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Full Length Research Paper

The use of circular dichroism spectroscopy to study protein folding, form and function

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Circular Dichroism (CD) is a spectroscopic technique widely used for the evaluation of the conformation and stability of proteins in several environmental conditions like temperature, ionic strength, and presence of solutes or small molecules. Circular Dichroism spectroscopy is non-destructive, relatively easy to operate, requires small amount of sample and few data collection. Additionally, data analyses are fast. Chiefly because of the advantages associated with the technique, CD is present in almost all laboratories involved with protein analysis even though it mainly provides low resolution information when compared with other techniques. However, this technique is sometimes not well appreciated due to some over or misinterpretation while relating Circular Dichroism with structure. Here we present important principles and other valuable tips to help experimentalists with the analysis and interpretation of CD data.

Key words: Circular dichroism, protein folding, protein stability, spectroscopy.

INTRODUCTION

Historical view and basic principles

Many macromolecules have molecular asymmetry, that is, its structure is not superimposable on its mirror image. In 1848, Louis Pasteur discovered that crystals can be classified according to their optical activity whether they rotate light clockwise (right) or anticlockwise (left). The discovery of this phenomenon, also known as chirality was very important because for the first time molecules were showed to be three-dimensional entities since two-dimensional structures are incapable of showing such phenomenon. CD uses a source of circularly polarized light, in which the vector oscillates rotationally to the right or to the left, forming a helix around the axis of propagation. To compare, when light is depolarized the electromagnetic vector oscillates in any direction perpendicular to the direction of propagation and when light is linearly polarized in a plan the vector oscillates on a single plane in the direction of propagation.

To understand circular dichroism, as any spectroscopic method, it is important to be familiar with the Beer-Lambert law (Schellman, 1975). Briefly, a beam of light with intensity I_0 passing thru a sample is absorbed and continues its trajectory with intensity I. Absorbance A at a determined wavelength is:

$$A = \log_{10} (I_0 / I) \quad (1)$$

A is related to physical-chemical properties of the sample:

$$A = \varepsilon \times l \times C \quad (2)$$

Where ε is the molar extinction coefficient (an intrinsic property of the molecule) in $M^{-1}.cm^{-1}$, l is the optical path-length in cm, and C is concentration in M.

Chirality, a property of molecules related to the structural conformation, is studied mainly by two methods. One is polarimetry, in which the sample is analyzed by linearly polarized monochromatic light. Sample optically

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Abbreviations: Mb; Myoglobin, **WT**; wild-type, **TFE**; trifluoroethanol, **CD**; circular dichroism **[θ]**; mean residue molar ellipticity, **A**; absorbance and **UV**; ultraviolet.

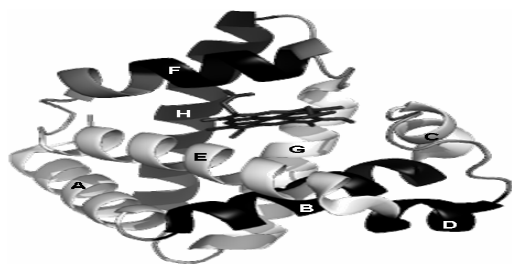

Figure 1. Three-dimensional structure of sperm whale myoglobin. Myoglobin is an all α-helical protein involved in oxygen transport. It has 8 α-helices named A to H. The helices are shown as cartoon and the heme group as stick. The figure was created using the software Pymol (Delano Scientific LLC) and an Mb structure from PDB (2JHO).

active rotate the plane of the light and the angle of rotation, α (in milidegrees), is measured. The polarimetry also follows Beer-Lambert law:

$$[\alpha] = \alpha \times l \times C \quad (3)$$

Where [α] is the specific rotation. In this case data can also be measured as a function of the spectrum, and this method is known as optical rotatory dispersion or ORD.

The other method is Circular Dichroism in which circularly polarized light both to the left and to the right, pass through a sample and the difference in absorption of the components of left and right is measured. An optically active sample will absorb differently left and right circularly polarized light, also obeying the Beer-Lambert law:

$$\Delta A = A_L - A_R = \varepsilon_L \times l \times C - \varepsilon_R \times l \times C = \Delta\varepsilon \times l \times C \quad (4)$$

Where *L* is left and *R* is right. The difference in absorption results in a true light which is elliptically polarized and its angle, named Ψ and usually represented by θ, is measured as milidegrees. Elipticity and absorption are related by the equations:

$$\Delta A = \frac{\theta}{32980} \quad (5)$$

$$\theta = \frac{2.303\ (A_L - A_R)}{4l} \quad (6)$$

For more detailed reviews on the principles of circular dichroism see Johnson (1985), Kuwajima (1995), Kolowski et al. (2000) and references therein.

CD and proteins: Principles

Circular Dichroism is chiefly used for the study of the secondary structure of proteins, although it can also be used to study the conformation of peptides and nucleic acids (Johnson, 1985; Woody, 1995; Woody, 1996). This technique is widely used because requires low sample concentration (1 - 10 mg/ml), allows the study in a wide range of variables (temperature, pH, etc) and gives information on the effect of added ligands. For proteins the results are usually expressed as the mean residue molar ellipticity, [θ]:

$$[\theta] = \frac{\theta \times 100 \times M}{C \times l \times n} \quad (7)$$

Where θ is the ellipticity in degrees, *l* is the optical path in cm, *C* is the concentration in mg/ml, *M* is the molecular mass and *n* is in the number of residues in the protein. The mean residue molar ellipticity [θ] is given in $deg.cm^2.dmol^{-1}$. Such standardization allows independent comparison with results obtained from different batches of samples or from measurements taken in different laboratories or using different spectropolarimeters.

