TECHNICAL REPORT N° 52 **November 2001**

CD PROTEINS COURSE

The enclosed document refers to a practical training course which will be held this month in Brazil.
The target is to train a few people, potentially interested in CD, but not specialist of the technique, to assist them in getting the best from their samples..
User education is a main target today, since CD is becoming more and more a complementary (ancillary if you want) technique for *protein people.*
The text will be left as memoranda to all students, while several lectures will touch more specifically the scientific aspects behind.
It's curious that such an initiative comes from Brazil and not from one of the many countries in which CD is popular since many years!
We must thank prof Alberto Spisni and dr Calos Ramos of LNLS of Campinas for this interesting opportunity.
The enclosed text may well be used by other people in other locations.

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Circular Dichroism of Proteins

A Basic Practical Introductory Training School

Program of the course

1st morning

1.1- Introduction
1.2- CD as spectroscopic way to measure chirality
1.3- CD hardware
1.4- Nitrogen flushing
1.5- Spectra collection
1.6- Search of optimal scanning parameters
1.7- Choice of sample concentration and cell pathlength
1.8- Sampling cells and their care
1.9- Nomenclature

1st afternoon

1.A- Instrument start up & self-diagnosis
1.B- A guided tour inside the spectrometer
1.C- Collecting spectra and baseline with different parameters
1.D- Processing the acquired data

2nd morning

2.1- CD spectra of proteins
2.2- How to prepare a sample
2.3- Typical measurement parameters
2.4- Typical data manipulation
2.5- Estimation of secondary structure

2nd afternoon

2.A- Reference spectra to check performances
2.B- Running a protein sample
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3rd morning

3.1- Folding and unfolding experiments with temperature
3.2- folding and unfolding experiments by titration
3.3- Stopped-flow
3.4- MCD
3.5- Other applications of CD in biochemical field

3rd afternoon

3.A- Practical test running an unknown protein sample and getting results

Rev 1, Nov 2001

1.1 Introduction

There are at least two categories of Circular Dichroism (CD) users.
The technique specialists: Organic, Inorganic, Physical Chemists mainly.
They built their academic career with CD, their best students did the same …., you'll find these people at main CD events, as the CD conferences held every two years.
Most of the customers are different: they are practical users, which means they take advantage of the technique for complementary information.
People dealing with protein structure are the most representative of the second category: by far the majority of the CD spectrometers sold today are purchased by them, not by CD specialists.
This means that CD is reaching laboratories where young people will find no past tradition to learn from.

This basic training is dedicated to them.
Main aim is to transfer some confidence on the technique, on its possibilities and on its limitations; at a plain, introductory level. The course includes a minimum of theory combined with practical experiments.
Level will be kept as simple as possible, however a written trace may be useful to remember the topics in the future: we prepared this document for the target.

1.2 CD as a spectroscopic way to measure chirality

There are two main optical spectroscopy methods to measure chirality:
-Polarimetry (and spectro-polarimetry = Optical Rotary Dispersion = ORD)
-Circular Dichroism

In **polarimetry** you feed the sample with monochromatic linearly polarized light, optical active sample will rotate the plane of (linear) polarization and you measure this effect.
How? Typically you fill your cell with the solvent and orient a polarizer (called analyzer) placed after the cell to extinguish the light passing through.
Than you replace solvent with your sample: since it's optical active some light will pass through; you than rotate the analyzer until no light is allowed to pass anymore. The angle of rotation (α) is what you measure.
α is linearly related to sample concentration and cell pathlength, very often it's also sensitive to the experimental temperature.
So in chemical products catalog, for chiral ones you'll find the value $[\alpha]_D^{xx}$ where:

D means the analytical wavelength of the Na line at 589nm
xx the temperature in degrees cent

and:

$[\alpha] = \alpha/100C'l$

where α is the measured optical rotation in millideg
C' is sample concentration in g/ml
l is the pathlength of the cell in cm

You can extend polarimetry to spectro-polarimetry (**ORD**), measuring α not at single wavelength, but all over the spectral range.

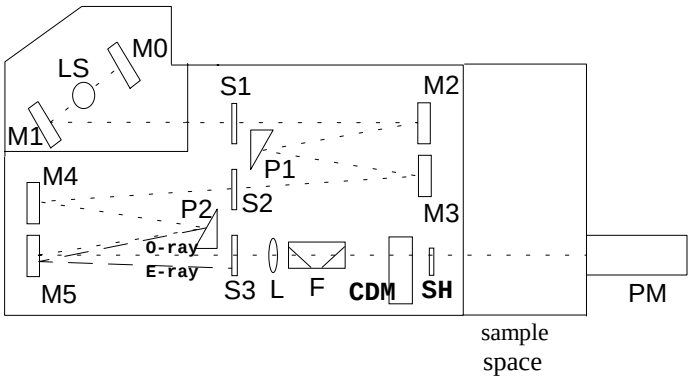
And what about **CD**?

CD is the alternative optical method to measure chirality. You feed your sample with left and right circularly polarized light and you measure the difference in absorption for left and right handed components.
This can be expressed as ΔA or more frequently as Θ (in millideg) which is the ellipticity of the beam outgoing from the sample (see section 1.9).
CD takes place at two conditions:
- sample must be optical active
- it must have a chromophore nearby the chirality center

CD and ORD spectra contain the same sort of information, you may switch from one to another by a mathematical treatment (Kramers-Kronig transformation).
As a matter of fact CD replaced ORD in most of the applications, since the resulting spectra are far easier to interpret and also more simple to compare.

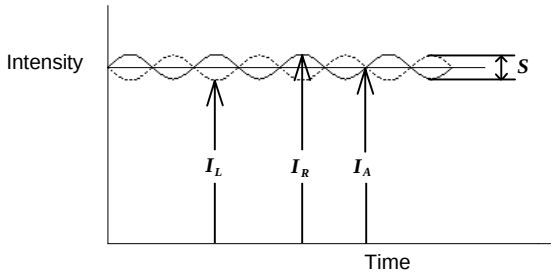
1.3 CD hardware

A CD spectrometer is basically similar to a single beam UV-VIS spectrophotometer.
It uses a single light source[1] and a double prism monochromator[2]. Monochromator output is linearly polarized and passing through the piezoelastic modulator it's converted into a modulated (50KHz) circularly polarized radiation which passes through the sample and it's detected by the photomultiplier tube.



LS	*light source (DC xenon lamp, 150W)*
M_1	*elliptical mirror*
M_0	*spherical mirror*
S_1, S_2, S_3	*entrance, intermediate and exit slits*
M_1, M_2, M_3, M_4	*collimating spherical mirrors*
P_1, P_2	*quartz prisms*
L	*quartz lens*
F	*filter (quartz plates to clean the linear polarization)*
CDM	*CD modulator (piezoelastic element operated as an achromatic quarter wave plate)*
SH	*shutter*
PM	*photomultiplier tube*

The sample (if optically active and absorbing light at the given wavelength) will absorb in different way the left and right circularly polarized light components.



At the detector output we will have therefore a waveform as shown above, with:
- an alternated component of amplitude S proportional to Circular Dichroism
- an average DC component (I_A) proportional to efficiencies of light source, monochromator and photomultiplier tube, as well as transmittance of the sample at the specific wavelength.

The correct CD signal is S/I_A.
Pls note that S amplitude is order of magnitudes smaller than I_A.

