APPLICATIONS OF CIRCULAR DICHROISM (CD) SPECTROSCOPY AS EDUCATIONAL TOOL FOR UNDERGRADUATE STUDENTS AND MONITORING PROTEIN CONFORMATIONAL CHANGES INDUCED BY LIGAND BINDING

by

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ABSTRACT

APPLICATIONS OF CIRCULAR DICHROISM (CD) SPECTROSCOPY AS AN EDUCATIONAL TOOL FOR UNDERGRADUATE STUDENTS AND MONITORING PROTEIN CONFORMATIONAL CHANGES INDUCED BY LIGAND BINDING

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The work reported here provides an overview on the applications of circular dichroism (CD) spectroscopy for the study of protein secondary structure. This project has two specific goals. First, to develop and validate an undergraduate experiment for CHEM 3175 (biophysical chemistry laboratory) to demonstrate the use of CD spectroscopy for the determination of protein secondary structure. In an extension of this work, the same principles of analysis were applied to the study and characterization of the protein-ligand interaction of MiaE (a non-heme iron hydroxylase) and its substrate, tRNA.

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CHAPTER 1

INTRODUCTION

1.1 Background

Circular dichroism (CD) is a valuable spectroscopic method for studying macromolecules that differentially absorb clockwise (right handed) or counter-clockwise (left handed) circularly polarized light as the result of structural asymmetry. While other methods of protein structural analysis are available, the high sensitivity of CD spectroscopy allows for characterization of macromolecules at concentrations not feasible for higher resolution techniques such as X-ray crystallography (XRD) and nuclear magnetic resonance (NMR) spectroscopy. Moreover, CD measurements can be made under a variety of experimental conditions (including physiologic), which is not typically possible for conventional XRD or NMR methods[1,2]. Historically, CD spectroscopy has been employed to monitor protein stability (thermal, pH, ionic strength or solvent), compare conformational differences in protein from various expression systems or species, observe changes in structure upon protein-protein or protein-ligand interactions and to confirm proper folding during purification processes[3,4,5]. Other advantages for the use of CD include the speed of analysis which makes time resolved measurements possible[2].

1.2 Theory of Measurement

When two waves of plane polarized light of equal amplitude and wavelength meet perpendicular to each other but 90° or -90° out of phase the resulting wave is circularly polarized. As shown in Figure1.1 (A), when the resulting left and right handed circularly polarized waves are recombined they form a plane-polarized wave.

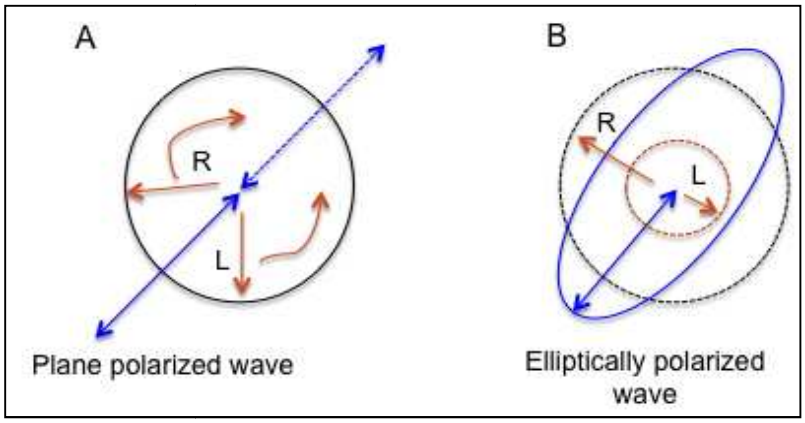


Figure 1.1 (A) Plane polarized wave (B) Elliptically Polarized Wave. Plane polarized (blue) is formed by combination of right and left circularly polarized light. Elliptically polarized wave (blue) is caused by absorption of the left circularly polarized light.

When a chromophore interacts with circularly polarized light it may absorb the left or right circularly polarized waves to different extents resulting in the transmission of light that is elliptically polarized[1]. In, Figure1.1 (B) the differential absorption (termed circular dichroism) of the left circularly polarized light resulting in an elliptically polarized wave is demonstrated.

The spectropolarimeter, or the CD instrument, measures the difference in absorbance, ΔA, between the left (A_L) and right (A_R) circularly polarized components ($\Delta A = A_L - A_R$)[6]. The signal can also be reported as ellipticity (θ) in degrees and is related to ΔA, simply by a numerical factor, θ (deg) $=(2.303*\Delta A*180)/4\pi$. The ellipticity ($\theta$) is the tangent of the ratio of the minor to major axis of the ellipse[2]. A basic diagram of the JASCO J-810 model is shown in Figure 1.2. This model and its predecessor, J-710, use a modulator, CDM, to switch from left and right circularly polarized light. The absorption of each component by the sample is detected sequentially by the photomultiplier[1].

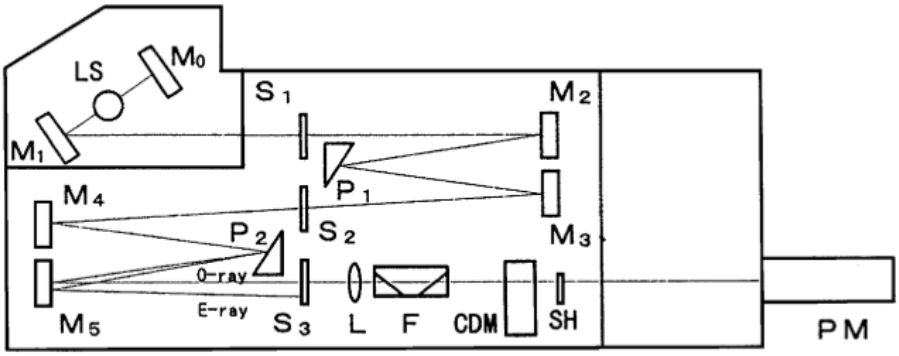


Figure 1.2 Jasco J-810 Diagram. A Xe light source is directed by mirrors (M1-M5) to prisms P1 and P2 to select the wavelength, followed by focusing, L, and modulation, CDM, before entering the sample compartment and detection by the photomultiplier, PM. This figure was taken directly from Biochimica et Biophysica Acta.2005, 1751 (ref 1).

A signal is observed for chromophores that are chiral either intrinsically due to its structure, through covalent bonding to chiral centers or when in asymmetric environments due to a 3-dimensional structure such as proteins and oligonucleotides[1,6]. For example, proteins typically absorb UV-light due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions associated with peptide amide bonds and aromatic residues, respectively. In particular, the amide $n \rightarrow \pi^*$ transitions observed between 190 – 240 nm are highly sensitive to their local symmetry and thus are useful for interrogating a proteins secondary structure[1,6]. As illustrated in Figure 1.3, protein secondary structures such as alpha helix and beta sheets can be identified by characteristic CD absorption features observed from these transitions[6]. In the spectral region above >240 nm chromophores such as the aromatic residues can contribute to observed ellipticity. However, these features are typically much less intense[1]. In some cases information about the tertiary structure can be determined from the intensity of these spectral bands. Furthermore, ellipticities can also be weakened when the chromophore resides on highly mobile side chains within a globular protein[1].

