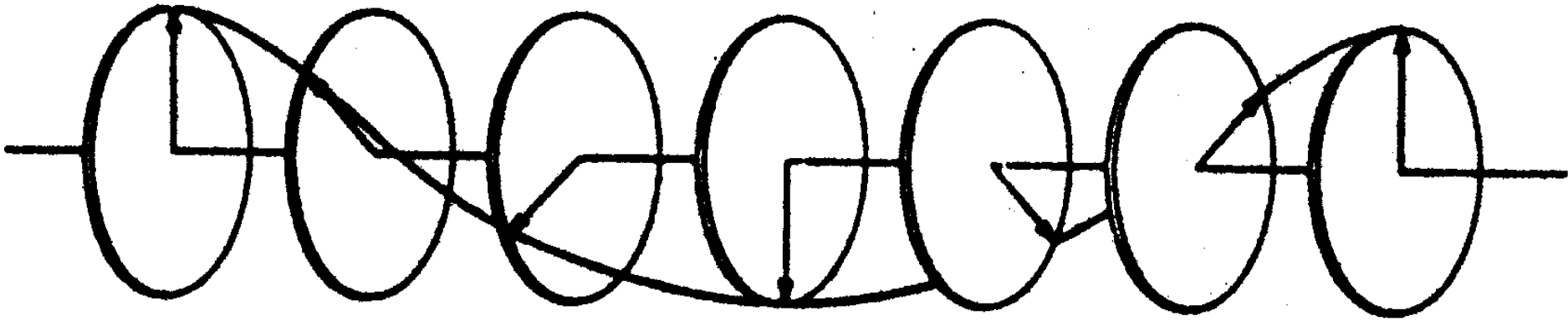
旋光色散

- 旋光色散常用摩尔比旋[Φ]表示。

$$[\Phi] = [\alpha]*M/100$$

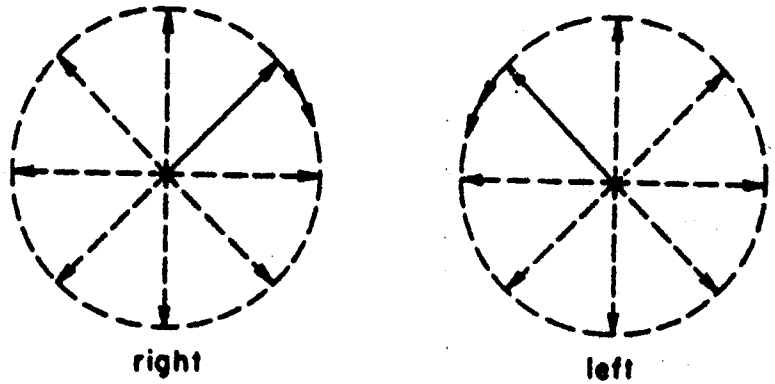
M为旋光介质的分子量

圆偏振光(Circular polarized light)



振幅保持不变，而方向周期变化，
电场矢量绕传播方向螺旋前进

圆偏振光



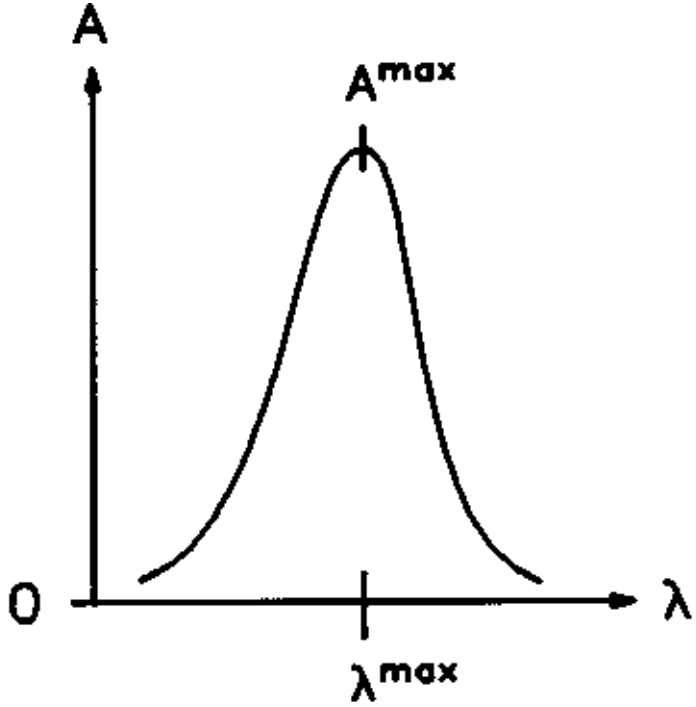
朝光源看，

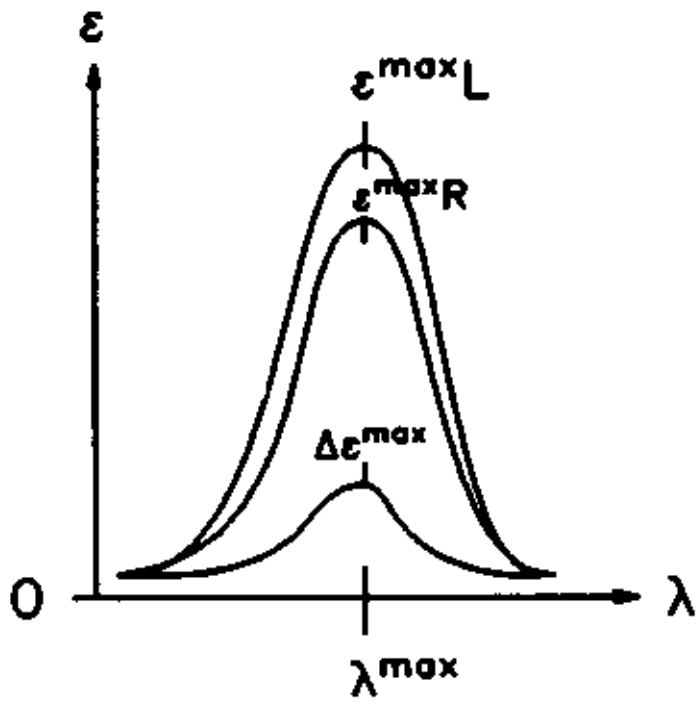
电场矢量方向按顺时针方向旋转的，称为右圆偏振光；

电场矢量方向按逆时针方向旋转的，称为左圆偏振光。

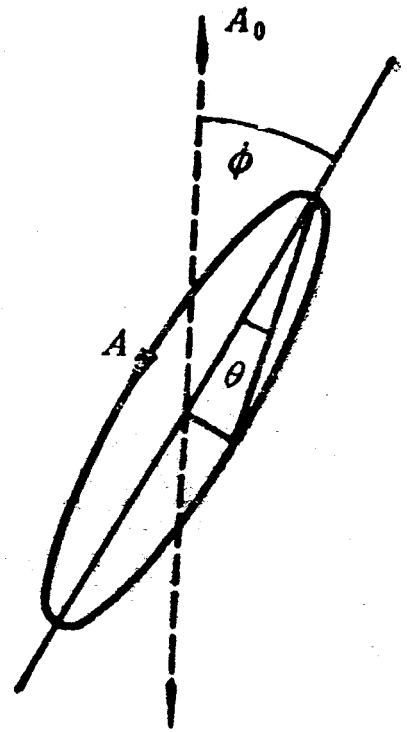
圆二色性(circular dichroism, CD)

- 光学活性分子对左、右圆偏振光的吸收也不同，使左、右圆偏振光透过后变成椭圆偏振光，这种现象称为圆二色性。


A
A^{max}
0
λ
λ^{max}


ε
$\varepsilon^{max}L$
$\varepsilon^{max}R$
$\Delta\varepsilon^{max}$
0
λ
λ^{max}

圆二色性的表示



- 吸收(率)差

  $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$

  $\Delta A = A_L - A_R$

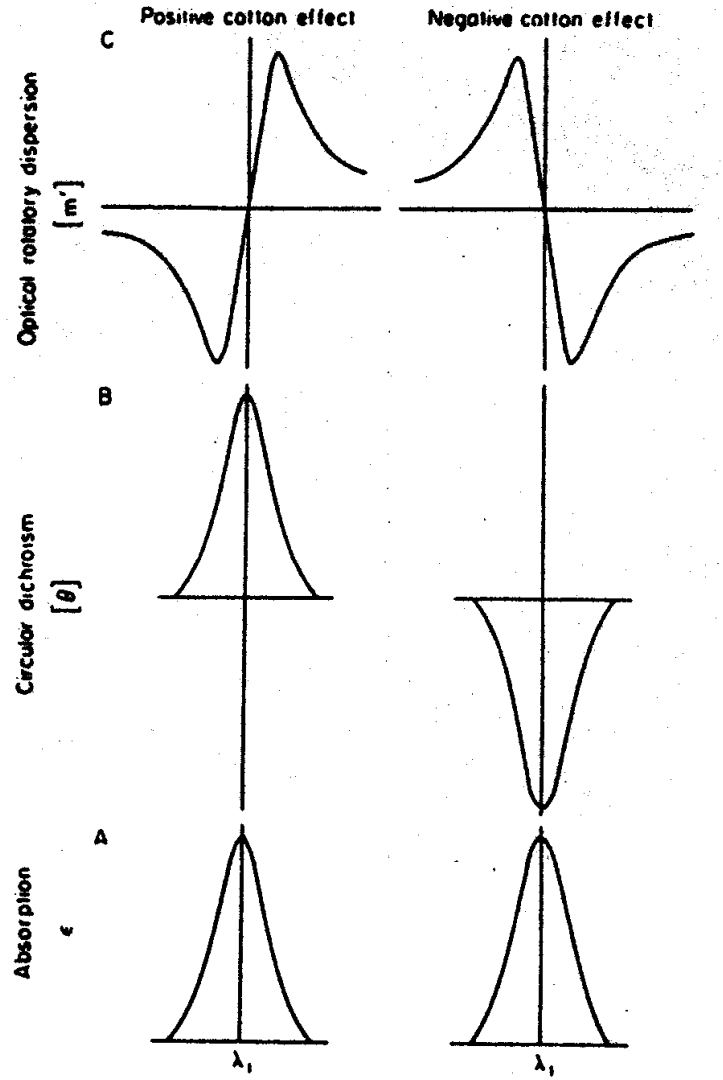
- 椭圆度θ，摩尔椭圆度[θ]