In normal operational mode the I_A is kept constant by dynode feedback, i.e. changing the gain of the photomultiplier, increasing or decreasing the high voltage applied onto it, while CD (normalized S amplitude) is measured simply by a lock-in amplifier

1 *a Xe lamp is used in CD rather than D_2 and halogen sources as in most UV-VIS. This because the short arc of the Xe lamp is more intense than these sources*
2 *a double monochromator is necessary to remove the stray-light of the intense Xe source. Double prism rather than dual grating since with prisms you induce no stray polarization (even more in this case prisms are used to generate a linearly polarized output), prisms efficiency is superior to grating one in the low UV, furthermore with prisms you avoid order sorting filters and their induced artifacts*

1.4 Nitrogen flushing

Flushing the optics with dry nitrogen is a must:
1. fitted Xe lamp has a quartz envelope, so if operated in air it'll develop a lot of ozone, harmful for the mirrors
2. below 195nm oxygen will absorb radiation

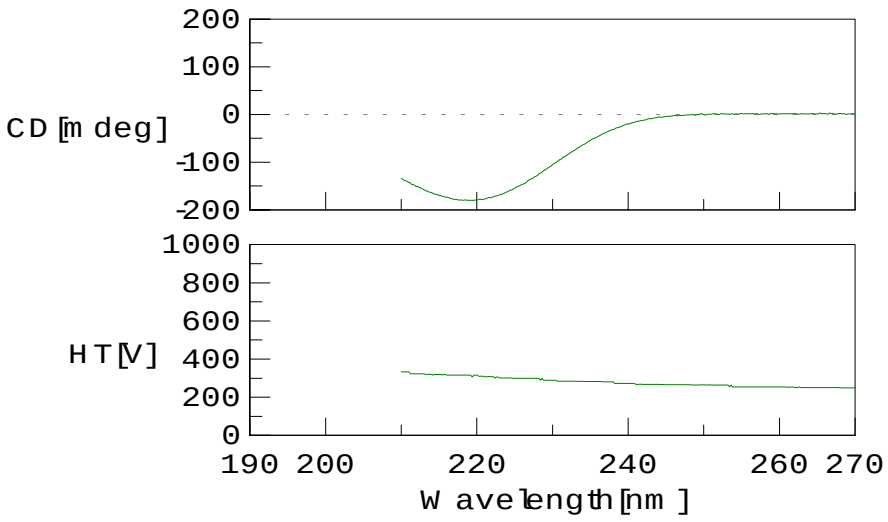
So it's a must to flow continuously the unit with well dried, high purity (99.99% min) nitrogen gas.
A flow rate of 3 l/min is enough to keep optics relatively oxygen free, but to operate in the low UV the flow should be increased roughly as follows:

-down to 190nm 3 l/min
-from 190 to 185nm 5 l/min
-from 185 to 180nm 10 l/min
-below 180nm up to 50 or even more l/min

As a good practice it's necessary to switch on nitrogen flow a few minutes before turning on the source.
A suitable nitrogen flow-meter (if not supplied with the spectrometer) is an absolute must to keep flow under control.
When you change the sample keep compartment cover open as shortly as possible, in order to speed-up the flushing. Make also sure that the quartz window to insulate sample compartment from monochromator optics is properly installed.

1.5 Spectra collection

In contrast with most optical spectroscopic techniques two channels[1] are usually simultaneously acquired during spectra collection.
CH1 - showing CD intensity (basically the output of the lock-in amplifier see 1.3)
CH2 - showing the high voltage (HT) applied to the PM tube (which is used to keep constant I_A as from 1.3)



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.

1 a modern unit like the Jasco J-810 allows to collect 4 channels: signals from other detectors (fluorescence, pH, temperature ….

1.6 Search of optimal scanning parameters

As we said above a CD spectrometer can be seen as a special type of UV-VIS spectrophotometer, so in the selection of the scanning parameters, old, well known, rules apply.
We want to stress once more here that in CD technique we are usually measuring very weak bands, consequently measurement is rather more slow than in UV-VIS spectroscopy.

-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.

-Response time
Signal to noise level is proportional to the square root of the integration time, the simplest way is to improve spectra quality is increasing the response time*.
** response ≅ integration time is equivalent to time constant used on old analog photometers multiplied by 5. 1 sec r.t. ≅ 0.2 sec of t.c.*

-Scanning speed selection
Once we select SBW and RT values, the maximum scanning speed allowed not to distort the spectra is given by relation SBW/response time of the system.
For example using slits of 1 nm and response time of 1 sec the maximum scanning speed is 1nm/sec = 60 nm/min

-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.

-User choices
we explained above the basic criteria, but several users prefer *to see the noise* so they prefer to use substantially shorter than necessary response times. The net result will be a spectra with higher noise, but its random distribution will allow much easier visual or software post run smoothing.

1.7 Choice of 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 0.5 to 1. It's usually difficult to get proper data when absorbance (*of sample + solvent*) is over 2 O.D.

-Problems:
-Particularly scanning in the low UV it may be difficult to know the exact absorbance level. Most modern spectrophotometers are limited to 190nm, in any case reliable measurements below 200nm may be a problem. Getting absorbance measurement directly with the CD spectrometer is usually to be suggested.
-Many solvents and buffers absorb strongly in the UV, since the total absorbance level is what it matters, this must be well considered, see table below as a rough reference

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*

1.8 Sample cells and their care

In CD spectroscopy you must use only quartz cells of known manufacturer, up to a few years ago CD cells were only cylindrical type (22mm O.D.), these are the ones which give less birefringence. Today most of the people are using rectangular cells, which quality substantially improved.
The wide use of Peltier thermostatting devices is another reason why this type of cells took over.
These cells are the same used for UV-VIS spectroscopy (but some manufacturer will offer *special CD selection*), they are cheaper and require less sample volume. Since Z is typically 15mm and beam size is about 9 mm diameter, minimum volume in a 10mm path rectangular cell is around 2ml, this volume can be further reduced lifting the cell base with a spacer.
Rectangular cells are commercially available with Teflon lids or stoppers, the latter type is best for measurement with variable temperature.
Minimum pathlength is 1mm.
This is a rather severe limitation since many spectral experiments in the far UV are carried on with shorter paths. In these cases you must use either cylindrical cells (available with path down to 0.01mm) or demountable rectangular ones.
Ultra-short path cylindrical cells are therefore still the best choice where very low UV range is required, but in this region more artifacts are potentially present.
Best way to check new cells is to fill them with distilled water and run spectra all over the range of interest to be compared to air baseline run in same conditions. As a rule it'd be better to use cell always in same orientation, but in initial testing it may pay to rotate them 180° to see if the two spectra are significantly different.

Use of semi-micro cells (4 mm width) is possible, but cells must have black side walls, using or not using beam condensing systems. Also ultra-micro cells (such as Hellma 105.200 with 8x2mm aperture and nominal 160µl capacity for 10mm path) can be used with care.
It's however imperative to pretest them (and related beam condensing system) for birefringence artifacts as indicated above.