As an example to illustrate the above we can use the properties of sperm whale myoglobin (Figure 1), an all α-helical protein involved in oxygen transport. Usually, 1 micromolar (~ 17 mg/l) of the holoprotein (153 residues, ~17 kDa) in a cell of 1 cm gives -38 milidegrees at 222 nm (Ramos et al., 1999; Ribeiro-Jr and Ramos, 2004). Using Equation 7:

$$[\theta]_{222} = (-0.038 \times 100 \times 17{,}000) / (0.017 \times 1 \times 153) = -24{,}840\ deg.cm^2.dmol^{-1}.$$

The same approach can be used to calculate [θ] for other wavelengths generating [θ] for an entire spectrum. Also very important, CD is a nondestructive method and often used to study protein folding and the processes of protein-protein interaction and protein-DNA interaction (Johnson, 1985; Sprecher et al., 1979).

Protein structure

Secondary structure

CD probes the secondary structure of proteins because the peptide bond is asymmetric and molecules without a plan of symmetry show the phenomenon of circular dichroism. The amide chromophore of the peptide bond dominates the CD spectra of proteins below 250 nm. Amides have 2 electronic transitions of low energy that are well characterized with transitions $n \rightarrow \pi^*$ and $\pi_0 \rightarrow \pi^*$ showing circular dichroism at 215 - 230 nm and 185 - 200 nm, respectively (Woody, 1995; Woody, 1996; Woody and Koslowski, 2002). These two transitions dominate the phenomenon of circular dichroism at far-UV (<240 nm). The transition $n \rightarrow \pi^*$ is electrically prohibited but is magnetically permitted, being primarily responsible for the negative bands at 222 nm, characteristic of the α-helix spectrum, and 216 - 218 nm, characteristic of the β-sheet spectrum. The transition $\pi_0 \rightarrow \pi^*$ is primarily responsible for the positive band at ~190 nm and the negative band at 208 nm, characteristic of the α-helix spectrum, and for the positive band at ~198 nm, characteristic of the β-sheet spectrum.

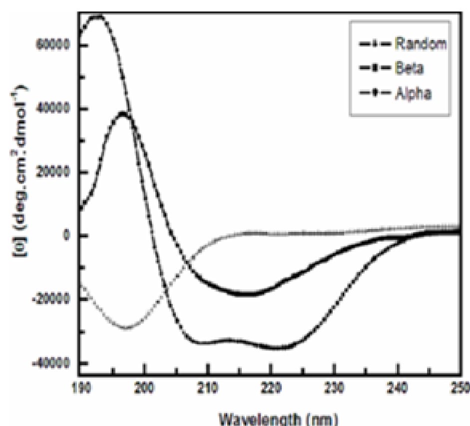

Figure 2. Characteristic far-UV CD spectra for an all-α-helix, an all-β-sheet and a random coil pro-tein. The spectrum for an all-α-helix protein (Alpha) has two negative bands of similar magni-tude at 222 and 208 nm, and a positive band at ~ 190 nm. The spectrum for an all β-sheet protein (Beta) has in general a negative band between 210-220 nm and a positive band between 195 - 200 nm. The spectrum for a disorderly (random) protein has a negative band of great magnitude at around 200 nm.

In conventional CD instruments a far-UV spectrum is collected from 180 to 250 nm when well-behaved samples are used. However, further information is available in the vacuum UV region (<180 nm) which can be collected using synchrotron radiation CD, or SRCD. This is not discussed here and there are many good reviews in this area (Wallace and Janes, 2001).

Figure 2 shows characteristic far-UV CD spectra for an all-α-helix, an all-β-sheet and a random coil protein. In a typical CD spectrum, the absence of signal means absence of circular dichroism, a negative signal means absorption of left circularly polarized light, and a positive signal means absorption of right circularly polarized light. The spectrum for an all-α-helical protein has two negative bands of similar magnitude at 222 and 208 nm, and a positive band at ~ 190 nm (Figure 2). The band at 222 nm is related to the strong hydrogen-bonding environment of α-helices and is relatively independent of their length. The spectrum for an all β-sheet protein has, in general, a negative band between 210 - 220 nm and a positive band between 195 - 200 nm (Figure 2). Spectra for β-sheet proteins are more diverse than those for α-helical proteins because β-sheets may be present at parallel, anti-parallel, or mixed conformations, and can be twisted in many ways. The spectrum for a disorderly (random coil) protein has a negative band of great magnitude at around 200 nm (Figure 2).

Tertiary structure

Aromatic residues (tryptophan, tyrosine and phenylalanine) can also exhibit circular dichroism with $\pi_0 \rightarrow \pi^*$ absorption at between 250 and 300 nm. Although the aromatic residues contribute mainly to the near-UV (> 250 nm) they also contribute to the far-UV spectra of a protein. In general the contribution is very small but when the content of these residues is very high the estimation of secondary structure is complicated. Dissulfide bonds also have circular dichroism related to a transition $n \rightarrow \sigma^*$ at approximately 260 nm. Generally, the peak for a disulfide bond is wider than that for an aromatic residue.