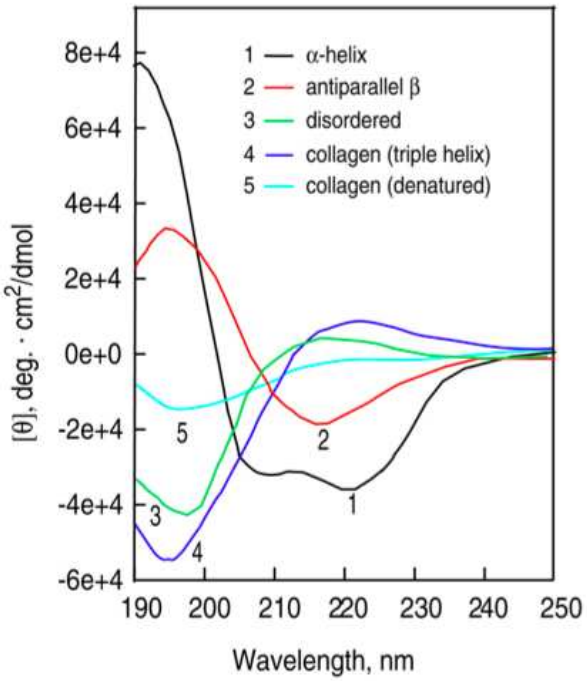


Figure 1.3 CD spectra of Secondary Structural Elements. Poly-L-lysine in different conformations, lines 1-3, and collagen in its native, line 4, and denatured form, line 5. This Figure was taken directly from Nat. Protoc. 1(6), 2006 (ref 2).

1.3 Experimental Considerations

As with any analytical technique, collection of reliable data is dependent on proper sample preparation, instrument usage and data analysis. The instrument should be calibrated to ensure the wavelengths and magnitudes are accurate. The reagent 1S-(+)-10-camphorsulphonic acid (CSA) is commonly used for protein applications since it has spectral bands at 290.5 and 192.5 nm. At these wavelengths, the expected ellipticity for a 0.06% (w/v) solution of CSA in distilled water with a 1 cm pathlength is +202 mdeg and -420 mdeg. respectively[1]. The ratio of the intensities should be between 2.05 and 2.08 and is reflective of the instrument performance and is independent of the concentration or pathlength[1,2]. The ammonium salt of CSA can also be used, sometimes preferred because it is less hygroscopic. The expected ellipticity of a 0.06%w/v solution of the ammonium salt should be +188 mdeg and -391 mdeg at 290.5 and 192.5 nm, respectively, using a 1 cm pathlength[1].

The Jasco J-710 spectropolarimeter, used in this study is similar to the currently manufactured model, J-815. It is a benchtop instrument with a footprint approximately four by two feet and needs a continuous source of nitrogen gas to flush the system preventing ozone formation from ionization of oxygen at the UV lamps. A xenon lamp generates ultraviolet light, which is directed through a series of shutters and prisms to select, polarize and focus plane polarized light to the modulator. The modulator contains a piezoelectric quartz crystal subjected to an alternating electric field that induces structural changes in the crystal and results in selection and transmission of left or right circularly polarized light. The alternating left and right circularly polarized light is transmitted through the sample compartment followed by detection at the photomultiplier. The instrument outputs the raw data in millidegrees (mdeg) using the Spectra Manager software. In Spectra Manager the spectra can be processed and manipulated. For example, operations such as baseline subtraction, curve smoothing, peak detection and unit conversions are readily performed.

Selection of the buffer system can greatly impact the quality of data generated. The absorbance intensities in the far UV (<220 nm) for common solvents and buffers should be considered as most have increasing absorbencies in the lower wavelength range. A short list of buffers and suggested wavelength limits are shown in Table 1.1. When the high tension voltage (HT[V]) exceeds an acceptable threshold, generally greater than 700 V, poor signal to noise prohibits accurate measurements. The HT[V] is the voltage applied to the photomultiplier and is usually traced simultaneously during the CD scan for convenience. Methods to minimize spectroscopic interference include using alternative salts other than chloride (sulphates or fluorides), lowering the concentrations of buffer ions, filtering all solutions to remove particulates and using cuvettes with shorter pathlengths. As demonstrated in Figure 1.4, the detrimental effect of buffer interference on the CD spectra of α-chymotrypsin is clear, with increasing absorption the signal to noise ratio is poor resulting in signal artifacts and unreliable data.

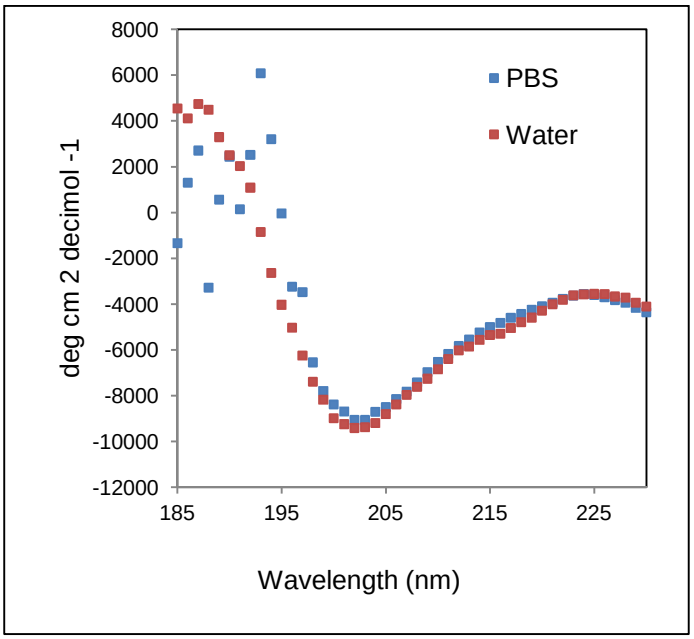
Phosphate buffered saline (PBS) contains both NaCl and KCl, which absorb strongly below 200 nm, in contrast to water, which remains below the HT(V) threshold at 185 nm.

Table 1.1 Wavelength Limits for Select Buffer Components

Buffer Components	Pathlength (cm)	Low Wavelength Limit (nm)
50 mM NaF	0.02	<185
50 mM boric acid	0.02	< 185
50 mM Na borate	0.02	180
50 mM Na acetate	0.02	190
10mM potassium phosphate, 100mM potassium fluoride	0.1	185
20 mM potassium phosphate, 100 mM NaCl	0.1	195
2 mM HEPES, 50 mM NaCl, 2 mM EDTA, 1mM DTT	0.1	200
50 mM Tris, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT	0.1	201
20 % glycerol	0.1	200
6 M Guadinium-Cl	0.02	210

Compiled from information presented in both Kelly et. al.(2005)[1] and Greenfield(2006)[2]

Similar to UV-Vis spectroscopy, appropriate baseline scans are necessary to correct for background contributions, or in the case of CD measurements, to ensure the buffer does not have dichroic components. In general the solution components should be optically inactive, free of particulate matter and prepared in degassed or distilled water as oxygen can also absorb below 200 nm and contribute to poor signal-noise values[2].



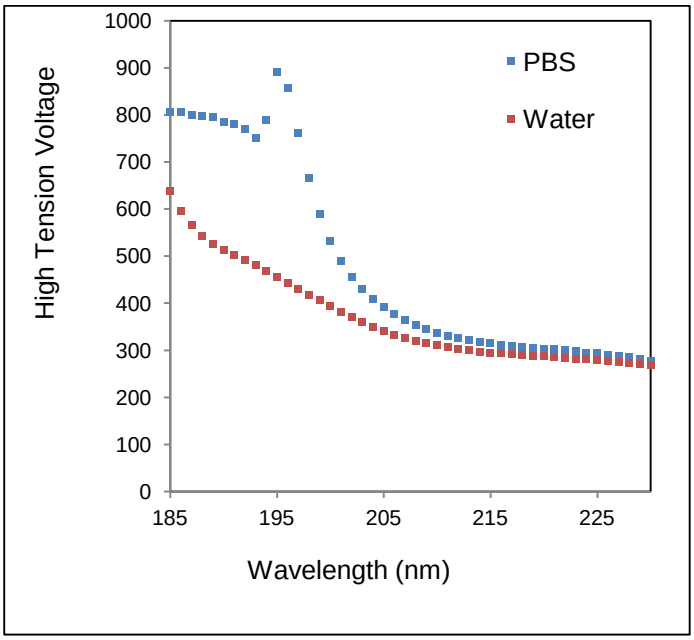


Figure 1.4 Alpha-Chymotrypsin and Buffer Effects. (A) MRE and (B) HT(V). α-Chymotrypsin was prepared in both water (0.19 mg/mL) and 10 mM PBS, pH 7.4 containing 137 mM NaCl and 2.7 mM KCl (0.3 mg/mL). Measured with JASCO J-710 in 0.1 cm cylindrical quartz cuvette and reported in mean residue ellipticity, MRE, degrees*cm2decimol-1 (A), the high tension voltage, HT(V), is also shown (B).