  $\theta = 2.303(A_L - A_R)/4$

  $[\theta] = 3298(\varepsilon_L - \varepsilon_R) \approx 3300\ (\varepsilon_L - \varepsilon_R)$

在蛋白质研究中，常用平均残基摩尔椭圆度

旋光色散与圆二色性

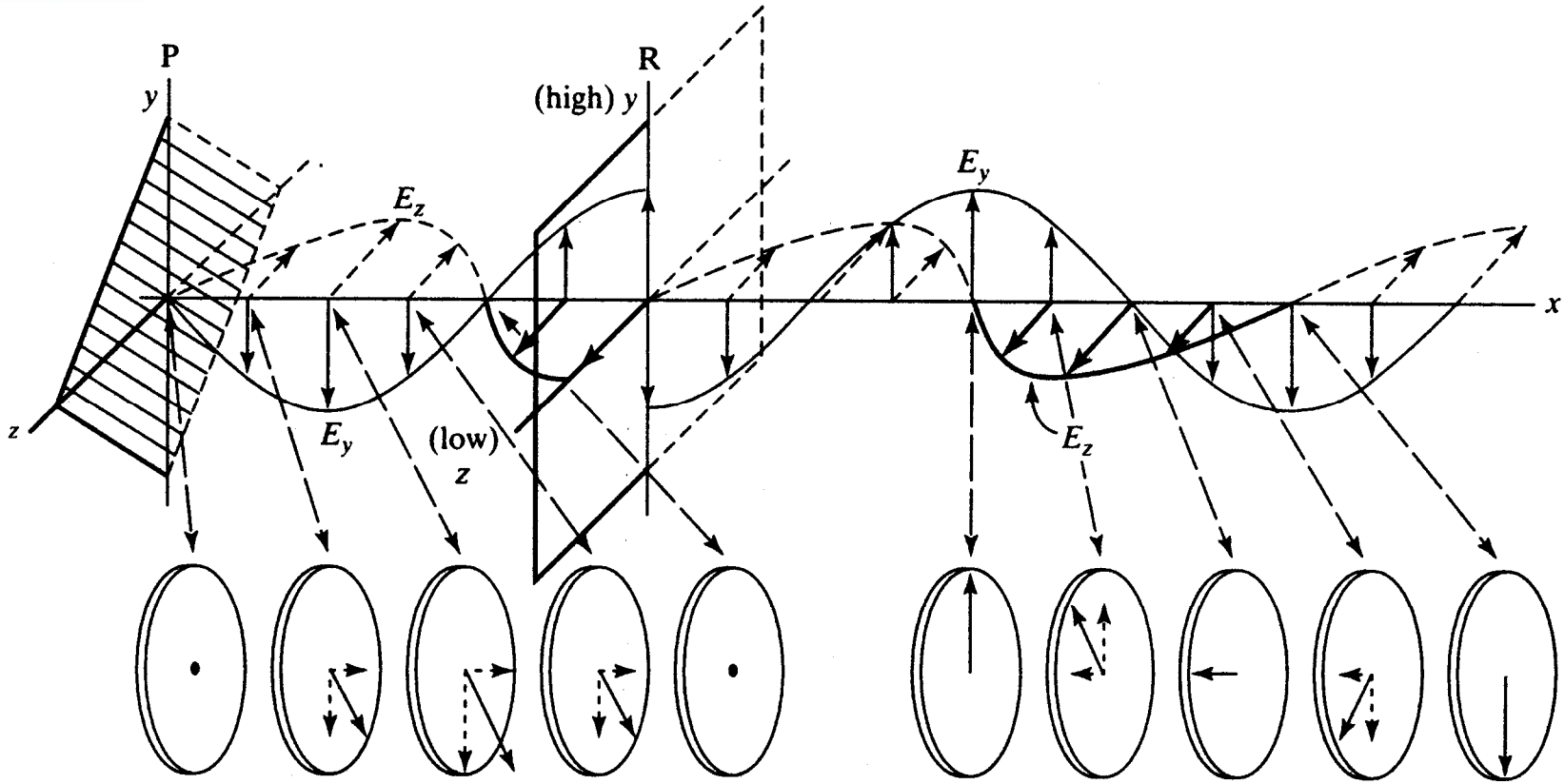

Positive cotton effect
Negative cotton effect
C
Optical rotatory dispersion
[m']
B
Circular dichroism
[θ]
A
Absorption
ε
λ1
λ1

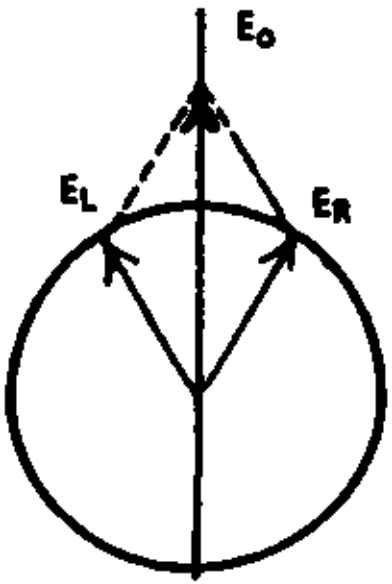
叠加原理

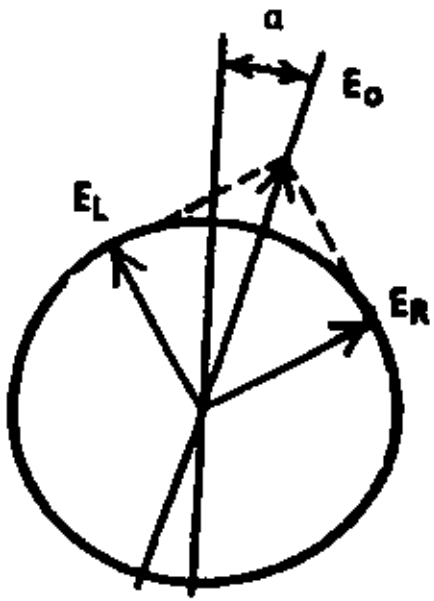
- 一束*自然光*可以分解或看作两束相互垂直而没有相位关系的平面偏振光的加和。

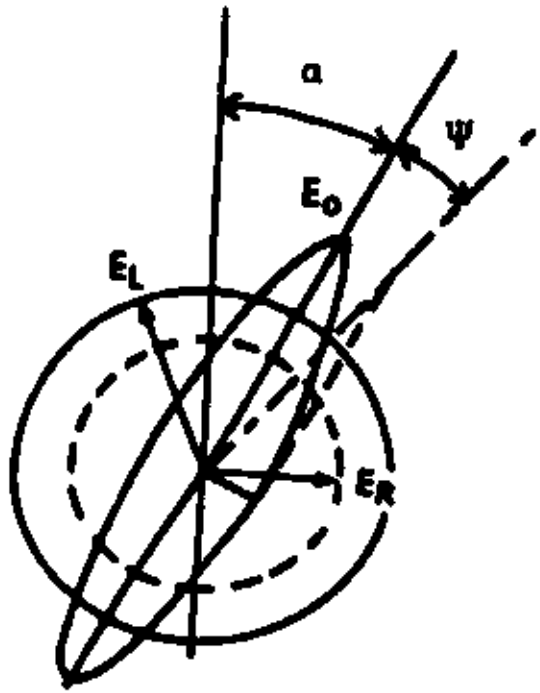
- *平面偏振光*可以分解成两束相位相等而旋转方向相反的圆偏振光的加和。
- 当振幅相等，并同步的左、右圆偏振光相加，则产生平面偏振光；如果这两束*圆偏振光*的振幅不等则产生*椭圆偏振光*(elliptically polarized light)