Secondary structure prediction

One of the main applications of CD for the study of proteins is the estimation of secondary structure of proteins. There is an intense progress in cloning and purification techniques that allows the production of a large number of proteins. However, due to present technical difficulties, structural information at high resolution from X-ray diffraction or nuclear magnetic resonance is sometimes difficult or even impossible. Obviously, CD cannot compete with these techniques to provide details about the structure of a protein, but can give a very good estimation of the fraction of the residues in the structure which are involved in α-helix, β-sheet or disorderly formation. This is extremely useful when information about the structure at high resolution does not exist. And even when such information exists CD gives important additional information about the effect of temperature (Hilário et al., 2005; Ribeiro-Jr and Ramos, 2005; Balan et al., 2006), pH (Ramos, 2004; Ribeiro-Jr and Ramos, 2004), charge (Ramos et al., 1999; Regis et al., 2005), ligands (Balan et al., 2005; Ribeiro-Jr and Ramos, 2005; Balan et al., 2006; Borges and Ramos, 2006; Ramos et al., 2007), etc., in the secondary structure.

Many methods have been developed to predict the secondary structure of a protein from its CD spectrum based on its primary sequence using statistical and/or physical-chemical information. In general, the fractional helicity, fH, or the mole fraction of helical backbone α-carbons within the peptide or protein, is usually calculateed as proportional to the experimental molar residue elipticity at 222 nm, $[\theta]_{222}$ (Marqusee and Baldwin, 1987).

One of the simplest methods, and yet fairly reliable, for estimating the quantity of α-helix is the evaluation of the signal at 222 nm using one or both of the following equations:

$$fH = ([\theta]_{222} - 3{,}000)/(-36{,}000 - 3{,}000) \quad (8)$$ (Morriset et al., 1973)

$$[\theta]_{222} = (fH - iK/N)\,[\theta]_{HX} \quad (9)$$ (Chen et al., 1974)

where $[\theta]_{222}$ is the mean molar residual elipticity at 222 nm (in deg.cm^2.dmol^{-1}), fH is the fractional helicity (in %), i is the number of helices in the protein, K is a constant at ellipticity when 100% of the structure is α-helical. $[\theta]_{HX}$ is equal to -34,686 deg.cm^2.dmol^{-1} and thus this value would be the theoretical maximum for an all α-helical pro-

Table 1. Some free programs on the web for secondary structure prediction.

Program	Web address	Reference
CONTIN-CD	http://s-provencher.com/pages/contin-cd.shtml	Provencher and Glöckner (1981)
SELCON	http://srs.dl.ac.uk/VUV/CD/selcon.html	Sreerama and Woody (1994a, 1994b)
DICHROWEB	http://www.cryst.bbk.ac.uk/cdweb/html/home.html	Whitmore and Wallace (2004; 2008)
DICROPROT	http://dicroprot-pbil.ibcp.fr/	Deleage and Geourjon (1993)
K2D2	http://www.ogic.ca/projects/k2d2/	Perez-Iratxeta and Andrade-Navarro (2008)

tein. Such information is useful to compare with the obtained experimental result. For example, if the result is much larger than that or equal to that when previous information point that the conformation of the protein should not be 100% helical, the calculation pre-experimental setups needs serious revision. Additional and valuable information is that the prediction of maximum value for a 100% β-sheet protein from its CD signal between 215 - 220 nm is around -20,000 $deg.cm^2.dmol^{-1}$.

In addition to the aforementioned, Greenfield and Fasman (1969) showed that both β-sheet and random coil would have low ellipticity at 208 nm of about -4,000 $deg.cm^2.dmol^{-1}$ whereas a α-helix would have a maximum at 208 nm equal to approximately -33,000 $deg.cm^2.dmol^{-1}$. Therefore, they suggested that an approximated estimation of the α-helical content may be made from $[\theta]_{208}$, the mean molar residual elipticity in $deg.cm^2.dmol^{-1}$ at 208 nm:

$$fH=([\theta]_{208}-4{,}000)/(-33{,}000-4{,}000) \quad (10)$$

There are other methods that use the whole spectrum of a protein to predict its secondary structure content. These methods establish statistical methods that use combinations of linear far-UV spectra based on reference proteins with known tertiary structure. However, careful analysis is needed in the evaluation because some uncertainty may arise from the fact that the circular dichroism of a protein depends not only on the quantities of structure, but also on the extension of the chain. Other programs, which use non-linear methods, have been created to try to improve the quality of prediction.

Table 1 shows a list of some programs used to predict the structure of a protein and which are available on the web. A wise strategy is to combine different programs for reaching greater confidence. Additional information can be found in (Greenfield, 2006). It is also important to emphasize those errors in calculating the concentration of the protein leading to errors of the same magnitude or even higher in predicting the structure.

Experimental parameters

Buffer choice

Ideally proteins should be diluted in pure water or at least in 10 mM phosphate, however many proteins are not soluble at these conditions requiring the presence of additives. Anyway, buffers used to dilute proteins should be as transparent as possible since information on secondary structure is obtained at wavelengths below 240 nm, in which many buffers absorb light. Therefore, the chemical reagents used in the preparation of buffers must be of excellent quality, without impurities that could scatter light. If necessary, buffers should be filtered to remove particles.