Proper cell cleaning is an imperative task, this may be not so easy, particularly proteins may not trivial to remove from windows surface. For washing use warm water with detergent* followed by mild acid rinse (Hydrochloric acid) and water rinse ……
** such as the HELLMANEX™ II*

1.9 Nomenclature

Current CD spectrometers measure CD in terms of ellipticity Θ, usually expressed in millidegrees.
This is, if you want, a rather strange way to express results, since CD is an absorption phenomena. Indeed the French school, which developed first commercial units, was using ΔOD as scale, but Americans took over many years ago and ellipticity Θ is the standard acquisition scale used today. Conversion is however very simple:

$\Delta A = \Theta/32980$
where:
ΔA in absorbance units
Θ is ellipticity in mdeg

Literature data are usually reported in **molar ellipticity [Θ]**:
$[\Theta] = \Theta/(10xCxl)$ or **$[\Theta] = (\Theta xM)/(cx\ lx10000)$**
where:
Θ is ellipticity in mdeg
C is the molar concentration (mole/l)
l is the cell path in cm
M is molecular weight
c is concentration in g/ml

since obviously
$C = (1000xc)/M$
[Θ] is expressed in deg x cm^2 x $decimole^{-1}$

For macromolecules such as proteins the mean residue molar ellipticity is used $[\Theta]_{MRW}$:
$[\Theta]_{MRW} = \Theta/(10xC_rxl)$
Formula is still the same, but C_r is the mean residue molar concentration
$C_r = (nx1000xc_g)/M_r$
where:
n is the number of peptide bonds (residue)
c_g is the macromolecule concentration (g/ml)
M_r is the molecular weight of the species

Notes:
-the Jasco software is not able to differentiate [Θ] from $[\Theta]_{MRW}$, so inputting concentration you must divide by the number of amino acids
-Jasco uses molecular ellipticity term in place of molar ellipticity
-most secondary structure estimation programs need $[\Theta]_{MRW}$

Another used way to report data in literature is **molar circular dichroism Δε**:
$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \Delta A/Cxl$
simple relations apply:
$[\Theta] = 3298x\Delta\varepsilon$ and **$\Delta\varepsilon = [\Theta]/3298$**
$\Delta\varepsilon_{MRW}$ is often used for macromolecules as mean residue molar circular dichroism, same rules as above

Note:
-Jasco software indicates Δε as molecular CD

Furthermore data are occasionally expressed as anisotropy factor **g**

$$g = \Delta\varepsilon/\varepsilon = \Delta A/A$$

which is independent from concentration and linearly related to the enantiomeric excess

1.A Instrument start-up & self-diagnosis

Modern units are fully PC controlled and only control on the main unit is the power switch.

- start nitrogen flow
- turn on power switch of the main unit
- start the PC , enter in the program and launch the application

unit will perform automatically a self-diagnostic routine:

- A/D check *a synthetic calibrated signal is sent to the A/D converter*
- Amp check *a synthetic signal of +18 mdeg of intensity is sent through the lock-in amplifier*
- HT check *unit will monitor the high voltage on PM tube at 300nm with 1nm SBW*
- PEM check *will monitor the 50KHz oscillation of the PEM modulator*
- Lamp check *is monitoring if current is passing through the light source*
- Shutter check *will detect if shutter is in ON position*

While these tests are not really enough to assure proper functioning of the apparatus, they are here to indicate more evident failures.
If diagnostic is not passed, for example because you left open the sample compartment cover, so HT will be zero and displayed as *too low*, you can push ignore and enter anyway in the measurement program.

1.B A guided-tour inside the spectrometer

Optical bench will be opened and the various main components will be shown. The light beam will be followed from the light source, through the monochromator, to the PEM, through the sample compartment and to the detector.
Slits and wavelengths will be changed to give a visual check of the system operation.
Seeing is believing!

1.C Collecting spectra and baseline with different parameters

A standard sample will be used to collect spectra with different parameters and built-up a minimum of confidence on the actual operation of the unit.

1.D Processing the acquired data

Main data processing capabilities will be shown to get a picture of software capabilities and of typical data treatment applied in CD spectroscopy.

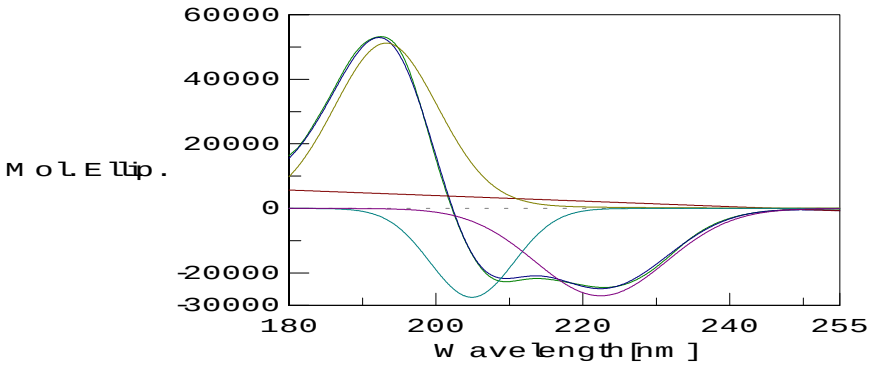
2.1 CD spectra of proteins

Far UV range 260 – 180 or lower
The far UV range is the one that gives information on secondary structure.

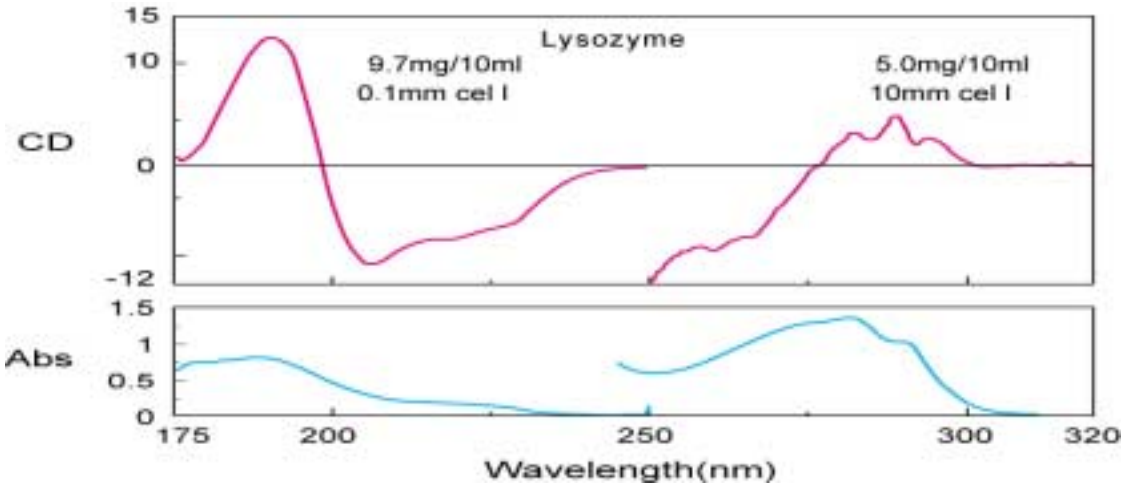
Random coil has a positive band at 212nm and a negative one at around 195nm

β-sheet shows a negative band at 218nm (π->π*) and positive one at 196nm (n->π*)

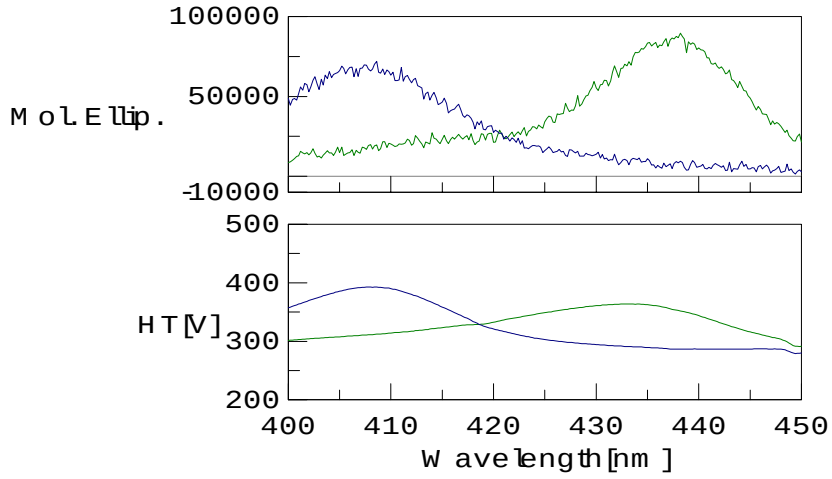
α-helix has negative (n->π*) at 222nm, parallel negative (π->π*) at 206nm and perpendicular positive (π->π*) at 190nm.
Figure below shows the fitting of a Myoglobin spectra (mainly α-helix) run on a modern unit with these three bands as suggested in 1965(!!) by Holzwarth using the curve-fitting program (JWCVF-485) of the Jasco J-810.