- 两束相互垂直而相位相差1/4波长的平面偏振光可以加和成一束*圆偏振光*


P
y
R
(high) y
E_z
E_y
E_y
(low)
z
E_z
z
x


E_O
E_L
E_R


a
E_O
E_L
E_R


a
Ψ
E_O
E_L
E_R

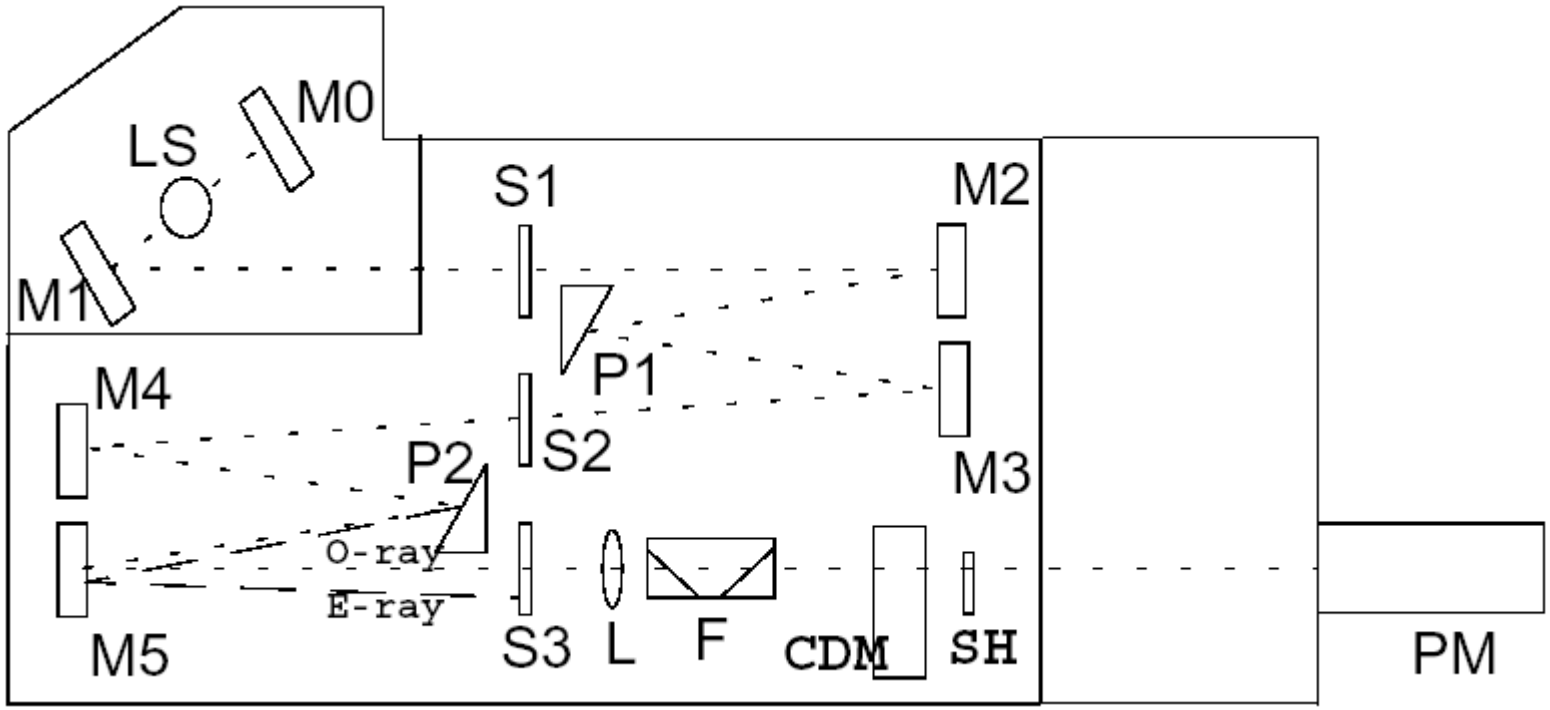
旋光色散与圆二色相互转换

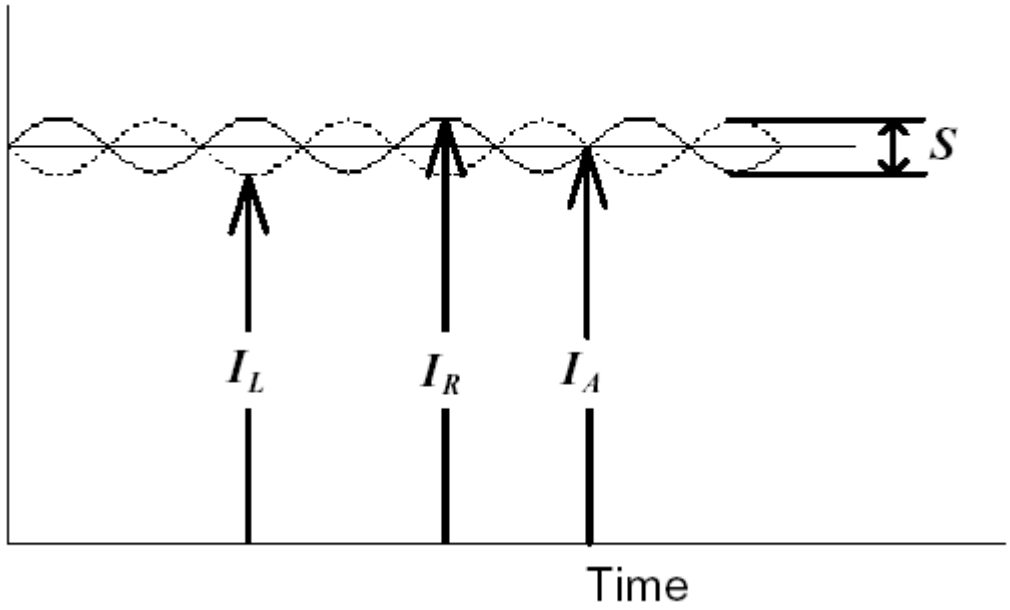
- 旋光色散和圆二色是同时产生的，他们包括同样的分子结构信息，并且可以由Kronig-Krammers转换方程相互转换。

$$[\Phi]_\lambda = \frac{2}{\pi}\int_0 \frac{[\theta]_\lambda \lambda_i'}{(\lambda^2 - \lambda_i^2)} d\lambda_i$$

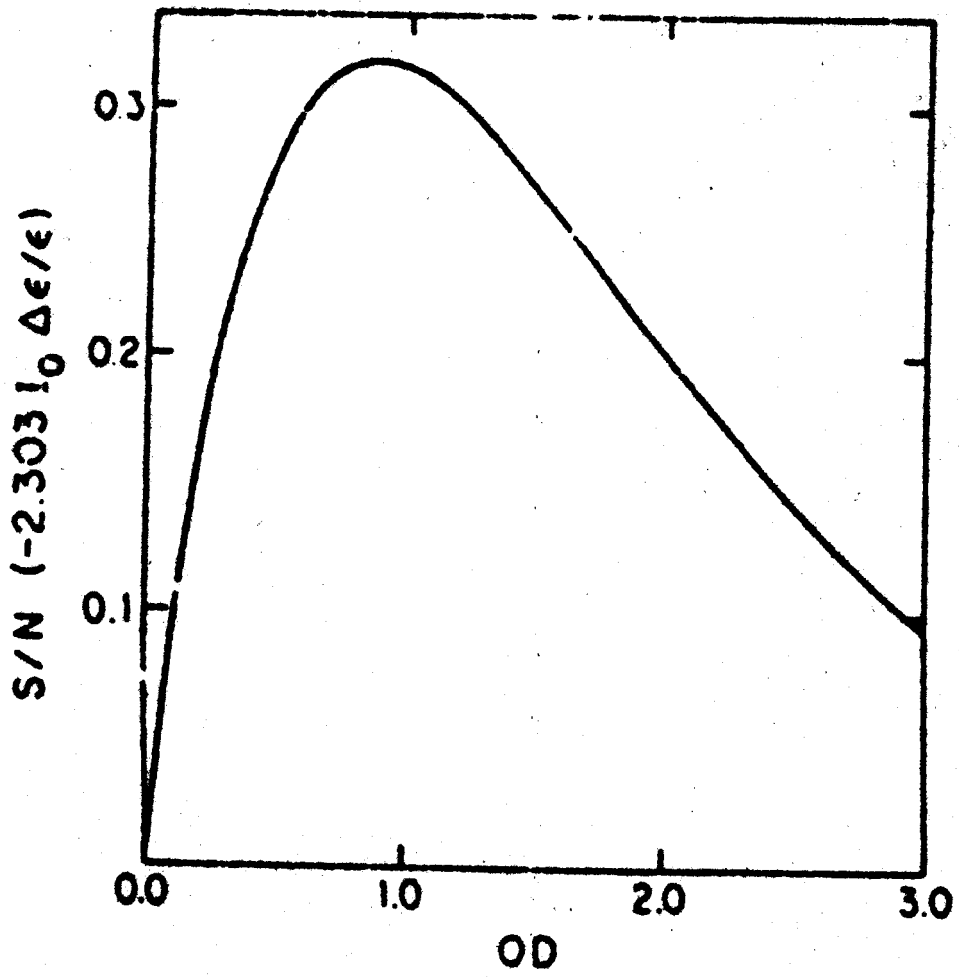
$$[\theta]_\lambda = -\frac{2}{\pi\lambda}\int_0^\infty \frac{[\Phi]\lambda_i \lambda_i^2}{(\lambda^2 - \lambda_i^2)} d\lambda_i$$

圆二色仪原理




S
I_L
I_R
I_A
Time

圆二色谱的噪音



CD实验的最佳吸光度在0.8左右

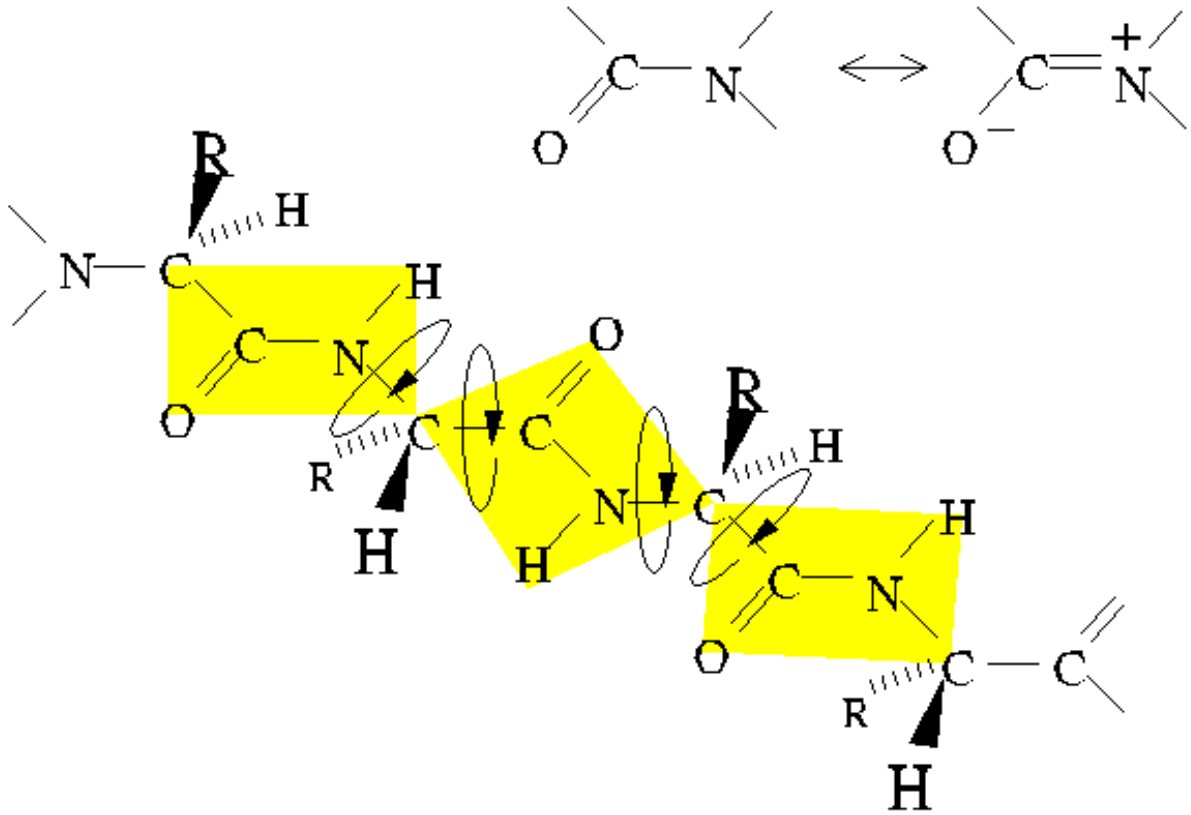
CD特点

- **CD signal is a very small difference between two large originals**.
- CD is only observed at wavelengths where absorbances of R & L components of circularly polarized light are not zero i.e. in absorption bands.