The indication of absorption of light is given by the dynode voltage module (in volts, V), which measures the response of the photomultiplier during the measurement and is an indicator of the quantity of photons that are not absorbed or are scattered by the sample. The higher the amount recorded, the greater the absorption or the scattering. A high absorption causes an increase in noise and consequently increases the errors of elipticity measurement. In general, the acceptable limit for reliable reading is 600 V, but acquisitions of up to 700 V are acceptable provided that the number of spectra collection and accumulation is highly enough to reduce the noise generated by absorption. It is advisable not to perform readings with voltage above 700 V as this may cause damage to the unit. The use of cells with thinner pathlengths decreases the number of photons that the sample is able to absorb. By using the Beer-Lambert law (Equation 2) one can vary the concentration of sample and the length of the optical path in order to optimize the measurement.

Table 2 presents the voltage obtained in a circular dichroism espectropolarimeter Jasco, model J-810, with a lamp with approximately 400 h of use, for a series of solutions (no protein). Since the effect is cumulative and the protein will also contribute to the signal, the Table presents the lowest wavelength to achieve about 400 V in each condition. Reagents of high quality were used and readings were taken in cells of 10 and 1 mm (indicated in the Table). Additionally, solutions of $NaClO_4$, NaF or KF, at 10 mM, are virtually transparent at wavelengths down to 170 nm when using a 1 mm cell (Schmid, 1997). Further information can be found elsewhere (Greenfield, 2006; Kelly et al., 2005).

Protein concentration

The circular dichroism (CD) spectrum of a protein depends on both its conformation and concentration. Equation 7 (above) shows that the mean residue molar ellipticity depends on the optical pathlength (which is known), on the protein concentration (which must be known with great accuracy), and on the degree of protein secondary structure (which can be altered by mis-folding, physical-chemical properties of the solution, drastic mutation, etc). An accurate measurement of concentration is fundamental for the prediction of secondary structure. Errors in the order of 10% in the concentration measurement will give the same factor of error in the structure prediction, such as, for instance, assigning β-sheet structure to an all α-helical protein.

Protein concentration is also an important parameter to reach the best signal to noise ratio. As a rough approximation for protein concentration start by using 0.1% (w/v) for 0.1 mm pathlength and 0.01% (w/v) for 1 mm pathlength (Schmid, 1997). Of course, the va-

Table 2. The lowest wavelength (cut off) to avoid absorbance higher than 400V for a series of solutions (no protein). The measurements were done using 10 mm and 1 mm pathlength cells as indicated. The effect is cumulative.

Buffer	Concentration (mM)	Wavelength (10 mm Cell)	Wavelength (1 mm Cell)
Sodium phosphate	20	205	190
	100	208	200
Sodium acetate	10	208	190
	50	-	200
Tris-Cl	10	212	200
	20	216	208
HEPES	10	220	208
NaCl	50	205	200
Na_2SO_4	20	-	210
Beta-mercaptoethanol	1	212	200
Ditiothreitol	1	222	205
Glicerol	(5%)	208	-
	(10%)	-	200
Guanidinium chloride	1000	212	-
	6000	-	212
Urea	2000	222	-

lues may vary depending on the folding state of the protein but it may work as a good set up for initial measurements.

Among the most common methods to determine the concentration of proteins is the reliable method of Edelhoch (Gill and von Hippel, 1989; Pace et al., 1995; Ramos, 2004), which was established in 1967 and has the name of its inventor. Other methods, such as biuret and its derivatives, weighing and analysis of amino acids can result in errors in the order of 10%. For a discussion about several methods for determination of protein concentration for CD experiments (Kelly et al., 2005).

Basically, the method of Edelhoch uses the absorption, as measured by conventional spectrometer, of aromatic residues in the region between 270 -300 nm, assuming they are completely exposed when in 20 mM sodium acetate pH 6.5 and 6.0 M guanidinium chloride (Edelhoch, 1967). For better results a solution of 25 mM sodium acetate pH 6.5 and 7.2 mM guanidinium chloride (Edelhoch solution) is used in a proportion of 5:1 to dilute the buffer (blank) and the samples. Duplicates of each sample and several protein dilutions will also help the confidence in the result. At 280 nm the ε280 for tryptophan is 5,690 $M^{-1}.cm^{-1}$, the ε280 for tyrosine is 1,280 $M^{-1}.cm^{-1}$ and the ε280 for cystine is 120 $M^{-1}.cm^{-1}$. Therefore, the absorption of a protein at 280 nm (ε280) under the conditions of buffer described above will be:

$$\varepsilon 280\ [M^{-1}.cm^{-1}] = nTrp \times 5{,}400 + nTyr \times 1{,}280 + nSS \times 120 \qquad (11)$$

Where;
n is the number of residues.
SS means a disulfide bridge (cystine).

It is worth mentioning that the most reliable data are obtained using absorption from 0.1 to 0.9, in which the signal to noise ratio S/N will be optimum. Dilutions should be used, if necessary, to work in this range of absorbance. Measuring at least duplicates is also very important. The stability of the spectrophotometer used for concentration calculations should be examined periodically. The absorbance scale can be calibrated with a solution of 0.04 mg/ml of potassium chromate in 0.05 M KOH at 25°C. The absorbance of the solution in a 1 cm cell should be 0.633, 0.712, 0.149, 0.987 and 0.124 at wavelengths of 260, 280, 300, 370 and 420 nm, respectively (Schmid, 1997).

When using the Edelhoch method it is advisable to check for possible contamination with nucleic acids that also absorb at the far ultraviolet. See Ribeiro and Ramos (2004) for a method to separate nucleic acids from a protein sample and an example of how inaccuracies in the measurement of concentration can cause misleading interpretations on the structure of proteins.