Near UV range 350 – 250 nm
The near UV region of CD spectra may give information on ternary structure: a missing three-dimensional structure will usually mean no CD signal in this region.
Signals are coming from tryptophan (300-280nm), tyrosine (290-270nm) and phenylalanine residues (270-250nm) as well as broad band disulfide bonds.
Intensity is much lower than in far UV, so typically 10mm path cells are used.
Spectra below refers to Lysozyme in far and near UV with different concentrations and cell pathlength.



Vis range
Metal proteins, for example hemoproteins exhibit good CD spectra in the visible region.
Spectra below refer to Soret band of Fe(III) Myoglobin and Fe(II) Myoglobin from extruded lyposomes.



2.2 How to prepare a sample

Choice of buffer
Particularly for low UV measurement choice of buffer is critical, since it must be as transparent as possible all over the wavelength range.
Typical buffer is 10mM potassium phosphate*.
If counter ion is needed use Na_2SO_4, KF or NaF, not NaCl.
Dithiothreitol, 2-mercaptoethanol and EDTA can be added to solutions at concentration of 1mM
Use high quality water, possibly degassed.
If possible filter sample with 0.2 or 0.5µm filter.
*Low concentration of perchlorate, Tris, Na-phosphate and borate are also reasonably transparent.

Sample concentration
For far UV measurement typically 1 mg/ml for 0.1mm path cells, or ten times less with 1mm cells.
Protein sample concentration should be known accurately for secondary structure analysis.
Spectrophotometric measurement at 280nm on fully unfolded protein (Extinction coefficients ε_{Trp}=5690 ε_{Thyr} =1280 liter mol^{-1}, cm^{-1}) or quantitative amino acid analysis as an alternative way if protein has no thyrosines or tryptophans.
1 mg/ml is typical concentration also for the aromatic region with 10 mm cells.

Cell pathlength
Far UV measurements are limited by buffer and sample absorption, while sample concentration may be changed, there is no way to change absorption of the buffer, this means that to extend the range shorter paths are necessary while increasing sample concentration.

Sample volume
This may be a concern, particularly when dealing with precious samples, see section 1.8

2.3 Typical measurement parameters

Please refer to section 1.6.
More specifically for protein analysis:

-Nitrogen flowrate
As from 1.4

-SBW
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

-Response time
Typically 2 or 4 sec, 0.5 sec for survey spectra

-Scanning speed
100 nm/min for survey spectra.
10, 20 nm/min for actual runs

-Accumulation
As many as necessary to get an acceptable s/n

-Spectral range
Start from wavelength in which no CD signal is by sure present (or where CD intensity is irrelevant compared to expected signal) and end up where buffer+sample are giving HT over 650V

-And ……
As a general suggestion pls run sample before baseline, since you must use exactly same parameters and it's better to optimize them on the sample.
However if you do not know exactly absorption of your buffer, pls run in advance a quick run of buffer baseline to sort out best cell path (lower UV limits are also strictly related to the health of the instrument you are using!).

2.4 Typical data manipulation

The following steps are usually required:

-Baseline subtraction
Sample and buffer baseline should be run with same parameters and possibly in the same cell oriented in same position.

-Zero shift
This should not be required, but occasionally for long experiments with weak signals, zero drift may be significative. It's a bad (but accepted and common) practice to compensate it shifting the baseline-subtracted spectra to zero at wavelengths where CD signal is by sure null.

-Smoothing
To improve s/n you can apply one of the several smoothing functions of the software.
You have visual presentation of both original and smoothed data: avoid to loose spectral details and/or to introduce artifacts (for example FFT will tend to create waves ….., not to be confused with bands!)

-Data conversion
For CH1 in [Θ] or Δε as required.
For CH2 in absorbance (if required)

2.5 Estimation of secondary structure

Once we have our spectra in proper format (baseline collected and converted in $[\Theta]_{MRW}$ mean residue molar ellipticity or $\Delta\varepsilon_{MRW}$ mean residue molar circular dichroism ($\Delta\varepsilon=[\Theta]/3298$) depending on software you are using) you can start estimate of the secondary structure of your protein.

A few essential remarks:
-In between the various main analytical methods to study protein secondary structure CD is the one which:
a- takes less time
b- requires less sample

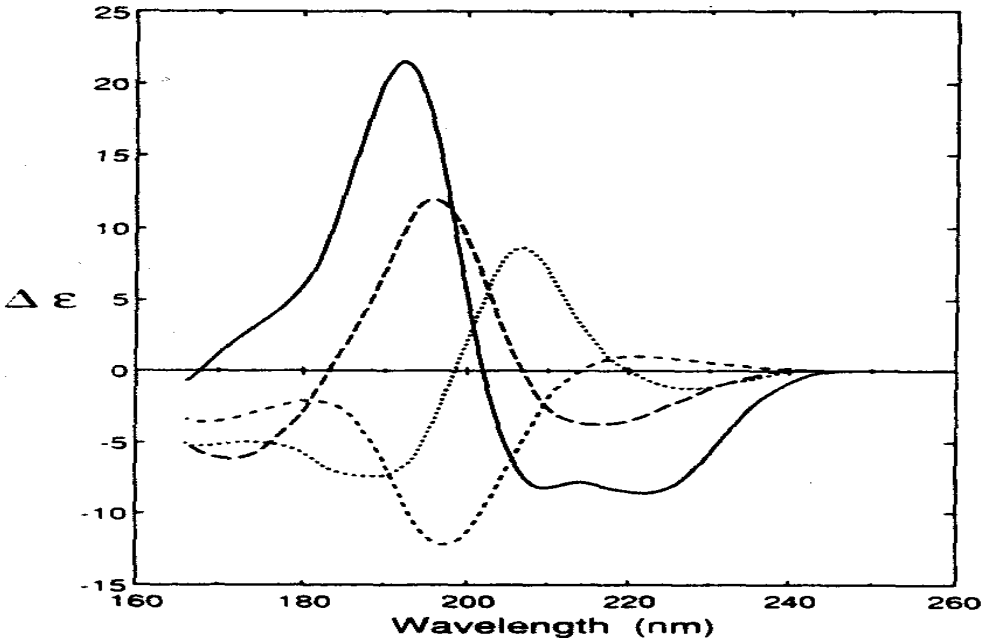
c- works on dilution closer to the actual environment in which proteins are practically acting
-But information from CD are limited, since obtained in a very restricted wavelength range, where spectra of all possible structures are different, but much overlapping (and many side factors may well influence spectral shape)

α helix
positive band around 192nm, negative ones at 209 and 222nm

β turn
positive band at 207 and negative one at 189nm

β sheet
negative band at 216 and positive one at 197nm

random coil
positive band at 212 and negative one at 198nm



-all estimation methods assume that the spectrum of a protein is the linear combination of the spectra of the secondary structural elements.
-many methods have been proposed, including
a- multilinear regression
b- singular value decomposition
c- ridge regression
d- convex constraint analysis
e- neural network
f- self-consistent method
Since many methods are existing (most of the related programs can be downloaded free of charge from Internet, where you can also find sites which from you can load your files for a fitting), this clearly indicates the difficulties behind

CD is de facto very sensitive and particularly well suited for α helix, indeed the simplest (and reliable) method calls for straight measurement of α helix content via a simple single wavelength measurement at 222nm.