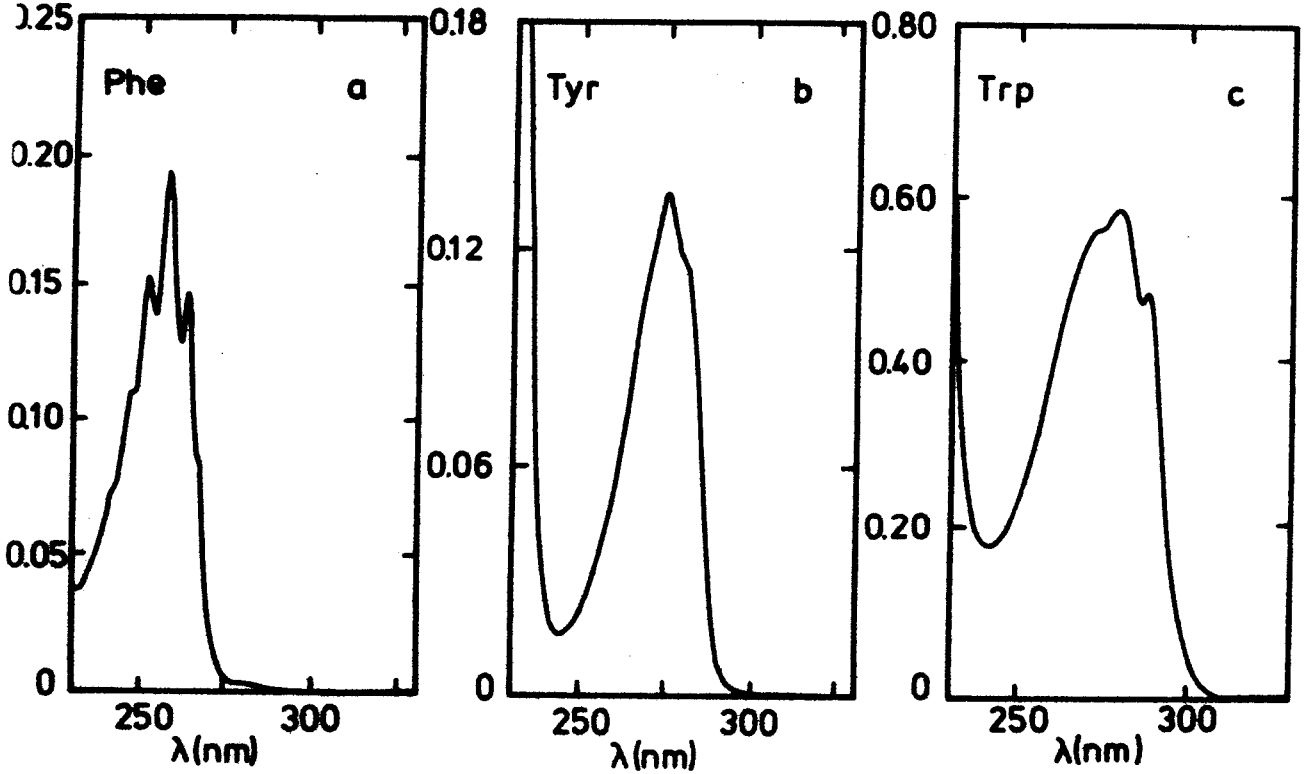
- In general $\Delta\varepsilon$ is much more conformation dependent than ε

蛋白质的CD谱

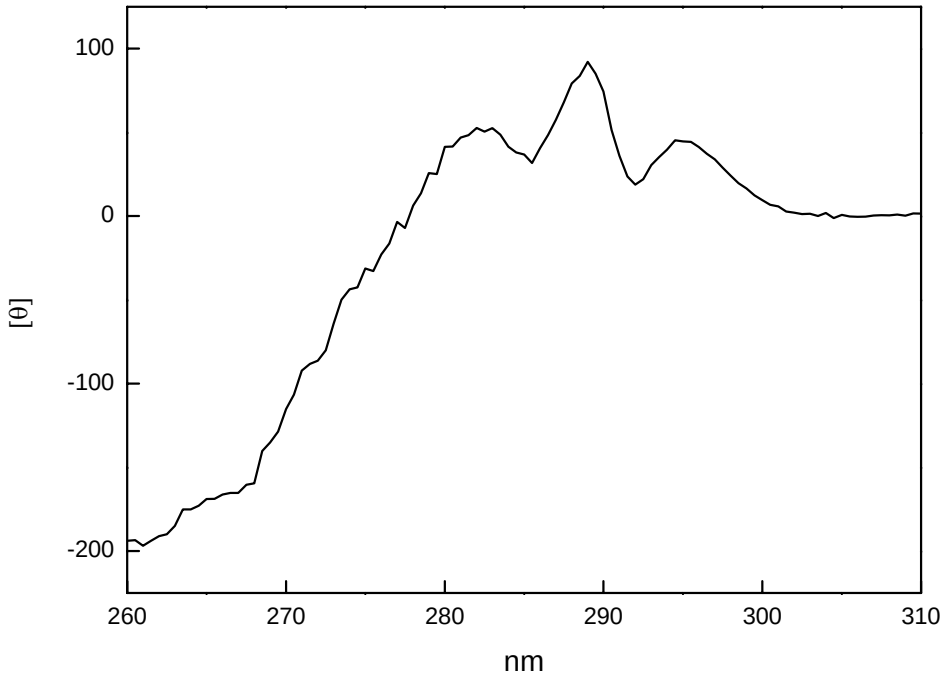
蛋白质的光学活性



The peptide bond is inherently asymmetric & is always optically active


0.25
0.20
0.15
0.10
0.05
0
Phe
a
250
300
λ(nm)
0.18
0.12
0.06
0
Tyr
b
250
300
λ(nm)
0.80
0.60
0.40
0.20
0
Trp
c
250
300
λ(nm)