Along with selection of appropriate buffer components the sample preparation is also a critical factor for reliable CD spectra. In particular, the protein concentration must be accurately

determined in order to evaluate the secondary structure content. The measured CD response is dependent on concentration, number of amino acids and cuvette pathlength[1,2,6]. For structural analysis the normalized result, mean residue ellipticity, is calculated accounting for the concentration and number of repeating units contributing to the observed CD signal. For proteins the repeating unit is based on the peptide bond. Similarly, the repeating unit for RNA and DNA are the individual mononucleotides joined by phosphodiester bonds. The mean residue weight, MRW, can be accurately determined by:

$$MRW = M / n \qquad [1]$$

where M is the molecular mass (Daltons) and n is the number of peptide bonds. The mean residue ellipticity (MRE) in {degrees cm^2 $decimol^{-1}$} can then be determined by:

$$[\theta]_\lambda = (MRW \bullet \theta_\lambda) / (10 \bullet d \bullet c) \qquad [2]$$

where θ_λ is the observed ellipticity (degrees) at λ_n, d is the pathlength (cm) and c is the concentration (g/mL). The spectra converted to MRE, $[\theta]_\lambda$, for a given sample should be the same regardless of the concentration tested. This normalization allows direct comparison to reference spectra for the accurate deconvolution into the secondary structure content[2]. In some cases, the MRE will vary with concentration and is indicative of aggregate formation or concentration dependent interactions. Methods for the determination of protein concentration include but are not limited to the bicinchoninic acid (BCA) method, Bradford method using Coomassie Blue and the Lowry method using the Folin-Ciocalteu reagent. In addition molar extinction coefficients specific to each protein or other molecules containing a chromophore can be used for the determination of concentration. The calculated value of the absorbance at 280 nm (A_{280}) as well as the extinction coefficients for many proteins can be found on vendor

specification sheets, from Protparam in the Expasy system (http://www.us.expasy.org/tools/protparam.html) or determined experimentally[1]. The range of concentrations applicable for CD measurements is broad and relatively high concentrations can be used as long as the absorbance of the sample (protein and solution components) is below the absorbance threshold, minimizing noise. Options for meeting these spectroscopic requirements include changing the pathlength of the cuvette, changing the concentration of the sample or both.

The selection of instrument parameters is also an important consideration prior to acquiring CD spectra. The requirements of the study, such as measurement speed or resolution, will determine the appropriate parameters. The settings that can be adjusted are bandwidth, time constant, scan rate, accumulation and sensitivity[1]. Bandwidth determines the precision of the wavelength selection. Increasing the bandwidth increases the amount of light incident on the sample. High-resolution measurements require smaller bandwidths but common settings are between 0.1-2 nm. The time constant is the time in which the data is averaged or time allowed for each data point collection[2]. Scanning rates or speed can vary between 10-100 nm/min depending on the experimental need (balance between resolution and speed). For example, lower scan rates generally improve the quality of data but may not be efficient for time resolved measurements or high throughput. The individual parameters are dependent on each other and must be adjusted accordingly to yield spectra with acceptable signal to noise levels. A suggested guideline follows that the product of the scan rate and time constant should be less than the bandwidth[1]. Another option for improving the quality of data is to increase the accumulation or number of scans averaged per spectrum. Averaging multiple scans is used for smoothing the spectrum for improvement of the signal to noise ratio. Again, increasing the accumulation will increase the time per measurement but can sometimes be alleviated by using a faster scan rate. Selection of these parameters should be investigated for each application

and will depend on time available, experimental requirements and stability of the sample and/or the instrument.

1.4 Data Analysis

Methods for deconvolution of CD spectra have improved greatly over the years due to better protein reference sets, optimized algorithms, and improvements in the acquisition of CD spectra[7]. Analysis methods assume the CD spectrum in the far UV range (190-240 nm) is a linear combination of the absorbance contributions from each of the secondary structural elements such that:

$$[\theta]_\lambda = \chi_a[\theta_a]_\lambda + \chi_b[\theta_b]_\lambda + \chi_o[\theta_o]_\lambda \qquad [3]$$

where the observed ellipticity $[\theta]_\lambda$ is the sum of the fractional composition of each structural element (χ_a, χ_b, χ_o) times the measured CD for homopolypeptides of pure alpha helix, $[\theta_a]_\lambda$, beta sheet, $[\theta_b]_\lambda$, or other defined structure, $[\theta_o]_\lambda$[8]. Initially estimates of secondary structure composition were based on least squared fits of experimental spectra to that of reference sets of poly-lysine in alpha, beta or random conformation[8, 9]. Today many algorithms exist that use collections of simulated CD spectra based on X-ray crystallography for reference protein data. In addition, alternative algorithms use collections of measured CD spectra of select proteins under defined conditions as the reference data set. The most simplest estimation of fractional composition for an unknown protein can be obtained using a set of at least three wavelengths and solving simultaneously a set of equations in the form of equation [3][10]. If three proteins of known structural composition are used as the basis set, estimations of $[\theta_a]_\lambda$, $[\theta_b]_\lambda$ and $[\theta_o]_\lambda$ can be generated representing these structural elements as they occur in typical protein[1]. Although straightforward, this method is only a crude estimation as there are many structural elements and they may not be represented well by the three selected reference proteins. In general estimations of alpha helices are typically the most reliable. Structural elements besides alpha

helices that may be present and underrepresented are parallel and anti parallel beta-pleated sheets, beta turns, poly(proline) type II helix, bends, bridges and extended forms[11]. Indeed estimation of beta sheet and other structural elements besides alpha helix is rather difficult and more complicated algorithms that utilize extensive reference data sets are often needed[7,11]. Accurate analysis of secondary structural content is highly dependent on the range of wavelengths used, the quality of the CD reference sets and the quality of the acquired CD data for the sample. Although a detailed description of these algorithms will not be discussed here, there are many reviews published that address the different methods available and the reader is encouraged to read them if interested (references 11, 12, 13, 14 and 15). A selection of available CD analysis programs, SELCON, VARSLC, K2D, K2D3 and LINCOMB CONTIN, can be found at the websites listed in Appendix A[2]. The neural network, K2D3, was used for the secondary structure analysis presented in this report, http://www.ogic.ca/projects/k2d3/index.html[15]. It uses a k-nearest neighbors (k-NN) like algorithm with predictive power based on thousands of reference CD spectra to estimate the alpha and beta-strand contribution of an unknown CD spectrum[15]. Regardless of the algorithm chosen, the determined alpha helix content is generally more accurate due to the stronger CD intensities and more consistent geometry of this structural element[11].

Despite limitations in accurately determining the secondary structure content for folds other than alpha helix, the quality of reference datasets are improving and with them a greater ability to identify the variety of structural elements that occur in protein. In addition, this limitation does not affect the quantitative aspect of CD. Quantitatively, changes in spectral intensities are directly proportional to physical changes in structure due to complex formation and is applicable to studies of protein binding or folding studies as a function of temperature, solvent and time[16].