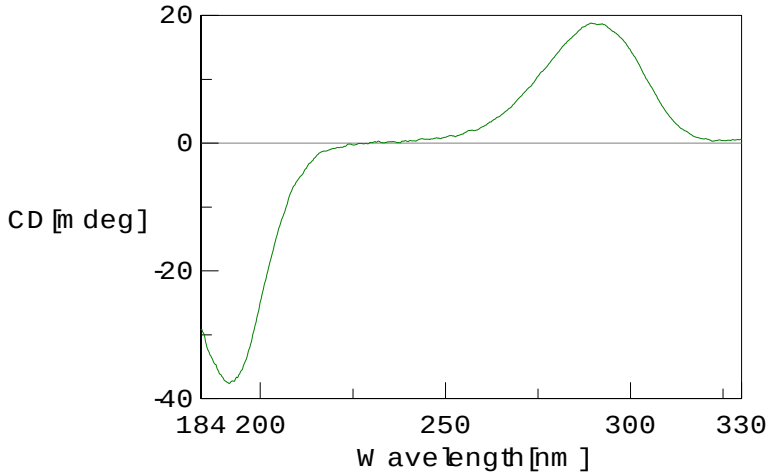
So generally speaking CD gives good data where representative spectra of similar compounds of well known structure are available*. But technique is very sensitive to small variations, so one of the main applications is to monitor minor structural changes.

** In between other complementary structural techniques:*
- *X-ray is by definition the main one, but it's devoted to specialists, it takes a lot of time, analyzes samples only in solid state*
- *High resolution NMR is invaluable, but here too sample concentration is very high and it's not fast*
- *Similarly to CD EPR is fast and can follow easily kinetics experiments, but it needs spin-labelling which may change structure*

- *IR (or FT/IR as said today) absorption is valuable too, particularly for β structures, but water is the worst solvent for IR range and Amide bands call for deconvolution to extract information and deconvolution tends to be a rather subjective task*
- *VCD (vibrational circular dichroism) has the advantage of IR and specificity of CD (we should call CD as ECD – electronic circular dichroism from now on ….), but here sensitivity is a main concern*
- *Plain Raman or ROA (Raman optical activity) are the alternative to IR and VCD, water is a good solvent for, but it's about all ….. here too (and even more) sensitivity is a main concern and data interpretation may be rather difficult*

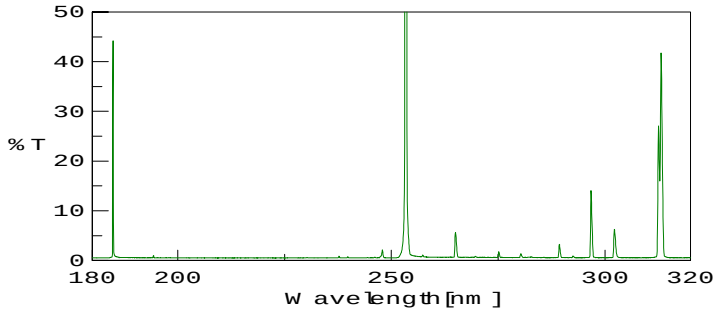
2.A Reference spectra to check performances

If you need to check performances of your unit before running critical CD spectra of proteins we would recommend to scan in advance a 0.06% aqueous solution of ammonium d-10-camphorsulfonate filled in a 1mm path cell.
Run also water baseline with same cell and subtract to the sample run.
Results should be as from following picture.



You can check here:
-absolute scale calibration (maximum at 290.5nm should be of 19 mdeg)
-proper PEM program (intensity ratio between 192.5nm and 290.5 nm bands should be around –2
-abnormal noise level particularly in the far UV will indicate other problems (lamp decay, mirrors degradation …)
Solution is rather stable for long time (keep in refrigerator anyway).
Some people keep a sealed cell permanently filled and use this standard run every morning; this is probably too much, but it's a good practice.

Another check (to be performed rather more rarely) is the wavelength calibration one. Secondary structure estimation requires proper accuracy.
What's better than the Hg spectra?:

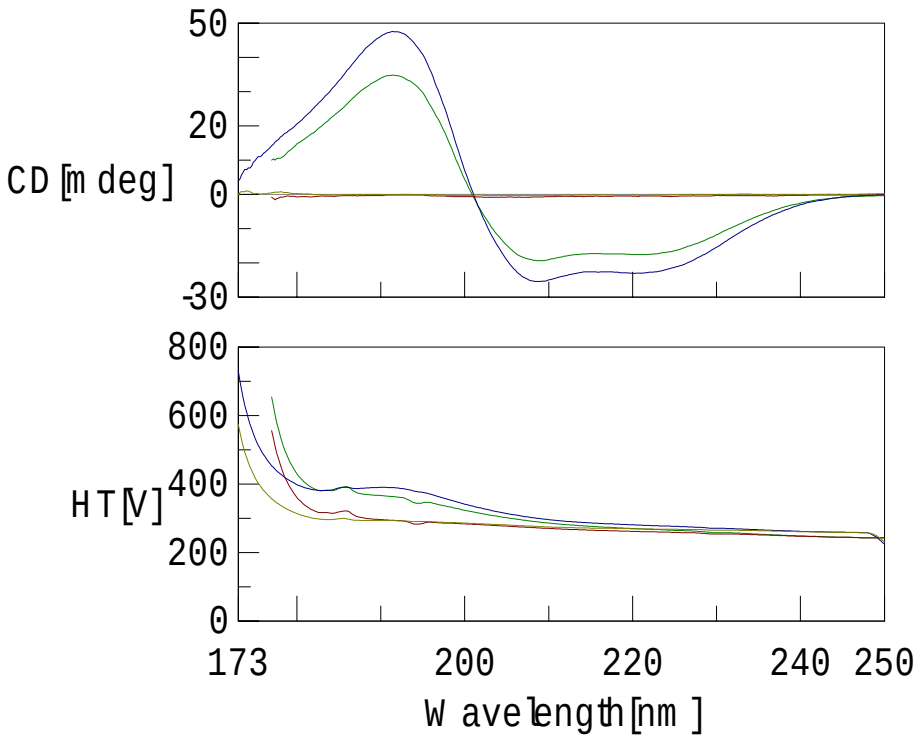


Several lines can be used:
313.16, 296.73, 289,36, 254.65, 184.90 nm ……
However to calibrate a CD spectrometer with a mercury lamp some skill and proper jigs are necessary, a job to be left to specialists.