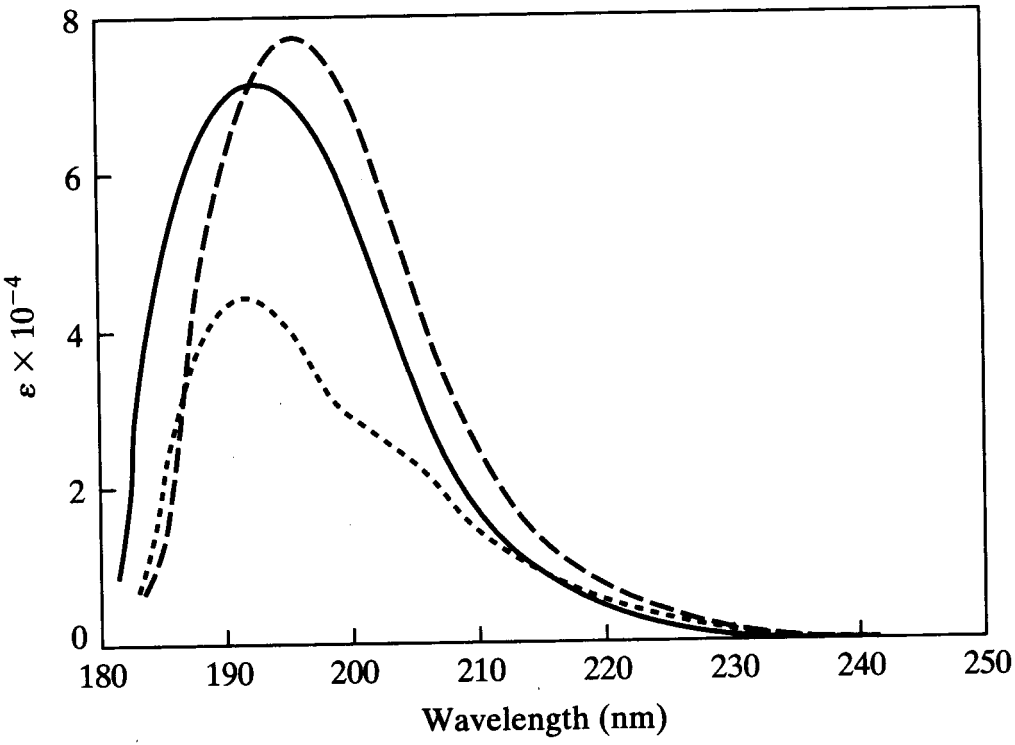
Near UV CD spectrum of Lysozyme

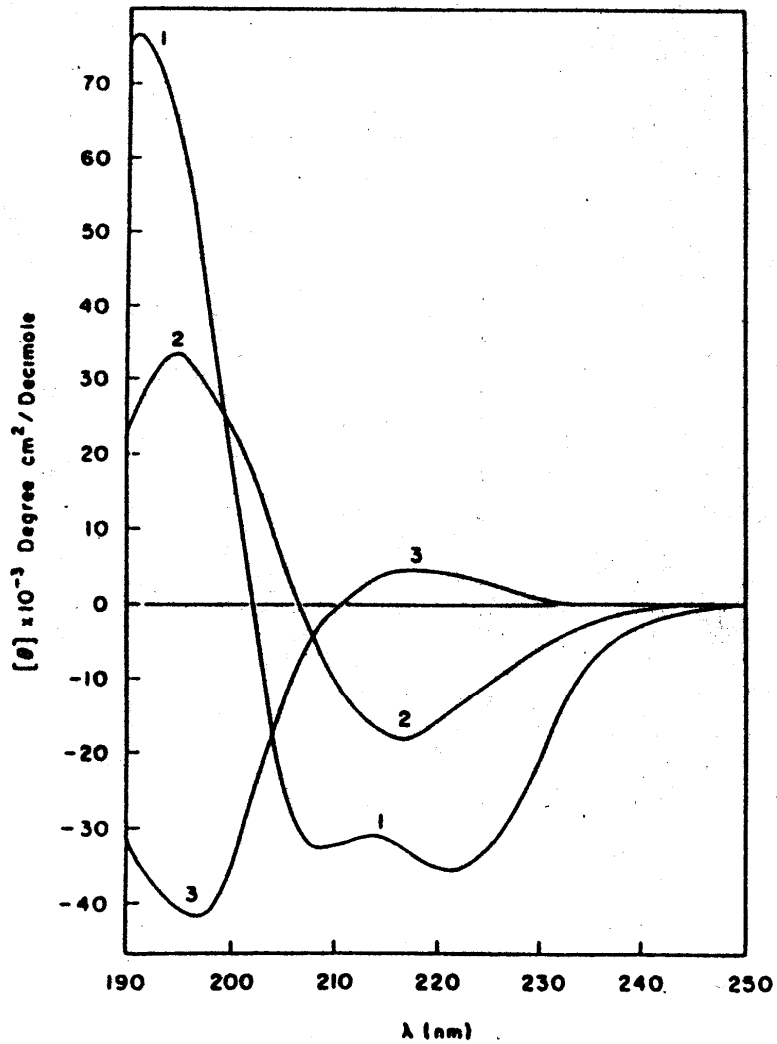


蛋白质的CD谱

- **CD spectra in the far UV region (180 nm – 250 nm) probes the secondary structures of proteins.**
- **CD spectra in the near UV region (~250 and ~ 350) monitors the side chain tertiary structures of proteins.**

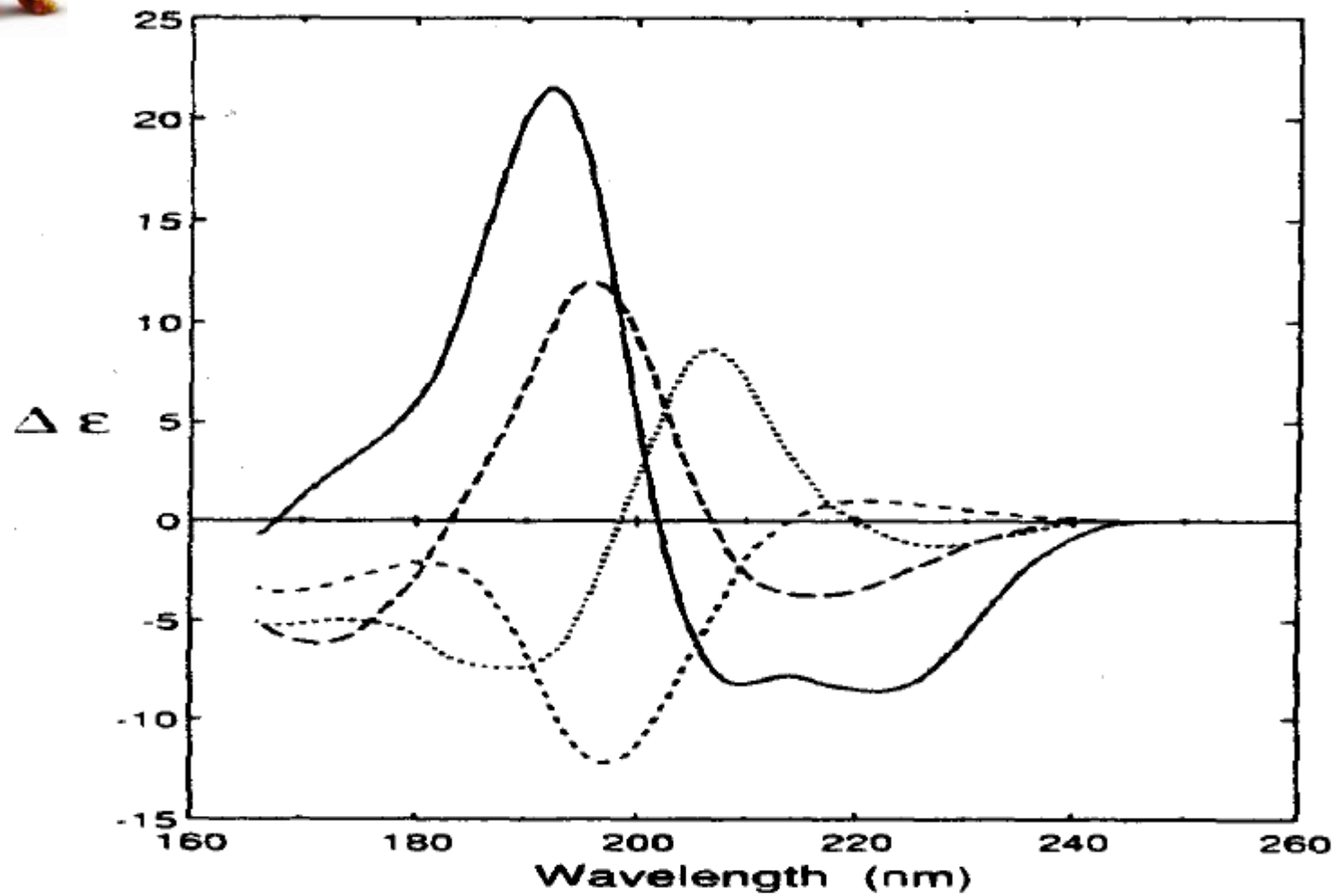
Far UV CD spectra of poly-L-Lys

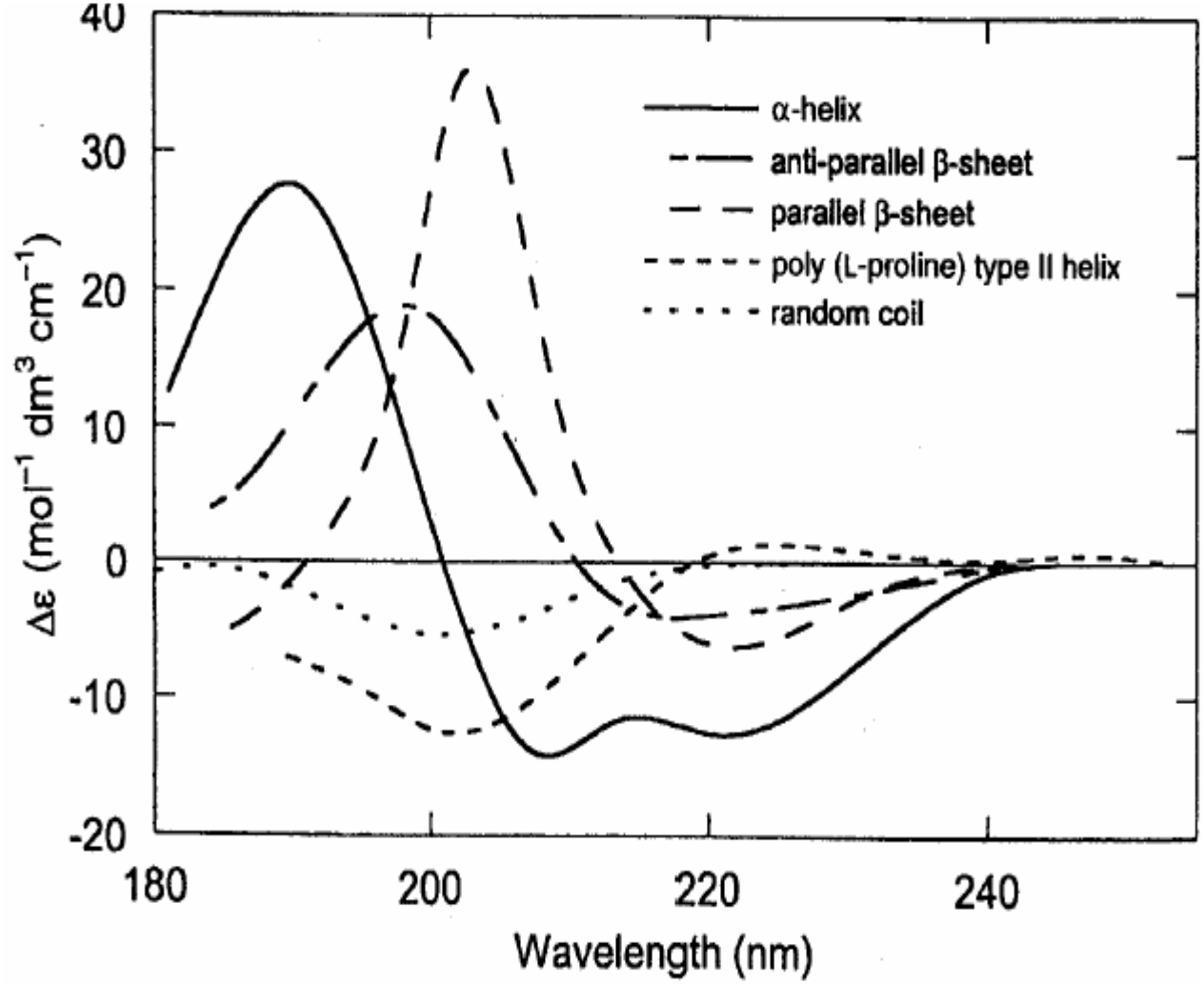




Main CD features of protein 2ndary structures

	- band (nm)	+ band (nm)
α -helix	222 208	192
β -sheet	216	195
β -turn	220-230 (weak) 180-190 (strong)	205
polypro II helix	190	210-230 weak
Random coil	200	212