CHAPTER 2

INTRODUCTION OF CD TO THE UNDERGRADUATE BIOPHYSICAL CHEMISTRY LAB

2.1 Introduction

The opportunity to teach the concepts of circular dichroism to undergraduates could yield benefits in expanding student understanding of molecular asymmetry, protein stability and thermodynamics while providing additional instrumental experience beyond UV-Vis spectroscopy[16]. CD in the undergraduate lab can give students a visual confirmation that proteins have unique structural elements identifiable by their spectral fingerprint. This property is a valuable tool for studying the dynamics of protein stability, binding dynamics and exploring the structure-function relationship. If instrumentation or time is not available there are online educational tools that can expose students to CD spectroscopy and its applications[8]. The UTA Chemistry and Biochemistry Department has a JASCO J-710 spectropolarimeter with associated nitrogen source and computer software for spectral analysis and was made available for the Biophysical Chemistry lab. A CD lab exercise was introduced encompassing the characterization of secondary structural content as well as the dynamics of protein folding. A copy of the lab outline provided to the students is included in Appendix B. The goal of the lab exercise was for students to learn the fundamentals of CD analysis, generate reliable data by including the proper controls, learn about data normalization and demonstrate protein folding dynamics. An example of the type of data that could be generated as a result of this lab will be presented.

2.2 Experimental Methods

Materials and Reagents: Buffer solutions were prepared from mono and di-basic sodium phosphate (Fisher Scientific) in Milli-Q purified water with minimal pH adjustment using 1N HCl and NaOH. In some cases PBS, phosphate buffered saline (10mM Phosphate, 137 mM NaCl, 2.7 mM KCl), was used (Omnipur, EMD Millipore). Glycerol (Sigma Aldrich G5516) was used for stability studies. All solutions were filtered with 0.2 µm PES membranes (Nalgene)

prior to preparing CD samples. Hen egg white Lysozyme (Rockland MB-109), Myoglobin equine heart (Sigma Aldrich M1882) and alpha-Chymotrypsin from bovine (Sigma Aldrich C4129) were prepared either in buffer, water or 5 M guanidine-HCl (GdnHCl). A JASCO J-710 spectropolarimeter and 0.1 cm cylindrical quartz cuvettes (J/31/B) were used for all CD measurements. The JASCO J-710 was calibrated on 7-18-12 using CSA by a JASCO technician.

Methods: Previous work was performed to determine the appropriate protein concentration for optimal signal/noise levels (data not shown). For the protein folding studies Lysozyme (0.4 mg/mL) was prepared in 10 mM sodium phosphate, pH 6.9 or 5 M Gdn-HCl in water. Each solution was diluted either 1:1 or 1:2 from the concentrated stock in ultrapure water for a series of solutions with and without Gdn-HCl. The CD spectrum was measured for each sample and a baseline containing the appropriate levels of buffer or Gdn-HCl was also collected and subtracted for each. Myoglobin (0.1 mg/mL) and α-Chymotrypsin (0.19 mg/mL) were each prepared in 10 mM sodium phosphate, pH 6.9 and water, respectively. Protein concentration was determined using the reported molar extinction coefficients for the absorbance at 280 nm; Myoglobin, ε^M =13980, Lysozyme, ε^M = 37970, Chymotrypsin, ε^M =50585 (ProtParam, Expasy).

Data Analysis: The parameters for measurements were standard resolution, 100mdeg, bandwidth, 2 nm, response time, 2 seconds, scan rate, 50 nm/min, and accumulation of 2 scans. The mean residue ellipticity, deg•cm^{2}decimol^{-1}, was used for the estimation of secondary structure content.

2.3 Results and Discussion: Lysozyme Study

The denaturing effect Gdn-HCl was used to demonstrate changes in protein structure and the ability of Lysozyme to re-fold upon the dilution of Gdn-HCl. An outline of the solutions tested is given in Table 2.1. For each solution an appropriate baseline scan of solutions without protein was subtracted from the collected sample raw data. The mean residue ellipticity, deg•cm^{2}decimol-1, for each sample was used in calculations of the percent alpha helix content.

Table 2.1 Sample Summary for the Lysozyme Folding Study

Control Series			Test Series		
Sample	Lysozyme (mg/mL)	Gdn-HCl (Mol/L)	Sample	Lysozyme (mg/mL)	Gdn-HCl (Mol/L)
1	0.4	-	4	0.4	5
2	0.2	-	5	0.2	2.5
3	0.13	-	6	0.13	1.67

All CD spectra were collected from 190-260 nm but only data from 210-260 nm was used for analysis due to strong absorption of Gdn-HCl below 210 nm limiting the usable data range. The CD data below 210 nm showed artifacts that coincided with HT(V) values above 700 V confirming data in that range was unreliable. Although not ideal, it was still possible to estimate the alpha helical content using this limited spectral range since some of the dominant characteristic bands of this structural element were still available (222 and 208 nm). At the highest concentration of Gdn-HCl a significant reduction in signal intensity was observed. As shown in Figure 2.1, the negative ellipticity around 210-240 nm appears to flatten indicating a loss of alpha helicity. After dilution of the Gdn-HCl, the curves for the previously denatured Lysozyme were similar to that of the buffer controls indicating Lysozyme had indeed re-folded. Prior to data analysis, the data must be normalized as previously described. This is a critical step (and learning objective) for the estimation of secondary structure content. The importance of normalization is due to the varying concentrations of lysozyme in each sample and can only be analyzed after normalization by converting the measured response in millidegrees to MRE (mean residue ellipticity) as well as baseline subtraction. The CD spectra for the control samples in buffer should match after normalizing to MRE. In addition, the absorption at 260 nm should be minimal and shifts of the spectrum up or down from this “baseline” could indicate the lamps have not fully warmed up or the instrument needs calibration.

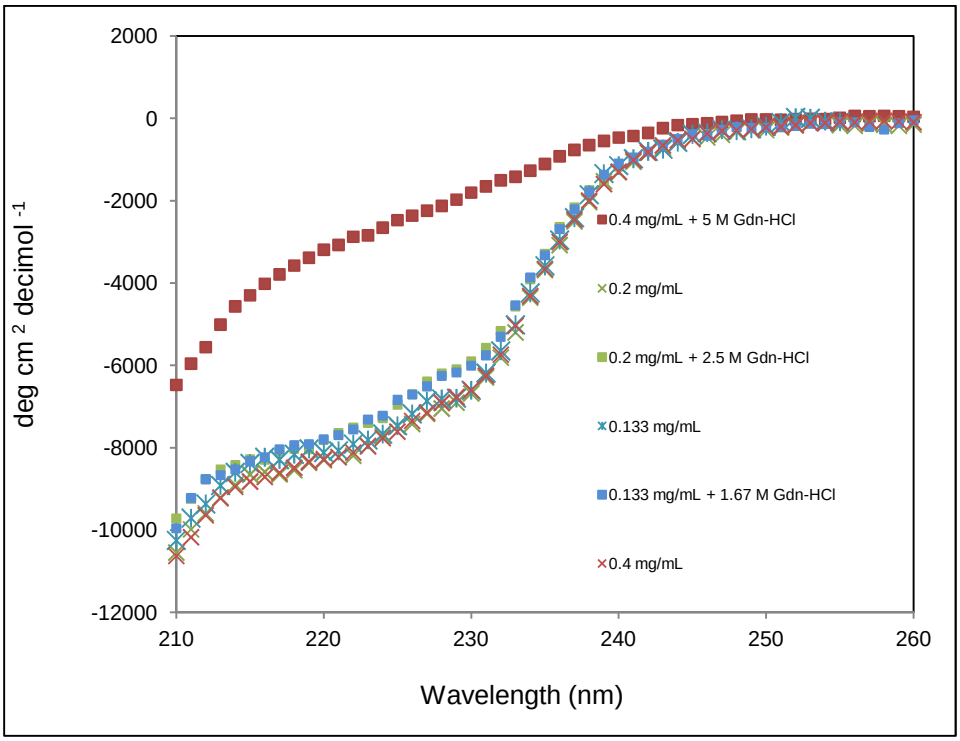


Figure 2.1 CD spectra of Lysozyme Denaturation. In solution with and without Gdn-HCl reported in MRE.