2.B Running a protein sample

As a common sample we test here BSA (Albumine, bovine serum), other alternative samples may well be used. Spectra reported below are samples and baselines run at following conditions:

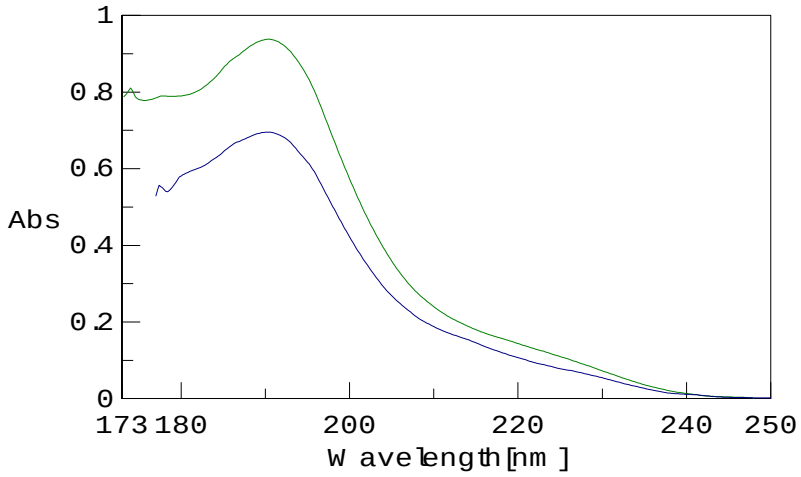
Concentration	0.05% and 0.4% BSA in 10mM K-Phos (pH6.9)
Cell path	0.2 mm and 25µ (demountable)
SBW	1 nm
Range	250-173 nm
Scanning speed	50 nm/min
Response	2 sec
Accumulation	4
Data pitch	0.2 nm



As expected, The use of shorter path cells allows some gain in the UV penetration.

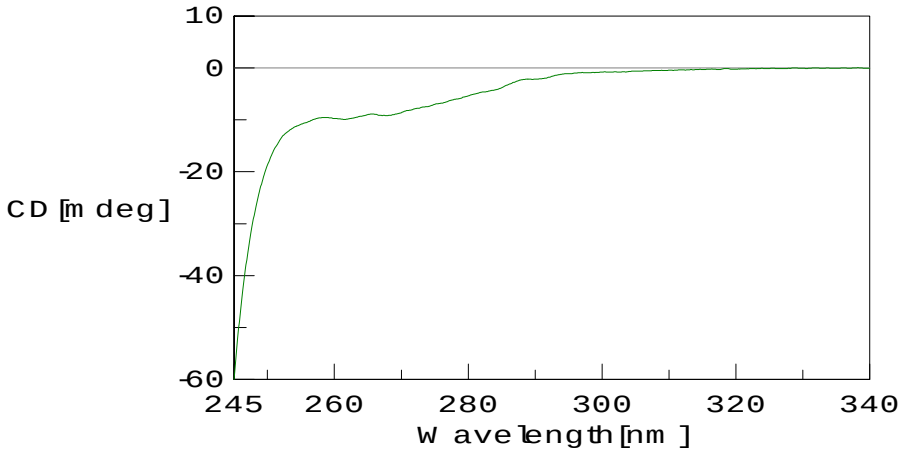
Exercise calls for collection of same spectra with different pathlength cells, changing concentration, and using alternative parameters.

Figure below show the calculated absorption spectra (HT→Abs conversion of all CH2 files, Sample – Baseline)



To explore the aromatic region 10mm path cell is the usual choice.
Spectra below is still related to Bovine Serum Albumine.

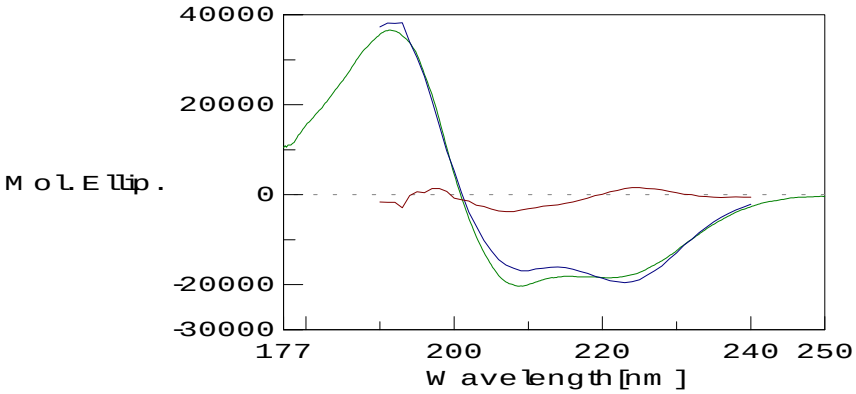
Concentration	0.1% BSA in 20mM Na-Phos (pH 7.0)
Cell path	10 mm
SBW	1 nm
Range	340-245 nm
Scanning speed	50 nm/min
Response	1 sec
Accumulation	5



2.C SSE by software

The collected spectra, after proper post-run manipulation must be checked for secondary structure content using one of the available software.
Spectra of BSA of section 2.B was converter in molar residue ellipticity $(\Theta)_{MRW}$ and analyzed using the Jasco JWSSE-480 software (least square method using reference by Yang).
This is a rather simple software, easy to use, but a bit outdated.

Results were, with restriction :
Helix 56.9%, Beta 0.0%, Turn 7.7%, Random 35.3% for a 100% total
or without restriction:
Helix 55.2, Beta 39.1, Turn 5.4, Random 41.9 for a total of 141.6



Using **DICROPROT** software (which includes different methods):

	Fasman	**Chen**	**Bolotina**	**Chang**	**Yang**
Helix	60%	54%	56%	36%	45%
Sheet	-7%	17%	18%	38%	12%
Turn	0%	0%	68%	1%	0%
Coil	21%	90%	24%	45%	26%
Total	74%	161%	166%	119%	83%

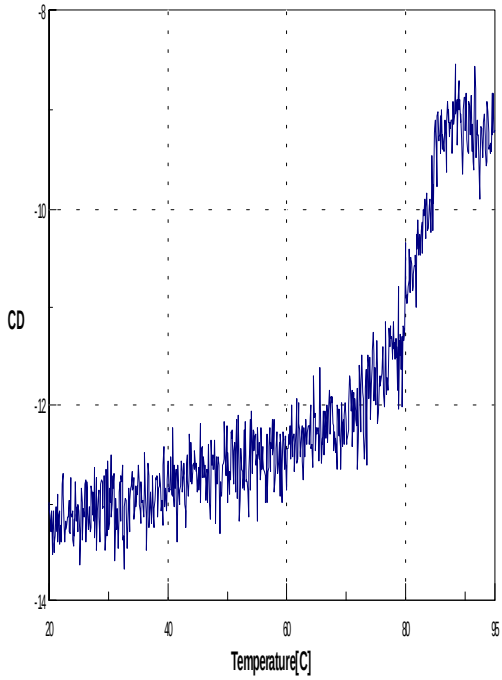
Using **CDNN** software:

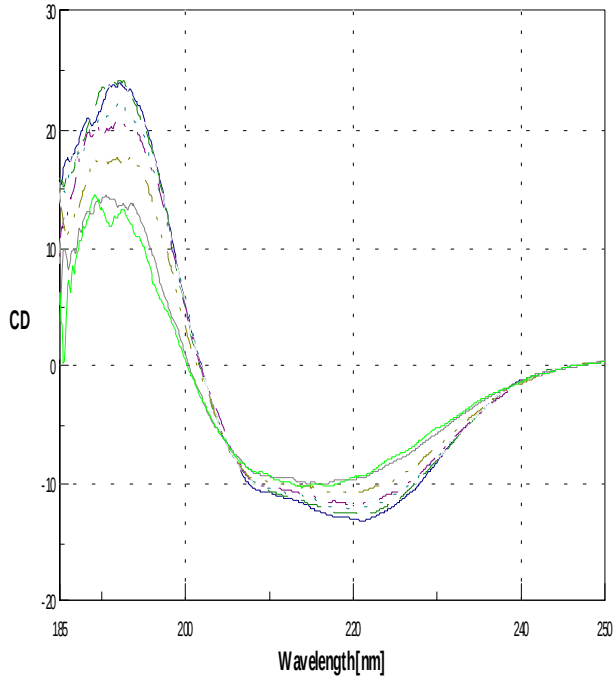
	180-260	185-260	190-260	195-260	200-260	205-260	210-260 nm
Helix	61,4 %	60,7 %	59,7 %	59,3 %	60,9 %	64,8 %	62,4 %
Antiparallel	1,0 %	1,0 %	1,2 %	2,6 %	3,6 %	3,4 %	3,4 %
Parallel	3,8 %	4,1 %	4,2 %	4,0 %	3,8 %	3,4 %	4,1 %
Beta-Turn	12,8 %	12,9 %	13,1 %	13,0 %	12,9 %	12,5 %	12,5 %
Rndm. Coil	14,9 %	15,2 %	15,2 %	16,2 %	16,5 %	16,3 %	18,0 %
Total Sum	93,9 %	93,8 %	93,3 %	95,1 %	97,7 %	100,4 %	100,4 %

While obviously the different software have not been used at the best, it seems clear that α kelix is the only component which for a good reliability is obtained in this case.

3.1 Folding and unfolding experiments with temperature

CD is a technique very sensitive to minor structural modifications. One of the most used applications is folding experiments versus sample temperature.
Application calls for the use of a cell holder thermostatted by a Peltier element or by a programmable bath with related software.
You can ramp temperature and collect spectra at predefined temperature while ramp is halted.
The example below shows such an experiment carried on Ovalbumin sample (20mM K-phos pH 7.0 in a 1mm cell) in the range 20-95°C, with a ramp speed of 50°C/hour and monitoring the data at 223nm.
Spectra were collected at 20, 40, 60, 70, 85 and 90°C.





In these experiments some specific cares should be followed:
-sampling with relatively long path cells (1 to 10mm) to minimize thermal gradients in the cuvette (with 10mm cells it's possible to insert a stirrer and a remote temperature sensor)
-slow ramp speed to assure thermal homogeneity in the cell
-proper equilibration time before starting spectra
By post-run data processing thermodynamic parameters (T_m, ΔH, ΔS) can be calculated.
Technique is well complementary to microcalorimetry.

Since experiments are very long it's today a common practice to put a second photomultiplier tube with a long pass filter at 90° to collect simultaneously fluorescence data. So with same run you can get CD, absorption and fluorescence melting curves.
More elaborate experiments (using different monitoring wavelengths, ramping up and ramping down temperature to follow hysteresis experiments, …..) are also possible.

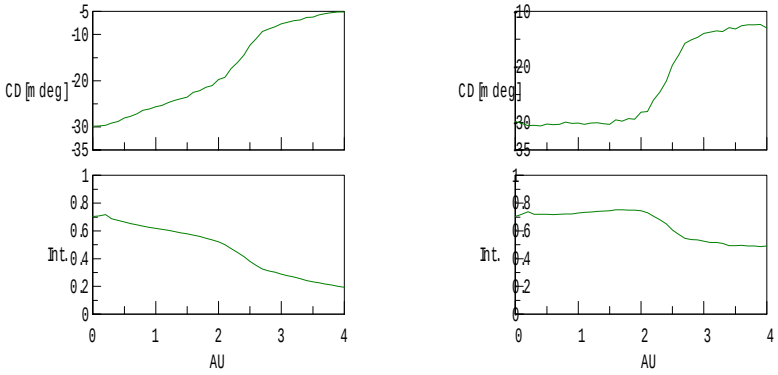
3.2 Folding and unfolding experiments by titration

CD can be used to monitor conformational changes by titration. In next example a CD spectrometer equipped with fluorescence accessory* was used to measure denaturation of BSA.

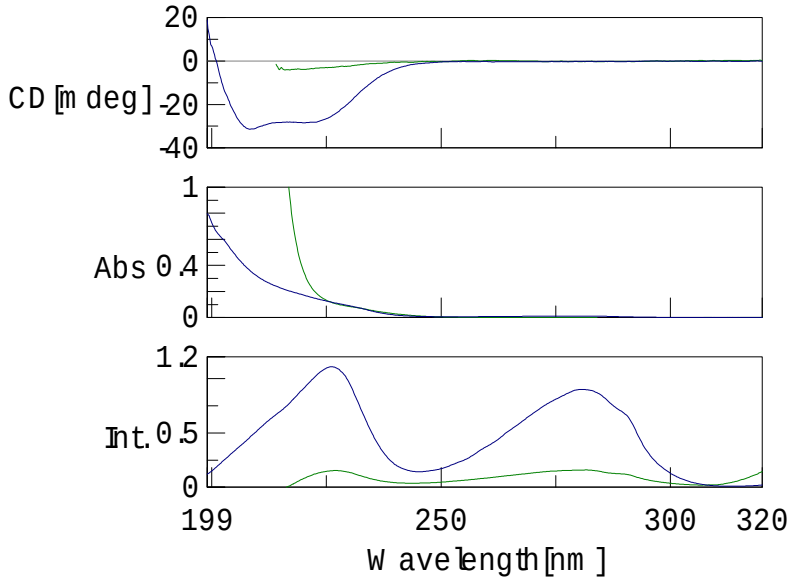
Starting solution:	0.002% of BSA in 50mM Na-Phos (pH 7.2)
Titrant:	6.6M Guanidine HCl
Cell:	fluorescence cell 10mm path
Monitoring wavelength:	222 nm
SBW:	1 nm
Response:	4 sec
Fluorescence filter:	high pass, 320 nm
Fluorescence PM tube:	HT 550V
Titration step:	0.1M (final GndHCL concentration in the cell 4M)
Temperature:	20°C

** layout used: J-810, CDF-426 Peltier fluorescence accessory, ATS-429 dual syringe automatic titrator*

Uncorrected and protein concentration corrected CD and Fluorescence titration curves



Actual CD, Absorbance and Fluorescence spectra (not concentration corrected) before and after titration

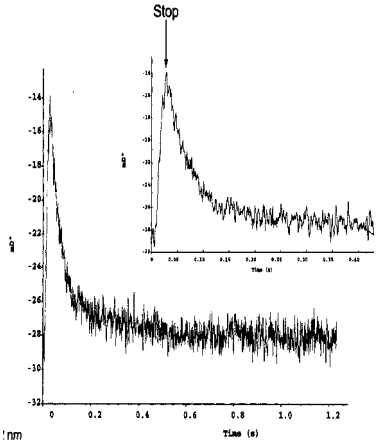


3.3 Stopped-flow

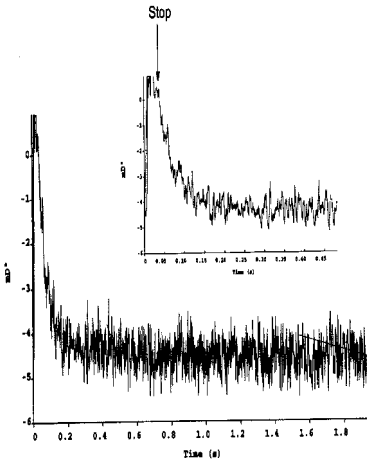
Folding and unfolding of proteins can be followed by stopped-flow CD.
Piezoelastic modulator based CD have a sampling frequency of 50 kHz so enough points can be collected even for very fast experiments. Stopped-flow is a good way to monitor transient intermediates, both related to secondary and tertiary structure. Possibility to measure simultaneously also fluorescence (and regular absorption) is a main feature of current apparatus.