25
20
15
10
5
0
-5
-10
-15
Δ ε
160
180
200
220
240
260
Wavelength (nm)


40
30
20
10
0
-10
-20
180
200
220
240
Wavelength (nm)
Δε (mol^{-1} dm^3 cm^{-1})
α-helix
anti-parallel β-sheet
parallel β-sheet
poly (L-proline) type II helix
random coil

估算蛋白质α螺旋含量

$$\%\,\alpha Helix = \frac{[\theta]_{222} - [\theta]^{R}_{222}}{-38000}$$

$$\%\,\alpha Helix = \frac{-[\theta]_{208} - 4000}{33000 - 4000}$$

仅适合α含量较高的蛋白质！

If we measure the CD signal for a protein of unknown structure we can find its proportion of 2ndry structures

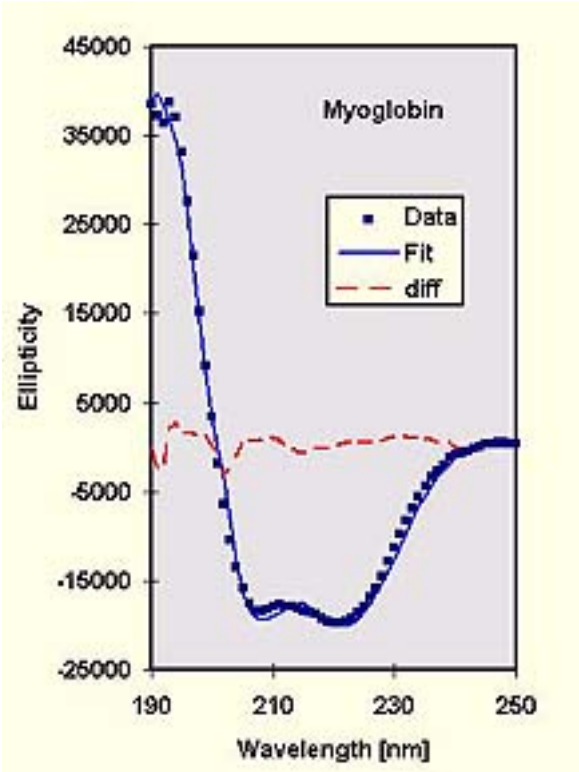
- Fit the unknown curve θ_u to a combination of standard curves.
- In the simplest case use the Fasman standards

$$\theta_t = x_\alpha\theta_\alpha + x_\beta\theta_\beta + x_c\theta_c$$

- Vary x_α, x_β and x_c
  to give the best fit of θ_t to θ_u
  while $x_\alpha + x_\beta + x_c = 1.0$

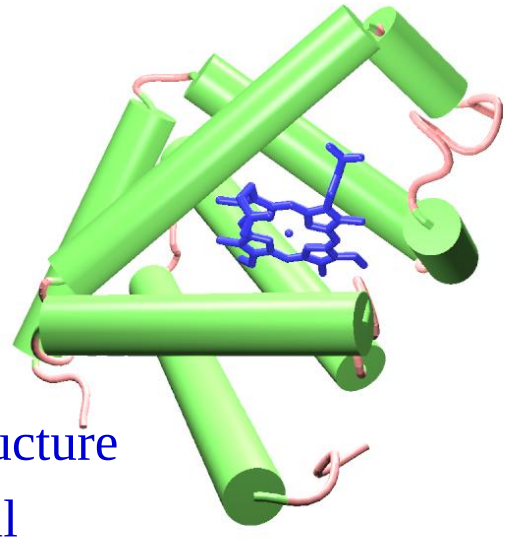
- Do this by least squares minimization

Example fit: myoglobin



- In this case:
- $\theta_t = x_\alpha\theta_\alpha + x_\beta\theta_\beta + x_c\theta_c$
- fits best with

$x_\alpha = 80\%$,
$x_\beta = 0\%$
$x_c = 20\%$



- agrees well with structure

78% helix, 22% coil

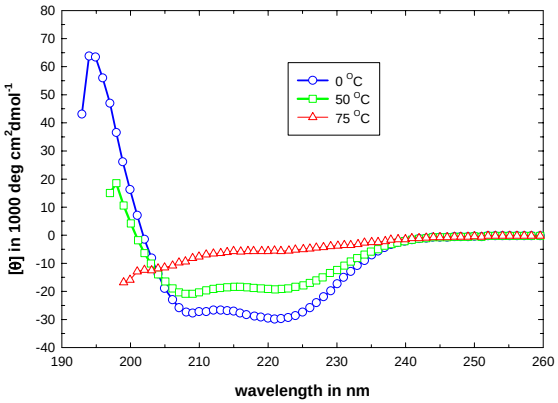
For further details:

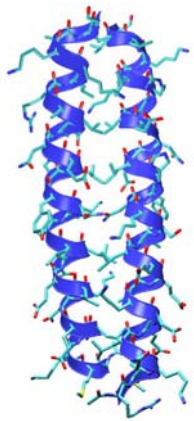
www-structure.llnl.gov/cd/cdtutorial.htm

Example fit (2): GCN4-p1

CD signals for GCN4-p1

O'Shea *et al.* Science (1989) **243**:538
figure 3: 34μM GCN4-p1 in 0.15M NaCl,
10mM phosphate pH 7.0





- At 0°C 100% helix

  75°C 0% helix

- Q: what about 50°C?

- $\theta_t = x_o\theta_o + x_{75}\theta_{75}$

  its best with

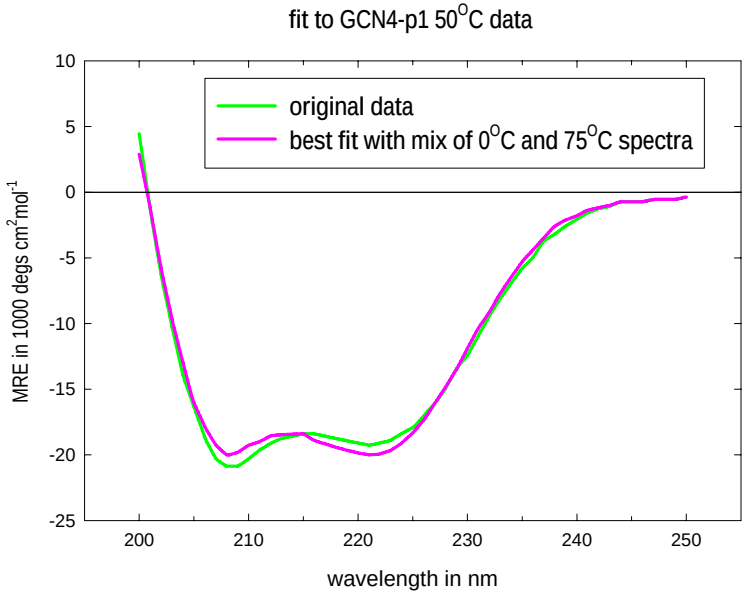
  $x_o = 50\%$,

  $x_{75} = 50\%$

- Shows that at 50°C

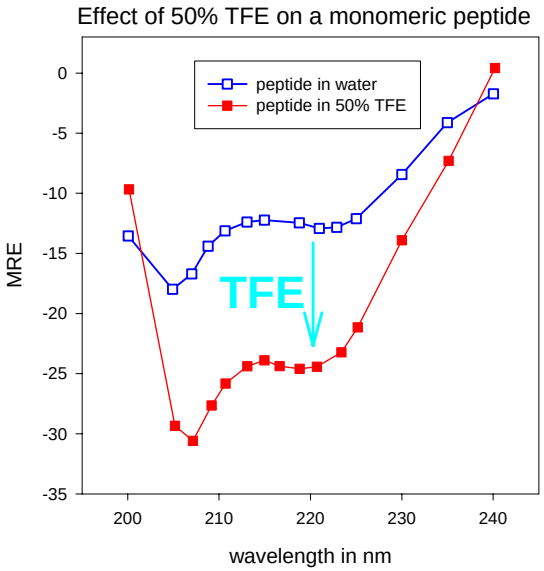
  1/2 of peptide α-helix dimer

  1/2 of peptide random coil monomer

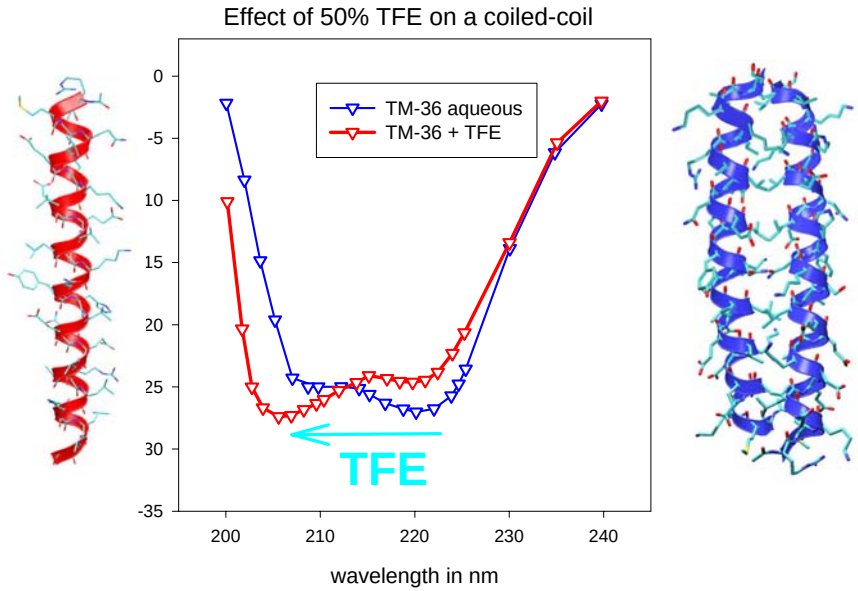


CD signals for same secondary structure can vary (a bit) with environment

- Can see this by looking at the effect of trifluoroethanol (TFE) on a coiled-coil similar to GCN4-p1
- TFE induces helicity in all peptides



- But on a coiled-coil breaks down helical dimer to single helices



- Although 2ndry structure same CD changes

Lau, Taneja and Hodges (1984) J.Biol.Chem. **259**:13253-13261

Recommended Methods

- For determination of globular protein conformation in solution: SELCON, CDNN and K2D.
- For determination of polypeptide conformation: LINCOMB with a suitable polypeptide set of references.
- For determining the effects of mutation s, ligands and perturbants on protein structure: LINCOMB.
- For evaluating the number of folding states giving rise to a set of spectra: The CCA algorithm and SVD.