There are several ways of representing the change in the alpha helix content. In one method, the change in ellipticity at 222 or 208 nm can be plotted versus Gdn-HCl concentration. This is a simple but effective method to observe the change in secondary structure during denaturation. Alternatively, the actual alpha helical content can be estimated using algorithms available online for academic use such as the K2D3 server[15]. The K2D3 estimated % alpha helix for the 0.4, 0.2 and 0.133 mg/mL buffer controls were 27, 26 and 26%, respectively. The alpha helix content of Lysozyme determined by X-ray crystallography is 28-30%, which agrees nicely with the data generated[10,11]. The graph shown in Figure 2.2 showing the change in alpha helix content as a function of Gdn-HCl concentration was based on the K2D3 estimation of alpha helix for each sample.

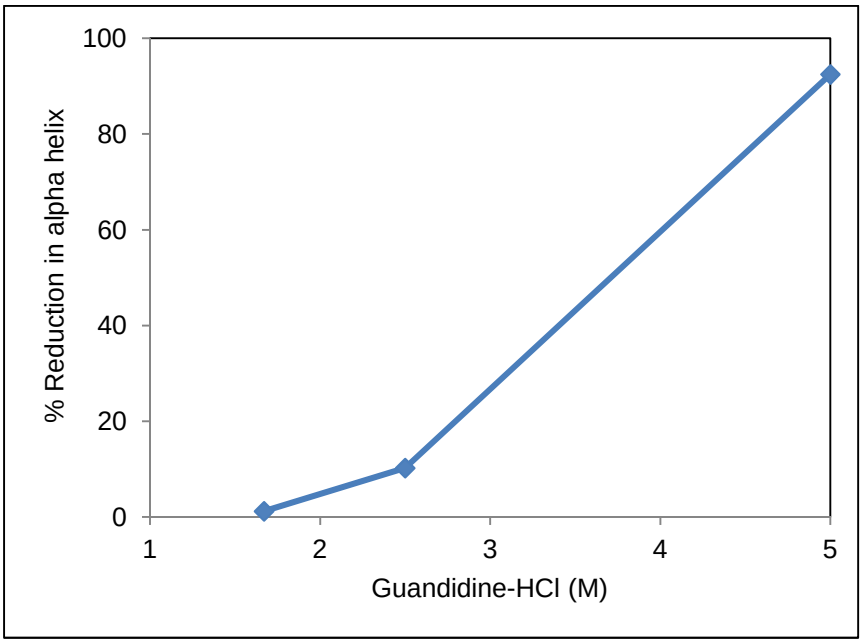


Figure 2.2 Reduction of Lysozyme Alpha Helix. Shown as a function of Gdn-HCl concentration

In an extension of the folding study, Lysozyme stability was evaluated by storing solutions either at 4° C or -20 ℃ for 24 hours. Ly sozyme was prepared in 10 mM sodium phosphate with or without 20% glycerol and the CD spectra was collected as described before. After storage for 24 hours the CD spectra was collected again and compared to the original scans. The CD spectra shown in Figure 2.3, clearly displays spectral overlap for each condition tested. The CD spectrum of Lysozyme stored in buffer solution at 4°C is identical to the original scan. Likewise, the Lysozyme sample stored at -20° C in 20% glycerol was equally stable as indicated by the spectral overlap. The observed offset in spectra between the two buffer conditions is most likely due to a slight change in secondary structure as a result of the glycerol addition. It is unlikely the shift is due to instrument drift since the spectra for both samples overlap so well with the previous day's scan. This short experiment nicely illustrates the ability of CD to confirm structure identity, show protein integrity during storage or even to ensure proper folding has occurred during purification.

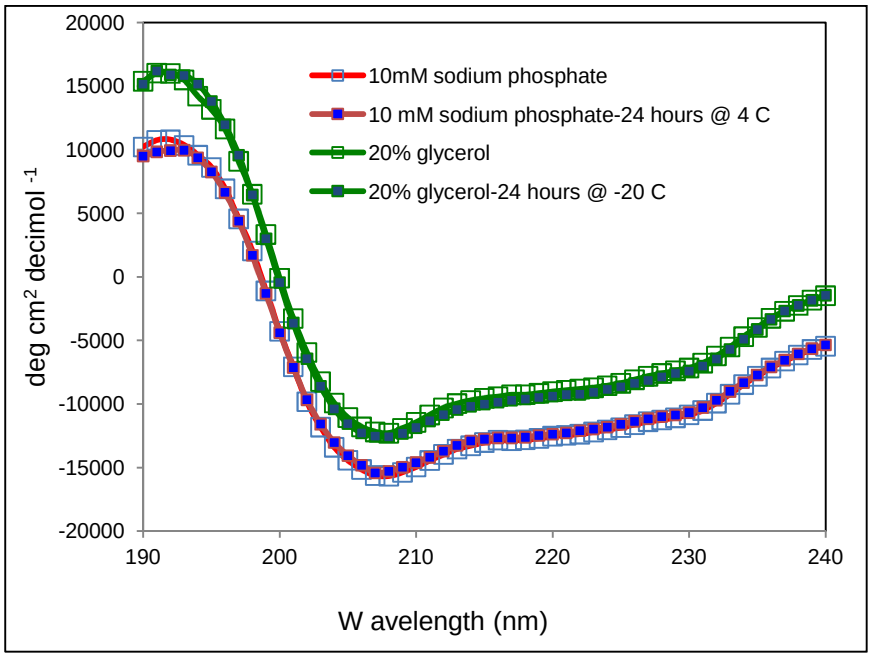


Figure 2.3 Lysozyme Stability. After storage in solutions at 0.18 mg/mL and 0.2 mg/mL in sodium phosphate buffer with and without 20% glycerol, respectively

As another requirement of this lab exercise, students were asked to generate the CD spectra for a reference set of three proteins with known secondary structure. The student objective was to demonstrate that the measured CD response is a linear combination of the individual contributions of each structural element. The CD spectra for the proposed reference set consisting of Lysozyme, Myoglobin and alpha-Chymotrypsin are shown in Figure 2.4. Given the CD spectra and fractional secondary structure composition of the three proteins (included in the lab outline) the student should be able to estimate the fraction of alpha helix content in an unknown protein. This can be done by solving simultaneously a set of equations such as that shown in equation [3]. A minimum of three reference proteins and three wavelengths are required and can be solved by recognizing that the mole fractions (or percent) of secondary structure (χ_a, χ_b, χ_o) and the ellipticity contributions for each structure ($[\theta_a]_\lambda$, $[\theta_b]_\lambda$, $[\theta_o]_\lambda$) can be written in the form of a 3x3 matrix and solved by the determinant method. A suggested first step

is solving for the ellipticity contributions ($[\theta_a]_\lambda$, $[\theta_b]_\lambda$, $[\theta_o]_\lambda$) of three wavelengths. An example of this system is shown in equation 4:

$$\begin{vmatrix} X_{\alpha(myo)} & X_{\beta(myo)} & X_{o(myo)} \\ X_{\alpha(lyso)} & X_{\beta(lyso)} & X_{o(lyso)} \\ X_{\alpha(chymo)} & X_{\beta(chymo)} & X_{o(chymo)} \end{vmatrix} \times \begin{vmatrix} \theta_{\alpha,\lambda} \\ \theta_{\beta,\lambda} \\ \theta_{o,\lambda} \end{vmatrix} = \begin{vmatrix} [\theta]_{\lambda,\,myo} \\ [\theta]_{\lambda,\,lyso} \\ [\theta]_{\lambda,\,chymo} \end{vmatrix} \qquad [4]$$

After calculating for the individual contributions ($\theta_{a\lambda}$, $\theta_{b\lambda}$, $\theta_{o\lambda}$) for each of the three selected wavelengths a similar system can be solved for the unknown protein.