There are several interesting methods that can give decent estimates of protein secondary structure content from circular dichroism (CD) spectra without any prior knowledge of sample concentration (Holtzer and Holtzer, 1992; McPhie, 2001; Raussens et al., 2003). However, the success of these approaches are less than that for currently available methods, but their simplicity and the fact that the concentration is not needed may be very attractive for the study of proteins and peptides for which an accurate concentration is not always available.

Other tips to improve measurement

Another important measurement is that of the absorption of the sample at the wavelength of interest prior CD experiments. The optimum absorbance is 0.87 because this gives the maximum signal to noise ratio. Absorbances higher than 1.2 should be avoided. See Kelly et al. (2005) for absorbance values of a protein solution of 1 mg/ml in a cell of 1 cm pathlength at various wavelengths in the UV. Constant flow of nitrogen should be used, usually 3 L/min for measurements at 260 nm, 5 L/min at 200 - 260 nm, 10 L/min at 180 - 200 nm, 50 L/min at 180 nm. The lamp should be let on for at least 30 min, however 60 min is the ideal total time, before measurements. The standard D(+)-camphorsulfonic acid is used for the calibration of the instrument: the concentration of 0.6 mg/ml gives an absorption of 188 milidegrees at 290.5 nm in a 1 cm cell and -38 milidegrees at 192.5 nm in a 1 mm cell. D(-)-pantolactone is also a good standard for the UV region and a 0.03% aqueous solution should give a signal of -186 milidegrees at 219 nm in a 1 cm pathlength cell. Some instrumental settings are important to reduce the signal to noise ratio. The slit width, which controls the spectral bandwidth should be as large as possible but not too large or the band will be distorted. A series of survey spectra with successively larger slit settings will help to determine the largest slit before a decrease in the measured absorbance. Additionally, signal to noise

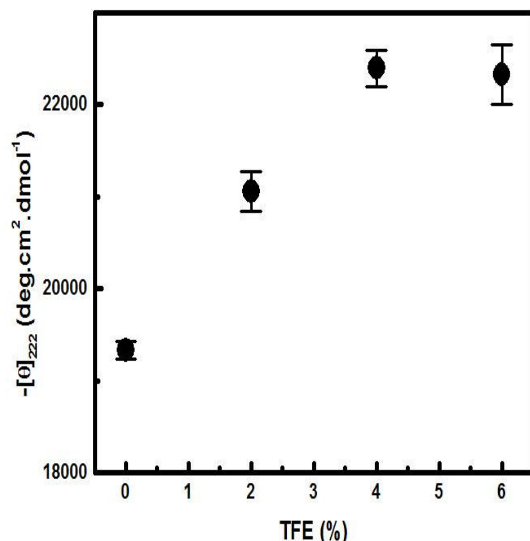

Figure 3. Ellipticity at 222 nm as a function of increased TFE concentration for apomyoglobin. Circular dichroism measurements were recorded using a Jasco J-810 spectropolari-meter with 10 mm path-length cell. The signal at 222 nm was measured and each point shown is the average of at least 3 independent measure-ments.

level is proportional to the square root of the integration time, thus the simplest way to improve spectra quality is increasing the response time. However, in this case the speed of reading needs to be reduced to avoid distortions in the spectrum. For that, the speed of reading multiplied by the response time must be less than 0.33 nm (that is, for a speed of 200 nm/min the response time has to be less than 0.1 s and for a speed of 50 nm/min less than 0.4 s).

Another way to reduce noise is by accumulating readings. In this case, to improve the signal to noise ratio by a factor X, the accumulation time will be X^2. The type of solvent, the concentration of the sample and the optical pathlength can also be changed to improve the quality of the measured spectrum. We may suggest initial measurement parameters for protein analysis: spectral bandwidth of 2 nm for the far-UV region and 1 nm for the near-UV region; response time of 1 s; and scanning speed of 20 nm/min. Accumulation should be as many as necessary and spectrum should start in a wavelength in which no CD signal is present and should end up where HT is about 700 v. Sample should be run before buffer baseline since the exactly same parameters are used to better optimize the subtraction.

Cells should be made of quartz (because the readings are in the ultraviolet wavelength). Cells should be handled with gloves. Good quality cells are almost totally transparent and a measurement of the empty cell should give the same measurement than the empty sample holder (just air). Cells can be cleaned with ethanol or organic solvent such as acetone, but every residue must be washed up, and there are also specific commercial detergents for cleaning. In some cases it will be necessary to maintain the cell at 50% nitric acid for several hours to remove proteins sticking to the quartz (Ramos, 1999).

CD devices available in the market are mainly from four companies: Jasco (http://www.jascoinc.com), Aviv (http://www.avivbiomedical.com), Applied Photophysics (http://www.photophysics.com) and Ollis (http://www.olisweb.com). It is easy to find them on the web and to get information on their spectropolarimeters and accessories.