Best fitting procedures use many different proteins for standard spectra

- There are many different algorithms.
- All rely on using up to 20 CD spectra of proteins of known structure.
- By mixing these together a fit spectra is obtained for an unknown.
- For full details see

  Dichroweb: the online CD analysis tool

  www.cryst.bbk.ac.uk/cdweb/html/

- Can generally get accuracies of

  0.97 for helices,

  0.75 for beta sheet,

  0.50 for turns, and

  0.89 for other structure types

(Manavalan & Johnson, 1987, Anal. Biochem. **167**, 76-85).

Determination of Protein Concentration

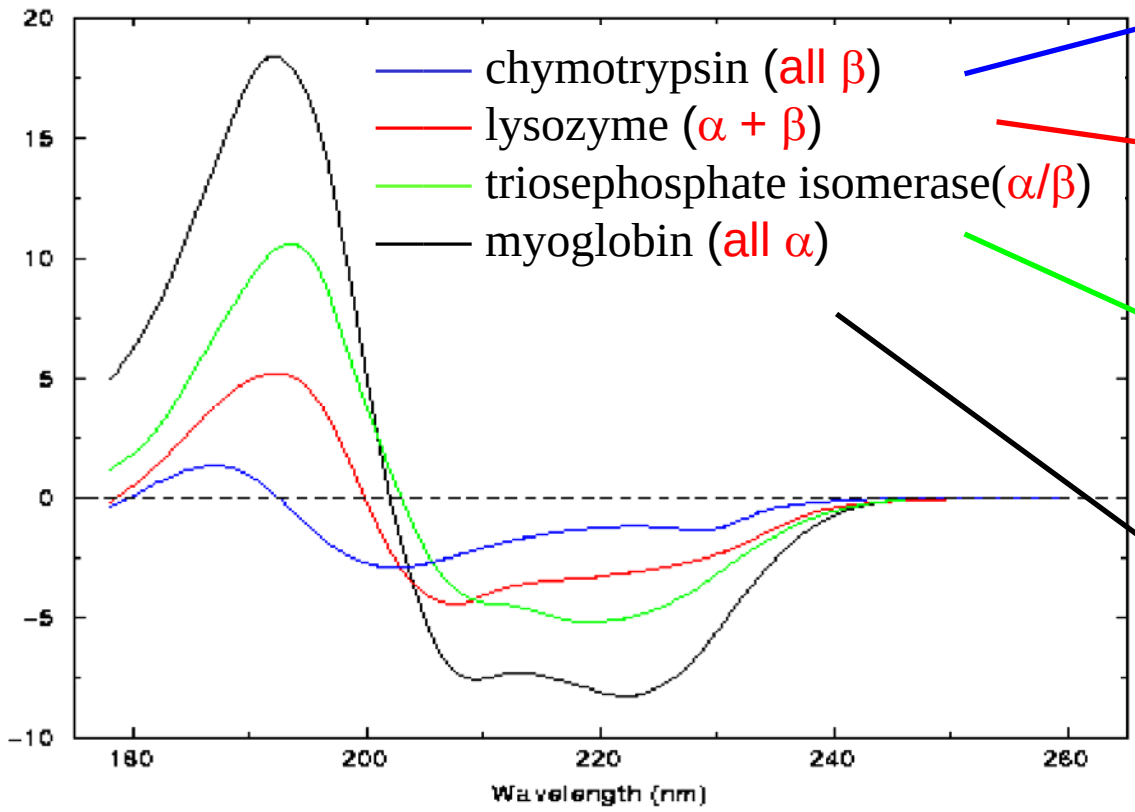
- Good Methods:
  1. Quantitative amino acid composition
  2. Determination of backbone amide groups using the microbiuret method.
  3. Determination of moles of tyrosine using difference spectroscopy under denaturing conditions.
  4. Determination of total nitrogen.

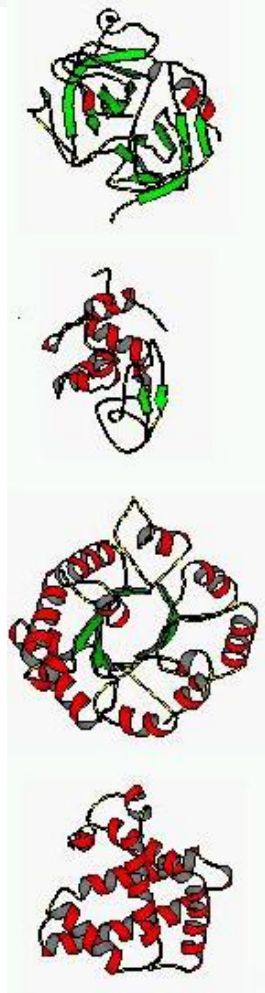
- Not Acceptable:
  1. Bradford Method.
  2. Lowry Method.
  3. Absorbance at 280 and/or 260 nm.

Limitations of CD secondary structure analysis

- The simple deconvolution of a CD spectrum into 4 or 5 components which do not vary from one protein to another is a gross over-simplification.
- The reference CD spectra corresponding to 100% helix, sheet, turn etc are not directly applicable to proteins which contain short sections of the various structures e.g. The CD of an α-helix is known to increase with increasing helix length, CD of β-sheets are very sensitive to environment & geometry.
- Far UV curves (>275nm) can contain contributions from aromatic amino-acids, in practice CD is measured at wavelengths below this.
- The shapes of far UV CD curves depend on tertiary as well as secondary structure.

CD signal of a protein depends on its 2^{ndary} structure





从CD谱分析蛋白质的结构类型

(Venyaminov & Vassilenko)DEF_CLAS.EXE:

对全α、 α/β和变性蛋白质的准确度为100%，

对α + β的准确度为85%，

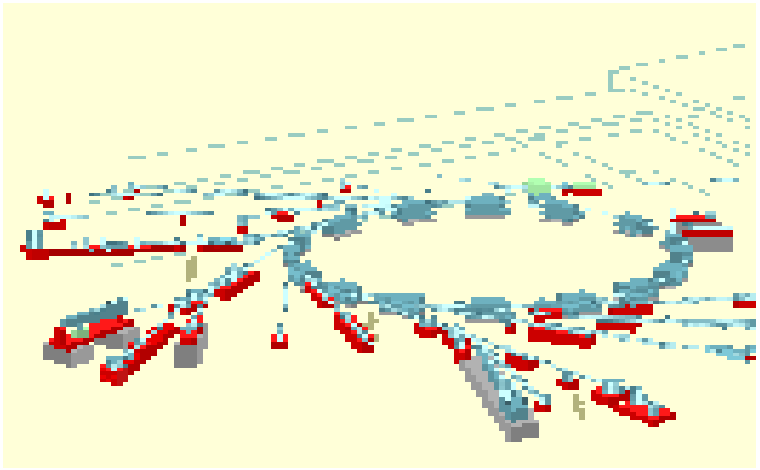
对全β的准确度为75%。

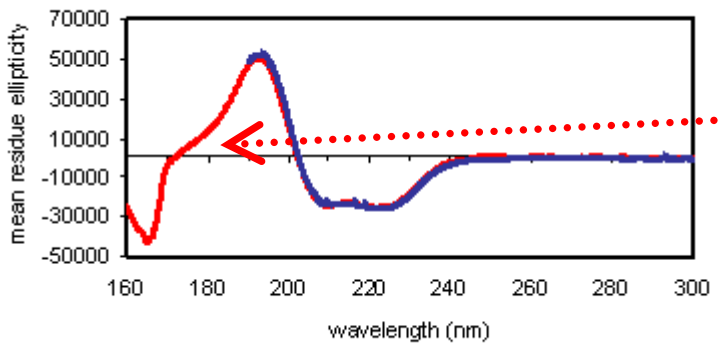
对多肽的判断较差！

CD is very useful for looking at membrane proteins

- Membrane proteins are difficult to study.
- Crystallography difficult - need to use detergents
  Often even when structure obtained:
  Q- is it the same as lipid?
- CD ideal can do spectra of protein in lipid vesicles.
- We will look at Staphylococcal α-hemolysin as an example

Instrumentation: synchrotron–based





- Synchrotron - whiz electrons around a ring.
- Can be used to produce very intense radiation by wiggling beam.
- Commonly used to produce X–rays (λ around 0.1nm)
- But can be used to push signals down to 160nm and below
- Great for fast stopped-flow to see rapid changes