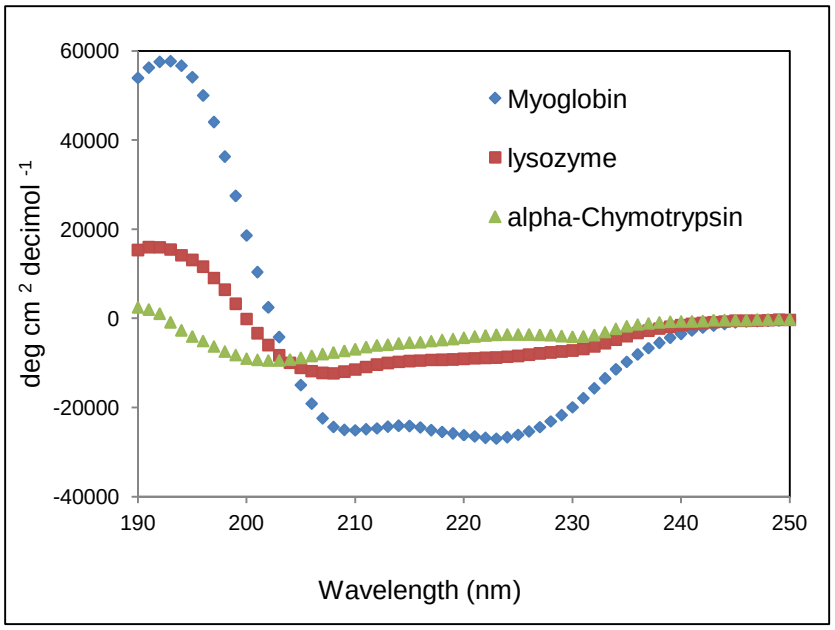


Figure 2.4 CD spectra of Reference Proteins. Myoglobin (0.1 mg/mL), Lysozyme (0.18 mg/mL) and alpha-Chymotrypsin (0.19 mg/mL) are shown.

In addition to the protein set, CD spectra of a homopolypeptide, such as poly-lysine, in 100% alpha, beta or random conformation can also be used in the similar manner to determine the unknown secondary structure content. Using the CD spectra of protein as reference sets is generally preferred if the unknown sample is a protein as they are more representative of native protein folding. However, if the unknown is a homopolypeptide then the poly-lysine set may be more suitable[2,10]. The ellipticity contributions of the alpha, beta and random structural elements

were calculated using both the reference protein set and a published poly-lysine data set[9]. As detailed in Tables 2.2 (A) and (B), the percent secondary structure content determined using both data sets showed comparable results of alpha helix when compared to the estimates by the K2D3 server. The unknown protein used in this study was MiaE, which was prepared separately and run over several days. This very simple approach to estimating the secondary structure content (SSC) is highly dependent on the wavelength selection but overall the estimates were fairly reasonable compared to the K2D3 server, which utilizes spectral data in 190-240 nm range. As illustrated in Table 2.2, the predictions using the poly-lysine set seemed to overestimate alpha helix content most cases. Calculations of alpha helix for the unknown protein, MiaE, using the protein set showed comparable estimates for half of the samples when compared to the K2D3 results. Beta-sheet content shown in Table 2.2 (B) shows that the poly (lysine) data set poorly predicted this structural element where the protein set showed more reasonable values. It's evident that using larger amounts of data for the estimation of SSC is a better option for obtaining more reliable values. The estimation of SSC using a 3x3 matrix was successful for most of the samples but differences did exist depending on whether the poly-lysine or protein reference sets were used. Nonetheless, this simple approach is valid and demonstrates the concept that the CD of different structural elements are additive. In addition, these results illustrate that there are multiple methods for estimating SSC.

Table 2.2 (A) Estimation of % Alpha Helix

	% Alpha Helix by Method			
	Poly(lysine) Set*	Protein Set*	K2D3	X ray Structure
Myoglobin	76	na	72	68[10],77[11]
Lysozyme	34	na	28	28[10], 30[11]
Chymotrypsin	15	na	4	8[10], 9.32[11]
#MiaE 91412	47	25	20	
MiaE 92112	49	34	38	
MiaE 92812	113	77	84	
MiaE 100112 (asl(Met))	81	45	64	
MiaE 100112 (asl(Leu))	67	45	65	
MiaE 100112 (asl(Trp))	69	47	67	

Table 2.2 (B) Estimation of % Beta Sheet

	% Beta Sheet by Method			
	Poly(lysine) Set*	Protein Set*	K2D3	X ray Structure
Myoglobin	0	na	2	0[10, 11]
Lysozyme	6	na	21	7.75[11]
Chymotrypsin	0	na	35	32.6[11]
#MiaE 91412	0	10	25	
MiaE 92112	0	7	20	
MiaE 92812	0	15	2	
MiaE 100112 (asl(Met))	0	5	3	
MiaE 100112 (asl(Leu))	0	5	3	
MiaE 100112 (asl(Trp))	0	7	2	

*The results presented are the average estimates from solving simultaneous equations for three sets of wavelengths. For example, the protein reference set was solved for the following wavelength combinations: A) 222, 208 and 200 nm, B) 203, 222, and 240 nm, and C) 195, 208 and 222 nm. The alpha helix content solved for in A, B and C were averaged. #Each MiaE result is for samples prepared on different days (date listed) or different preparations on the same day as indicated.

2.4 Conclusion

An example of the types of results that could be generated following the proposed laboratory exercise has been presented. The suggested procedures cover the basics of CD spectroscopy as applied to protein studies and emphasize fundamentals generating quality data that can be applied to other laboratory instruments. At completion students have examined the dynamics protein folding, performed data analysis for secondary structure content and gained experience with a new experimental technique. This lab procedure was written in a format that gives students the freedom to choose the type of data analysis performed. The estimation of alpha helix content from a protein reference set is possible using the linear combination theory presented above but could also be demonstrated by plotting the known fractional content for each protein versus the mean residue ellipticity at 222, 208 or 194 nm and applying this relationship to the unknown protein. This analysis is only possible using properly acquired data (including baseline subtraction) and by normalization to MRE, another important concept learned by participating in this lab exercise.

CHAPTER 3

CHARACTERIZATION OF MiaE BINDING OF THE ANTICODON STEM LOOP OF tRNA

3.1 Introduction

MiaE is a non-heme diiron monooxygenase that modifies the nucleoside in position 37 on the 3' side of the anticodon in transfer RNA (tRNA) that read codons beginning with uridine[17]. As outlined in Figure 3.1, in the presence of oxygen MiaE catalyzes the hydroxylation the isopentenyl group of 2-methylthio-N-6-isopentenyl adenosine (MS2i6A37)[17]. Modifications of tRNA introduce conformational variation, which is suggested to increase the fidelity of translation and stabilize or destabilize structures for greater regulation of the translational process[17,18]. The structure of MiaE, displayed in Figure 3.2, is predominately alpha helix, containing a ferritin-like 4-helix bundle fold that coordinates two iron atoms[17]. The quaternary structure is still unknown but it is structurally similar to other members of the diiron enzyme family that form homomultimeric complexes indicating a possibility that MiaE may also form multimers[17]. CD spectroscopy was used in this study to characterize MiaE binding of three different anticodon stem loop oligonucleotides (asl). The goal was to confirm previous work using EPR spectroscopy that showed a conformational change in the diiron cluster in the presence of asl substrate[19]. In addition, the secondary structure content was determined and the calculated binding constants for each substrate are reported.