Protein conformational changes

Additives for increasing secondary structure propensity

Some additives are often used to increase the propensity of certain polypeptides to form secondary structure. They are trifluoretanol (TFE), hexafluoroisopropanol, ethylene glycol, glycerol and others. Among these, the most widely used is TFE (Buck, 1998). Figure 3 shows the effect of increasing concentration of TFE on the $[\theta]_{222}$ of the apo form of sperm whale myoglobin. Apomyoglobin is myoglobin without the heme group and in this form its $[\theta]_{222}$ decreases from about -25,000 (holo form) to -19,000 deg.cm^2.dmol^{-1} (Ramos et al., 1999). It is a well studied monomeric protein with eight α-helices which has been cloned and purified (Springer and Sligar, 1987; Ribeiro-Jr et al., 2003; Ribeiro-Jr and Ramos, 2004). nmR studies (Eliezer et al., 1998) showed that apomyoglobin is structurally similar to myoglobin, except for an unfolded F helix (Figure 1). The addition of TFE increases the amount of secondary structure of apoMb (Figure 3) probably by enhancing the helical conformation of helix F. Although TFE is often used at high concentrations (up to 100%) for the study of peptides (Mares-Guia et al., 2007), the same procedure is not advised for proteins. Previous results have shown that concentrations much larger than 10% affect the tertiary structure of a protein and therefore its native state. Small viscogenic cosolvents alter protein stability (Timasheff, 1993). Ramos et al. (2007) have used far- and near-UV CD to test the effect of a series of small viscogenic cosolvents on the stability and structure of apomyoglobin at pH 4.2. The solvents were sucrose, glycerol, sarcosine and trimethylamine N-oxide (TMAO) from 0.25 to 2.1 M. Firstly, the effect of increasing concentration of sucrose on the far- and near-UV spectra was analyzed showing an increase in the amount of CD signal at the whole spectrum. The effect of the viscogenic cosolvent was specific on secondary structure since no effects on tertiary structure were detected by the investigation of 1 H one-dimensional nmR spectra (Ramos et al., 2007). Secondly, the effect on stability was investigated by following the urea-induced unfolding of the protein using $[\theta]_{208}$ to probe the conformation. Small viscogenic cosolvents increase the stability of apoMb pH 4 intermediate relative to the urea-induced unfolded form (Ramos et al., 2007). Since apomyoglobin at pH 4.2 is an intermediate, that is, a partially unfolded protein, viscogenic cosolvents can be used to stabilize protein preparations that are partially unfolded.

Isodichroic point

The isodichroic or isobestic point is the wavelength where the molar absorptivity is the same for two (or more) protein spectra (Johnson, 1988; Woody, 1996). For any peptide that exhibits an additive or temperature-dependent multiple CD spectra with an isodichroic point at 203 nm, fractional helicity is conventionally calculated as proportional to the value of $[\theta]_{222}$. An isodichroic point near 203 nm in the CD spectra of peptides or proteins has been the standard test for a local two-state dichroic model that is applied to ellipticities at neighboring wavelengths. As noted by Holtzer and Holtzer (1992), for either α-helical dimeric coiled coil proteins or a variety of helical peptides, a consistent ellipticity $[\theta]_{203}$ of –13,000 to –17,000 deg.cm^2.dmol^{-1} is observed. Therefore these authors propose its use as a convenient monitor of peptide concentration.

A 203 nm isodichroic point is often taken to validate a global two-state helix-coil ellipticity model that extends to 222 nm (Gans et al., 1991; Nelson et al., 1986). However, Wallimann et al. (2003) remind us that a CD spectrum sums local ellipticity contributions, and the existence of an isodichroic point at short wavelength does not rule out contributions of local dichroic chromophores that signifycantly perturb ellipticity only at longer wavelengths. Therefore, sometimes a more rigorous test of the two-state model is needed (Wallimann et al., 2003).