Applications of CD in structural biology

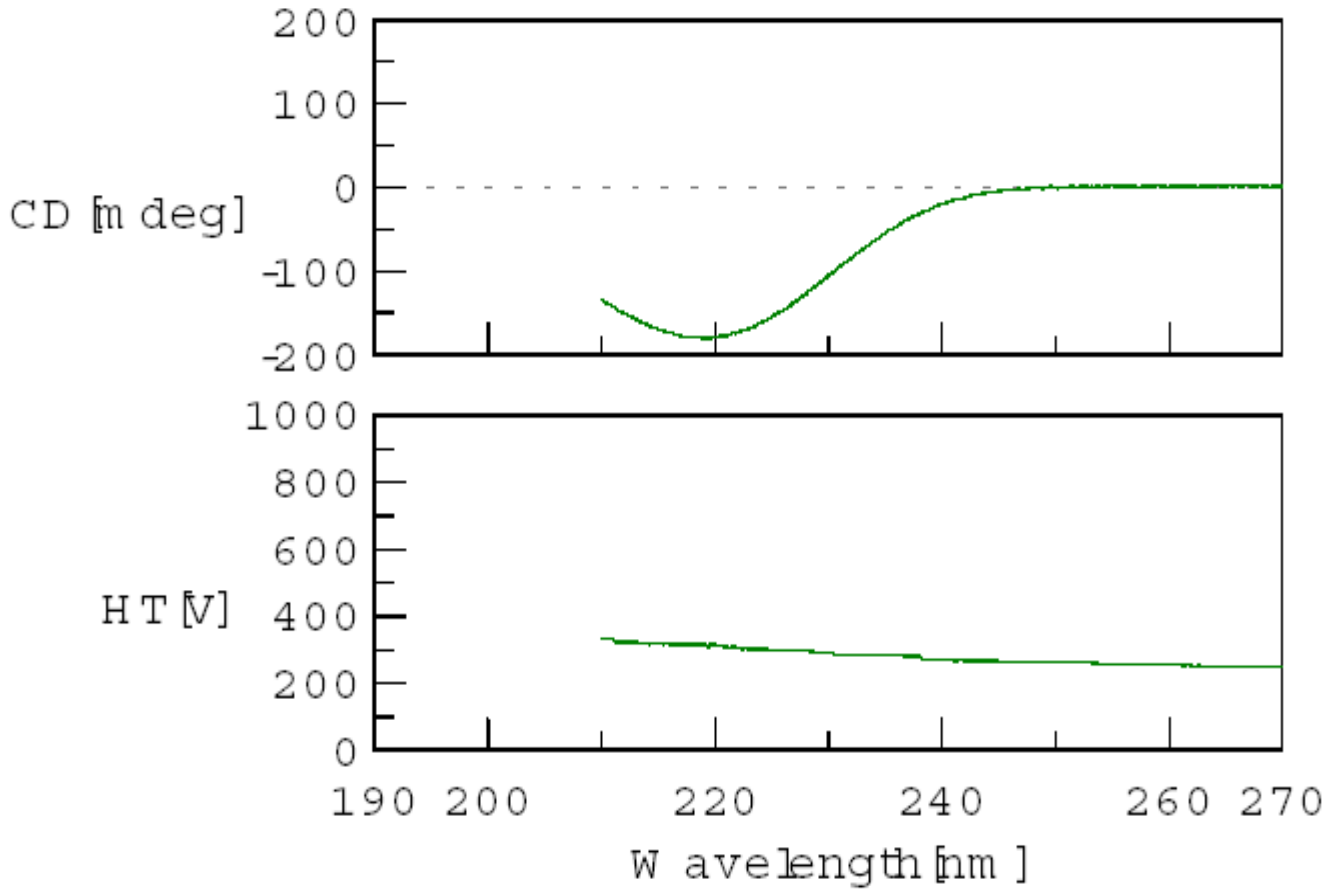
- Determination of secondary structure of proteins that cannot be crystallised
- Investigation of the effect of e.g. drug binding on protein secondary structure
- Dynamic processes, e.g. protein folding
- Studies of the effects of environment on protein structure
- Secondary structure and super-secondary structure of membrane proteins
- Study of ligand-induced conformational changes
- Carbohydrate conformation
- Investigations of protein-protein and protein-nucleic acid interactions
- Fold recognition

CD实验要点

Nitrogen flushing

Flushing the optics with dry nitrogen is a must:

- Xe lamp has a quartz envelope, so if operated in air it'll develop a lot of ozone, harmful for the mirrors
- below 195nm oxygen will absorb radiation


200
100
0
-100
-200
CD [m deg]
1000
800
600
400
200
0
HT [V]
190 200
220
240
260 270
Wavelength [nm]

HT plot

- The HT plot is very important, since readings **above 600-650V mean that not enough light is reaching the detector** so a sample dilution or the use of shorter path cell are required.
- Furthermore the HT plot is in realty a *single beam* spectra of our sample, since there is a direct relation between HT and sample absorbance. By data manipulation HT conversion into absorbance and buffer baseline subtraction is possible. Alternatively single beam absorbance scale can be used already in CH2 during data collection, loosing however a bit the alerting functions of this channel.

Bandwidth (SBW) selection

- Setting of slits should be as large as possible (to decrease noise level), but compatible to the natural bandwidth (NBW) of the bands to be scanned.
- As a rule SBW should be kept at least 1/10 of the NBW, otherwise the band will be distorted.
- If NBW is not known a series of fast survey spectra at different SBW will help proper selection. Trade in of accuracy versus sensitivity (i.e. the use of larger than theoretical SBW) is occasionally required.
- 2 nm in the far UV region
- 1 nm in the aromatic region (where fine structures may be present), optimal band-pass (as large as possible, but not loosing information) can be determined after a trial

Number of data point

- data pitch, i.e. number of data points per nm, will not directly influence the noise level. However if post run further data processing will be applied to reduce the noise, it’s advisable to collect as many data points as possible to increase the efficiency of the post run filtering algorithm

Accumulation

- another way to improve S/N is to average more spectra. Here too the S/N will improve with the square root of the number of accumulations.
- Averaging is very effective since it compensates short term random noise, but it'll not compensate long term drifts (mainly of thermal origin). So if long accumulations are used we recommend a suitable long warm-up of the system and/or the use of a sample alternator (to collect sequentially sample and blank and average their subtracted values).
- For long overnight accumulations it's essential that room temperature is well kept stable.

Sample concentration and cell pathlength

- A good suggestion is to run in advance an absorption UV-VIS spectra.
- CD spectroscopy calls for same requirements as UV-VIS: best S/N is obtained with absorbance level in the range 0.6 to 1.2. It's usually difficult to get proper data when absorbance (*of sample + solvent*) is over 2 O.D.

溶剂的吸收!!!

Table 3. Absorbance of various salt and buffer substances in the far-UV region[a]

Compound		*No absorbance above*	*Absorbance of a 0.01 M solution in a 0.1-cm cell at*			
			210 nm	*200 nm*	*190 nm*	*180 nm*
$NaClO_4$		170 nm	0	0	0	0
NaF, KF		170 nm	0	0	0	0
Boric acid		180 nm	0	0	0	0
NaCl		205 nm	0	0.02	>0.5	>0.5
Na_2HPO_4		210 nm	0	0.05	0.3	>0.5
NaH_2PO_4		195 nm	0	0	0.01	0.15
Na-acetate		220 nm	0.03	0.17	>0.5	>0.5
Glycine		220 nm	0.03	0.1	>0.5	>0.5
Diethylamine		240 nm	0.4	>0.5	>0.5	>0.5
NaOH,	pH 12	230 nm	≥0.5	>2	>2	>2
Boric acid, NaOH,	pH 9.1	200 nm	0	0	0.09	0.3
Tricine,	pH 8.5	230 nm	0.22	0.44	>0.5	>0.5
Tris,	pH 8.0	220 nm	0.02	0.13	0.24	>0.5
Hepes,	pH 7.5	230 nm	0.37	0.50	>0.5	>0.5
Pipes,	pH 7.0	230 nm	0.20	0.49	0.29	>0.5
Mops,	pH 7.0	230 nm	0.10	0.34	0.28	>0.5
Mes,	pH 6.0	230 nm	0.07	0.29	0.29	>0.5
Cacodylate,	pH 6.0	210 nm	0.01	0.20	0.22	

Usable short wavelength range (nm) of various solvents

	1cm cell	1mm cell	0.1mm cell
water	*185*	*180*	*175*
10mM Sodium phosphate		*182*	
0.1 M Sodium phosphate		*190*	
0.1 M Sodium chloride		*195*	
0.1 M Tris-HCl		*200*	
0.1 M Ammonium citrate		*220*	
n-Hexane	*210*	*185*	*180*
Heavy water		*175*	*171*
Trifluoroethanol		*177*	*170*
Cyclohexane	*210*	*185*	*180*
Isoctane	*210*	*185*	*180*
Dioxane	*220*	*210*	*202*
Benzene	*280*	*275*	*270*
Carbon tetrachloride	*250*	*240*	*230*
Chloroform	*240*	*230*	*220*
1,2-dichlororthane	*220*	*210*	*200*
Methanol	*210*	*195*	*185*
Ethanol	*220*	*200*	*190*
Trifluoroacetic acid	*260*	*250*	*240*
DMS	*264*	*252*	*245*
THF	*265*	*230*	*204*

Buffer Systems for CD Analyses

- Acceptable:
  1. Potassium Phosphate with KF, K_2SO_4 or $(NH_4)_2SO_4$ as the salt.
  2. Hepes, 2mM.
  3. Ammonium acetate, 10mM.

- Avoid: Tris; NaCl; Anything optical active, e.g. Glutamate

Summary

- CD is a useful method for looking at secondary structures of proteins and peptides.
- CD is based on measuring a very small difference between two large signals must be done carefully
- the Abs must be reasonable max between ~0.6 and ~1.2.
- Quarts cells path lengths between 0.0001 cm and 10 cm. 1cm and 0.1 cm common
- have to be careful with buffers TRIS bad - high UV abs
- Measure cell base line with solvent
- Then sample with same cell **inserted same way around**
- Turbidity kills - filter solutions
- Everything has to be **clean**
- **For accurate 2ndry structure estimation must know concentration of sample**

Typical Conditions for protein CD

- Protein Concentration: 0.2 mg/ml
- Cell Path Length: 1 mm
- Volume 350 μl
- Need very little sample 0.1 mg
- Concentration reasonable
- Stabilizers (Metal ions, etc.): minimum
- Buffer Concentration : 5 mM or as low as possible while maintaining protein stability