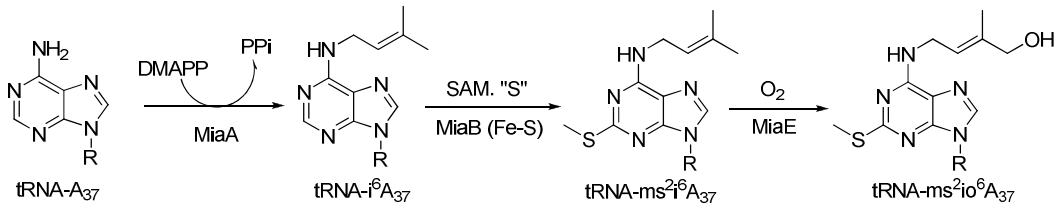


Figure 3.1 Modification of $MS^2i^6A_{37}$.

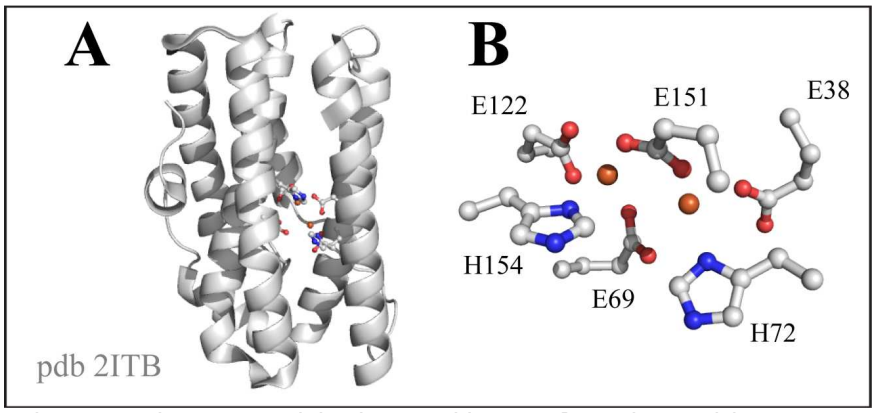


Figure 3.2 MiaE Crystal Structure. (A) MiaE Ferritin Core [Proteins. 70(1), 2008; 1-18] and (B) The Diiron Coordination Site. PDB 2ITB

3.2 Experimental Methods

The same instrument parameters were used for the MiaE testing as described above. MiaE (29057 Da, 254 amino acids, $\varepsilon^{M}_{370\ nm}$=8250) was purified from Salmonella typhimurium by Bishnu Subedi and dialyzed against 10 mM sodium phosphate buffer with 20 mM NaCl, pH 8.0 for all studies. The asl substrates, 17 base pair oligonucleotides, were confirmed hairpin structure by running baseline CD spectra. The sequence for the asl substrate coding for methionine, asl(Met), is CAUCACUCAUA^{37}AU where the modifiable residue is indicated. The asl substrate buffer contained 30 mM HEPES, 100 mM KCl and 50 mM ammonium acetate. The titration studies were performed by titrating 5 µM MiaE with concentrated asl substrate directly into the cuvette to minimize dilution and to observe the affected changes within the same MiaE sample. In addition, CD spectra of the asl substrates without MiaE were collected and subtracted from the spectra of the mixed samples. Data analysis was performed after this baseline subtraction on the normalized data in units of MRE, deg•cm^{2}decimol^{-1}. The binding constants were determined by assuming a one site binding model and that the change in ellipticity is directly proportional to the amount of complex formed. The K_d can be determined by fitting the fractional change in CD intensity over increasing concentrations of asl substrate, [B], to a square polynomial:

$$\frac{\Delta CD}{\Delta CDmax} = \frac{([M]tot+[B]+ Kd) - \sqrt{(([M]tot+[B]+ Kd)^2-4[M]tot[B])}}{2[M]tot}$$

Where $[M]_{tot}$ is the MiaE concentration and ΔCD_{max} is the maximum changed observed (theoretically equal to $[M]_{tot}$)[12,20].

3.3 Results and Discussion

Early studies were conducted to establish optimal concentrations and confirm the feasibility of using CD spectroscopy for binding studies. As seen in Figure 3.3, the initial studies established that changes in secondary structure could be seen with addition of asl substrate. Baseline scans of the asl buffer showed significant absorption below 200 nm and contributed to the observed noise in the CD spectra when higher concentrations of asl was used. To minimize noise, further work focused on optimizing the procedure for collecting data with minimal background absorption in the lower wavelength range.

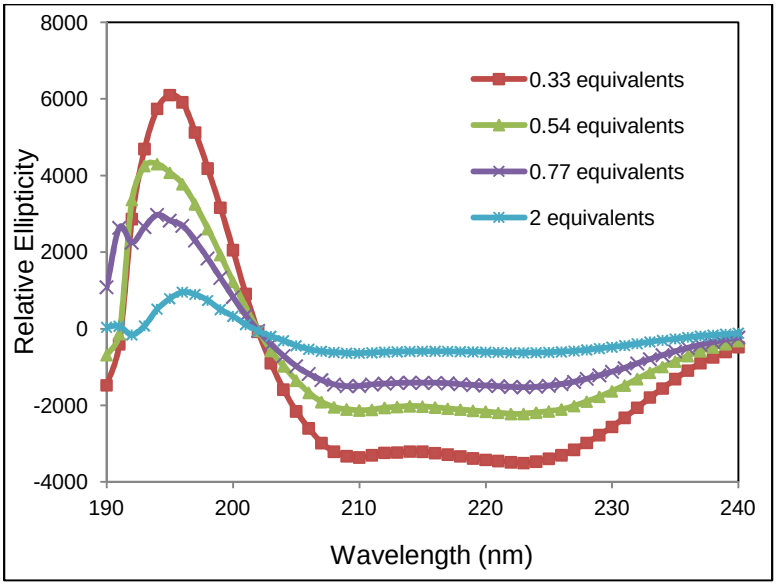


Figure 3.3 CD spectra of MiaE Normalized to asl(Met) Concentration. MiaE (10µM) with varying amounts of asl(Met) performed 21 Sept. 2012. The relative ellipticity did not have the asl baseline spectra subtracted but was normalized for the asl substrate concentration.

As a result of the optimization studies, a collection of MiaE scans were generated over several days. As illustrated in Figure 3.4, it became clear that MiaE was not stable once thawed. Differences in the CD spectra of MiaE observed within the same day show the sensitivity of MiaE structure in solution.