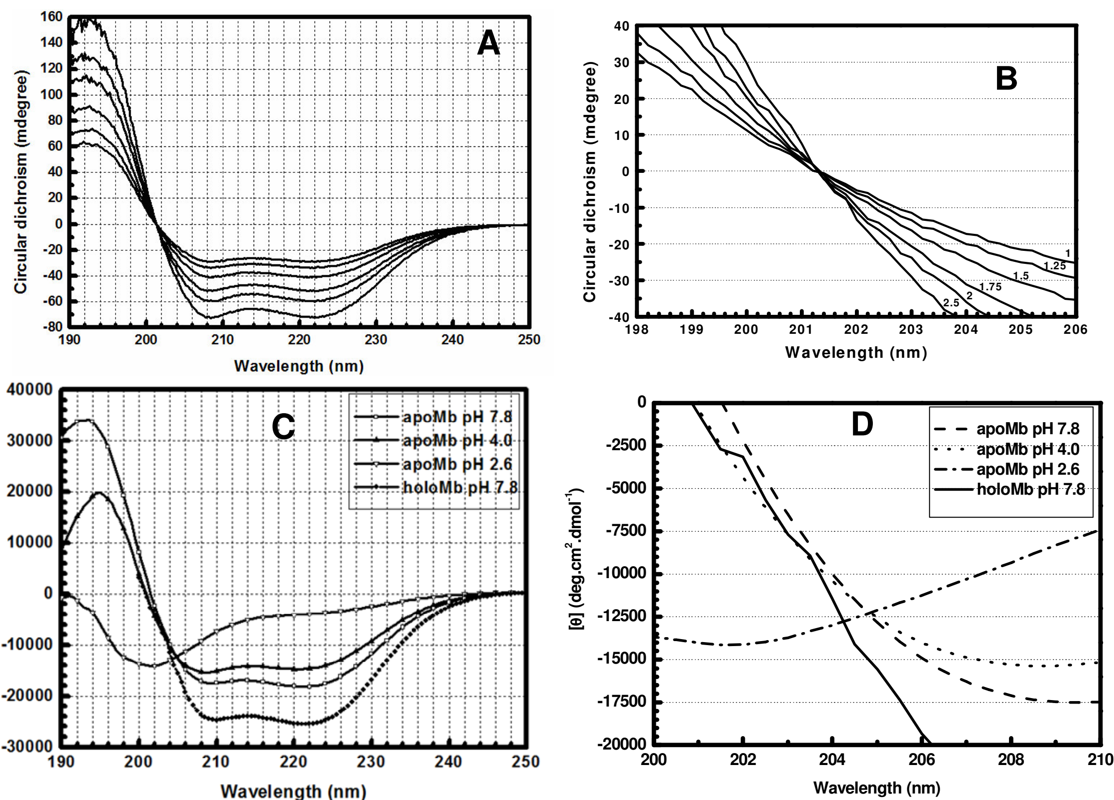

Figure 4. Circular dichroism spectra of apo and holo Myoglobin (Mb) as a function of concentration and pH for inspection of the isodichroic point. Circular dichroism measurements were recorded using a Jasco J-810 spectropolarimeter with 1 or 10 mm pathlength cell. Each spectrum was accumulated at least 16 times at room temperature. Dashed lines in the background are used to facilitate visualization. **A** and **B** Far-UV CD spectra of WT apoMb at concentrations of 1.00, 1.25, 1.50, 1.75, 2.00 and 2.50 µM. The isodichoric point is at about 201.5 nm with no absorption of circular polarized light. **C** and **D** Far-UV CD spectra of WT holoMb at pH 7.8 and apoMb at pHs 7.8, 4.0 and 2.6. The isodichoric point is at 204-205 nm with absorption of about -12,500 deg.cm^2.dmol^{-1}. B and D are zoomed from A and C.

The isodichroic point of native myoglobin, both in the holo and in the apo forms, at several concentrations differ both in the magnitude and in the wavelength from the spectra of the same protein at conditions known to cause conformational changes. Apomyoglobin maintains a folded structure at neutral pH, forms an intermediate at pH 4.2 and is almost completely unfolded at acidic pH (Jamin, 2005). The isodichroic point for the apoMb spectra as a function of increased concentration from 1 to 2.5 µM occurred at about 201.5 nm, with almost no absorption of circular polarized light (about 0 millidegree) (Figure 4A and B). On the other hand, native, intermediate and acidified apoMb have an isodichroic point at about 204 - 205 nm, with considerable absorption of circularly polarized light. When apoMb lost its native structure during pH unfolding, the CD spectra showed an isodichroic point at about 205 nm with an absorption of circular polarized light of about –12,500 deg.cm^2.dmol^{-1} (Figure 4C and D). The same was verified for holoMb, which gained structure upon heme binding with isodichroic point at about 204 nm and [θ] of about –12,500 deg.cm^2.dmol^{-1} (Figure 4C and D). To sum up, for α-helical proteins multiple spectra with decrease in concentration has isodichroic point at around 201 nm, with nearly no absorption of circular polarized light. The isodichroic point for a change in secondary structure conformation occurs at a different wavelength (near 205 nm) from the isodichroic point for a decrease in concentration, and there is absorption of circularly polarized light.

Potential applications for isodichroic analyses are:

i.) Researches working with proteins sometimes found that different preparations of the same protein have CD spectra that do not match each other. Usually the mismatch is caused by technical difficulties to measure the precise concentration of the protein. However, a correct diagnosis is crucial because spectra mismatch may be caused by modifications in the conformation of the protein and further, sometimes extensive, studies will be necessary to correct this problem. Concentration miscalculation were easily correct by using a spectroscopic method that relies on the absorbance of Trp and Tyr residues of proteins unfolded by high amount of denaturant and the mismatched CD spectra 'phenomenon' disappeared. As aforementioned the normalized spectrum depends chiefly on protein concentration.

ii.) The effect of additives and potential ligands on a protein. The addition of solutes will decrease the effective concentration of the protein and may also increase the scattering of light by the sample. In both cases it will reflect in a measured spectrum which will need correction for the effect of additives. In some cases may be difficult to differentiate concentration effect from conformational change just by looking at the shape of the spectrum. In this case analysis of the isodichroic point may help to identify if the ligand had effect on the structure. Note that the addition of heme to apoMb form holoMb and the spectra had characteristic isodichroic point of structural changes (Figure 4C and D). Isodichroic point analyses could be applied in high throughput tests where the effect of tenth or hun-