The following figures are related to a refolding experiment of ACBP

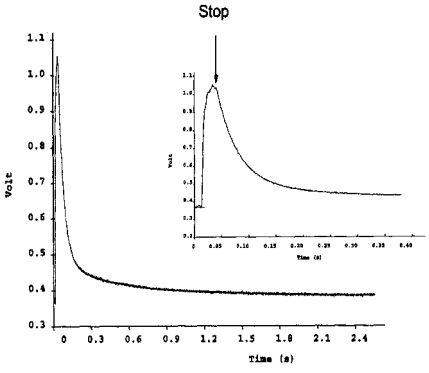
CD at 222nm
Syringe 1: 1mg of ACBP in 6M GndHCl,
20mM NaAc pH 5.3
Syringe 2: 20mM NaAc pH 5.3
Mixing ratio: 1:10
Cell: 2mm
Temperature: 5°C
Accumulation: 10 shots



CD at 286nm
As above but 3mg of ACBP
And 10mm path cell



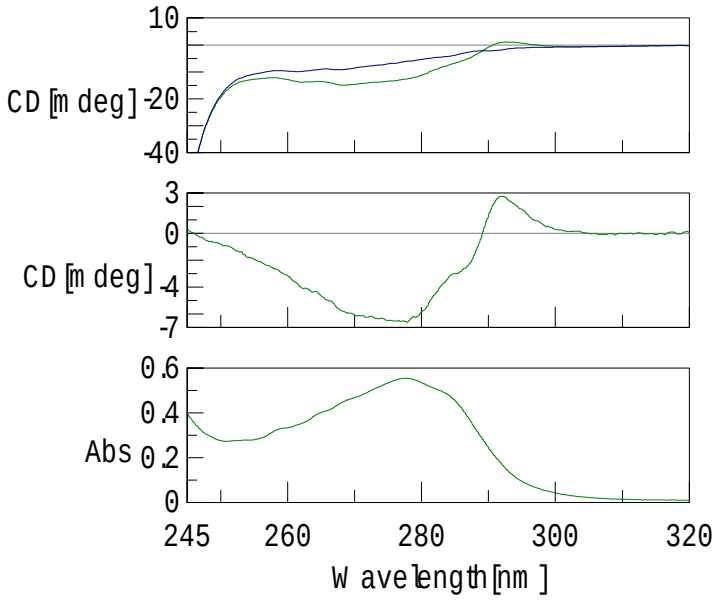
Fluorescence
As above, but
Ex λ: 222nm
Em λ >295nm
Cell: 2x2mm
Accumulation: 8 shots



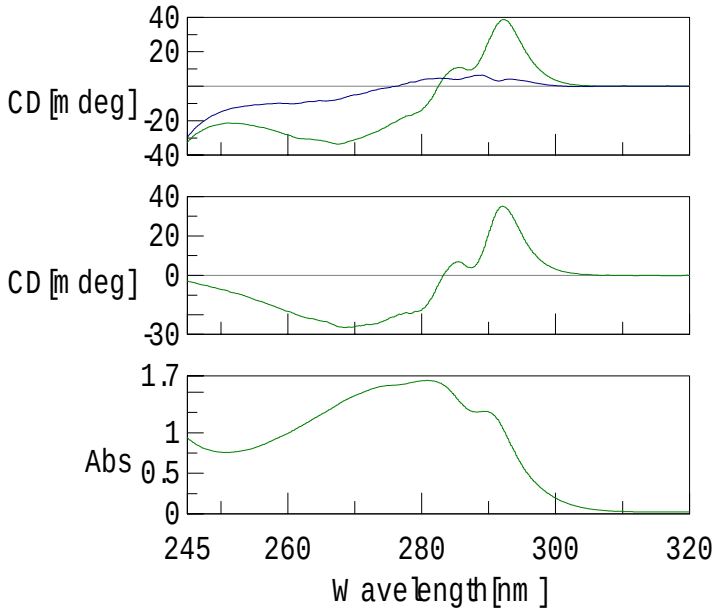
3.4 MCD

Magnetic circular dichroism (MCD) is measured putting sample in a magnetic field parallel to the direction of propagation of light.
MCD can be used to separate different transitions under a single/unresolved absorption spectra, since transitions of different polarizations may give opposed sign spectra.
In the aromatic region of protein spectra a sort of resolution enhancement is obtained as shown below.

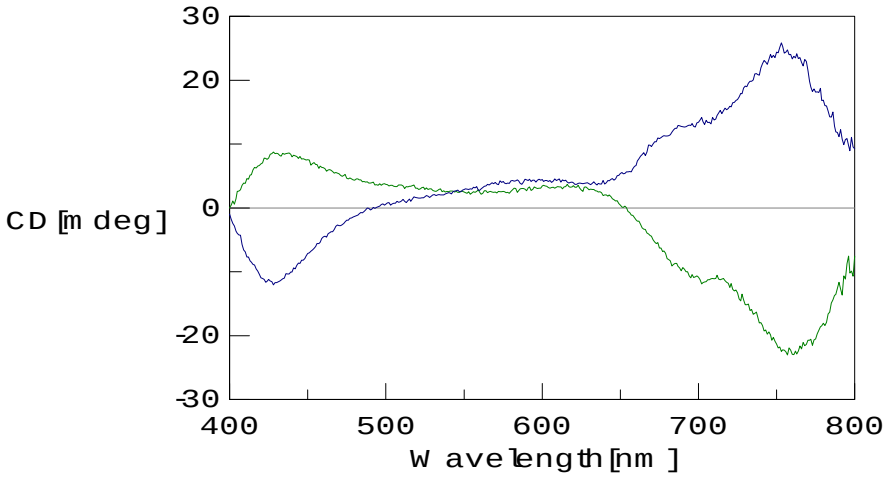
BSA
0.1% in 20mM Na-Phos
-CD & MCD (15kGauss)
-net MCD (MCD-CD)
-absorption
10mm cell



Lysozyme
0.061% in 20mM Na-Phos
-CD & MCD (15kGauss)
-net MCD (MCD-CD)
-absorption
10 mm cell



Nest example refers to MCD raw data of Cobalt-7-metallothien 2 (**Co7-MT2**) run at + and – 15kGauss at 530mM concentration in a 1mm path cell.



3.5 Other applications in biochemical field

These are virtually hendless
Interaction of proteins with drugs or other ligands, peptide analysis (particularly in support to peptide synthesis), polynucleotides (DNA and RNA) …..
In this respect it pays to remember here another technique you can apply with slightly modified CD hardware: Linear Dichroism (LD).
LD is the difference in absorption of light linearly polarized parallel and perpendicular.
LD = $A_{||} - A_{\perp}$
From an LD spectra we can measure either the polarization of a given transition or we can probe the molecular orientation if we know the polarization of a transition moment within the molecule.
LD is widely used for conformation of DNA and binding geometries of DNA-drug systems.
For these applications sample must be oriented (with electrical field or more commonly by flow), which imposes specific sampling arrangements in addition to the LD measuring capability (basically consisting only on a double frequency tuned lock-in amplifier and in a new program to the PEM to give half-wave modulation).