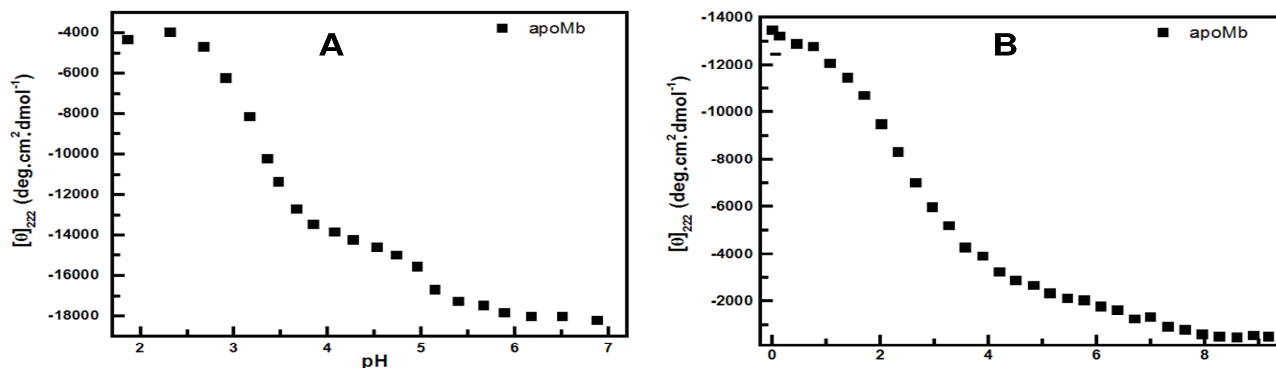

Figure 5. Circular dichroism spectra of apoMyoglobin (apoMb) as a function of denaturant conditions. Circular dichroism measurements were recorded using a Jasco J-810 spectropolarimeter with 10 mm pathlength cell. The signal at 222 nm was measured and each point shown is the average of at least 3 independent measurements. **A)** pH-induced equilibrium unfolding of sperm whale apoMb. **B)** urea-induced unfolding transition of sperm whale apoMb at pH 4.2.

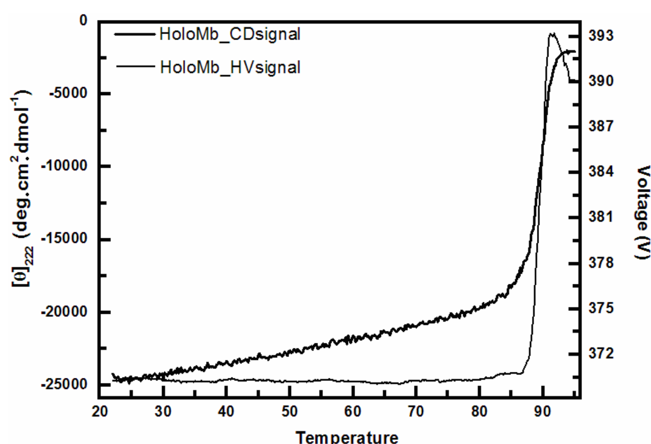

Figure 6. Circular dichroism spectra of HoloMyoglobin (Mb) as a function of temperature. Circular dichroism measurements were recorded using a Jasco J-810 spec-tropolarimeter with 10 mm pathlength cell following the signal at 222 nm and the dynode voltage V as a function of temperature. A heat-induced change in the dynode voltage is originated from variations in the light scattering which depends on the particle size and thus from aggre-gation. The transitions indicate that the protein unfolds as verified by the decrease in CD signal and aggregates as verified by the increase in the voltage which is related to light scattering. A non-aggregated protein would have shown a constant dynode voltage throughout the whole scan.

dred of tenths or hundreds of compounds are analyzed. In this case, the protein compound spectrum that has an isodichroic point with the native protein spectrum characteristic of conformational change can be select for further analysis.

iii.) Studies on the effect of site-directed mutagenesis. A mutation can affect a particular method for calculating protein concentration generating a CD spectrum that will be different from that of wild-type even though this hypothetical mutation did not affect the native structure. The analysis of the isodichroic point will distinguish a mutation that affects protein concentration calculation from that affecting native structure (Ribeiro-Jr and Ramos, 2004).

Following protein thermal-induced unfolding or aggregation

Circular dichroism spectroscopy is widely used to follow the stability of a protein by increasing denaturant conditions. The most common denaturants are temperature, chemicals (usually urea and guanidinium chloride) and extremes of pH (usually acidic). As the denaturant condition increases the stability of the protein decreases and then unfolds (Ramos and Ferreira, 2005). CD is a convenient technique to follow protein unfolding because the spectra of folded and random coil are quite different (Figure 2). The unfolding transition can be easily determined by choosing a wavelength where the difference in signal for folded and unfolded protein is large. For instance, α-helical proteins have a large CD signal at 222 nm in which unfolded proteins has none or little signal (Figure 2). Figure 5 shows two transitions for the unfolding of sperm whale apomyoglobin followed by CD at 222 nm, one induced by acidic pH (Figure 5A) and other induced by urea (Figure 5B). Note that the acidic-induced unfolding (Figure 5A) reveals an intermediate in the folding pathway of apomyoglobin which seems to unfold throughout a two-state like transition by urea-induced unfolding (Figure 5B).

CD can also be used to follow aggregation when heat-induced unfolding is followed (Figure 6). As discussed above the dynode voltage V (high-voltage applied to the photomultiplier of the UV detector to compensate for the reduction in the intensity light) is a result from light absorption or scattering and should be recorded for all CD measurements. For being related to scattering the recorded dynode voltage V can be used to probe the turbidity of the solution (Benjwal et al., 2006). Accordingly to the Beer-Lambert law a heat-induced change in the dynode voltage can only be originated from variations in the light scattering which depends on the particle size. Figure 6 shows the signal of CD at 222 nm and the dynode voltage V as a function of temperature. The curve profile shows that the CD signal starts to decrease at about 85ºC followed by a two-state like transition. This transition indicates that the protein unfolds as verified by the decrease in CD signal and aggregates as verified by the increase in the voltage which is related to light scattering. To sum up, as the protein aggregated its size increased and more light was scattered.

Conclusion

In conclusion, CD spectroscopy is an important technique to study protein folding, form and function. It is probably the most important technique for the initial investigation of

purified proteins. In addition to that, CD spectroscopy can give fast and reliable information about the folded conformation and the stability of a protein with little time expending and for an affordable cost.

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