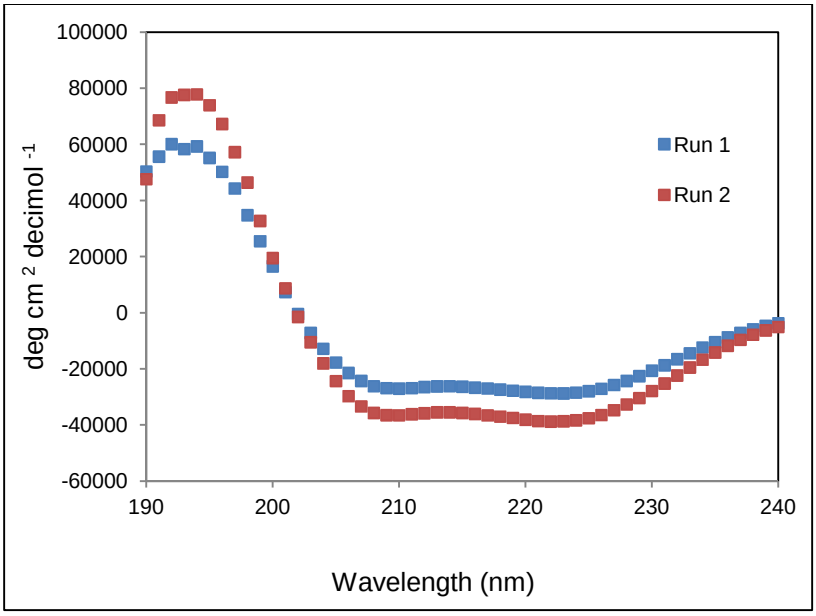


Figure 3.4 CD Spectra of MiaE Stability. MiaE (5µM) run 28 Sept. 2012

An overview of the conformational changes observed for MiaE over the course of this study is displayed in Figure 3.5. Except for the results on 28 Sept. 2012, the remaining spectra are much more similar. As previously demonstrated (Lysozyme stability, Figure 2.3), the CD spectra should overlap exactly if the protein has equivalent secondary structure. Despite the similarity of the spectra in Figure 3.5, there was clearly significant variation in MiaE structure between days. It should be noted that in the case of aggregate formation the MRE could change with concentration[2]. Since the studies were performed with different MiaE concentrations this may be indicative of aggregate formation but further studies are needed to confirm whether the observed changes in MRE are due to aggregate formation or caused by stability issues.

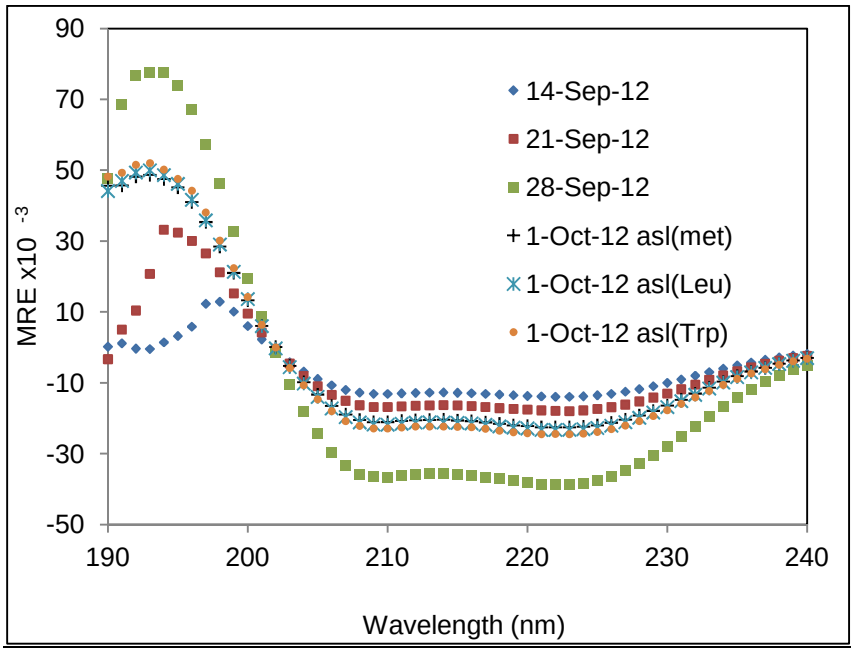


Figure 3.5 CD Spectra of MiaE Summary. MiaE in buffer solution at 26 µM (blue), 10 µM (red) and 5 µM (green, black, light blue and orange) reported in mdeg•cm^{2}decimol^{-1}.

The estimates for secondary structure content, previously shown in Table 2.2 (above), correlated with the variation seen in the CD spectra. For example, the percent alpha helix varied from 20 to 80% for the estimates using the K2D3 algorithm. Interestingly, the decrease in alpha helix content was matched by an increase in beta sheet content but also varied from 2-25%. In order to minimize error due to small changes in MiaE conformation the final study used a freshly thawed aliquot of MiaE and the titrations were performed directly in the cuvette by spiking increasing amounts of asl substrate directly to the MiaE sample. All data characterizing the MiaE/ asl binding are from a series of studies performed in this manner all on the same day (performed by Bishnu Subedi).

All three of the substrates, asl (Met), asl (Leu) and asl (Trp), are hairpin RNA and contain the anitcodons for methionine, leucine and tryptophan, respectively. The CD spectra for each asl substrate at each concentration was collected and used as the baseline spectra. The intensities of the spectral bands for the asl substrates were considerably lower (10 fold difference) than that of the MiaE spectra and show greater background noise, possibility due their low

concentration. CD spectra of hairpin RNA is highly dependent on the composition and stacking properties of the bases[21]. The published CD characteristics for hairpin RNA in the A (right handed) conformation are a positive peak around 260 nm, near zero ellipticity at 250-230 nm and a negative peak around 210 nm[21].

As illustrated in Figure 3.6, these characteristics are observed in the CD spectra for all of the asl substrates used in this study. The observed differences in the intensities of molar ellipticity for the three substrates are due to the slightly different nucleotides in their sequences.

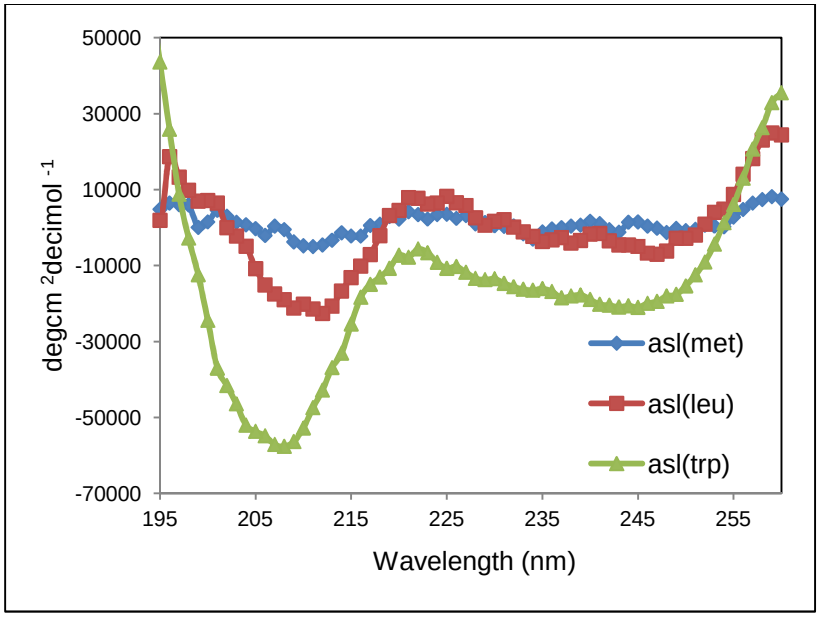


Figure 3.6 CD Spectra of asl Substrates. Molar ellipiticity for asl(Met), 3 µM, asl(Leu), 3 µM and asl(Trp), 5 µM.

The evident change in the MRE for MiaE, shown in Figure 3.7, as a function of the asl(Met) concentration is evidence of secondary structural changes. Quantification of the spectral changes is discussed below. However, the determination of secondary structure content for MiaE cannot be determined for this data because both asl and MiaE absorb in this spectral region[22]. Because the two components are contributing to the MRE in this region, the changes observed could be due to changes in MiaE alone or the combined structural changes of both MiaE and asl. Although not explored in this study, a method to determine whether asl also

changes conformation upon binding could be employed. Following similar methodology, the asl substrate is titrated with increasing amounts of MiaE and the ellipticity at 260 nm is observed. At this wavelength only the asl substrate contributes to the absorbance and changes observed are attributed to asl alone[22]. Regardless, this study does confirm that all three asl substrates induce structural changes in MiaE. The CD spectra for the asl(Leu) and asl(Trp) are included in the Appendix C.

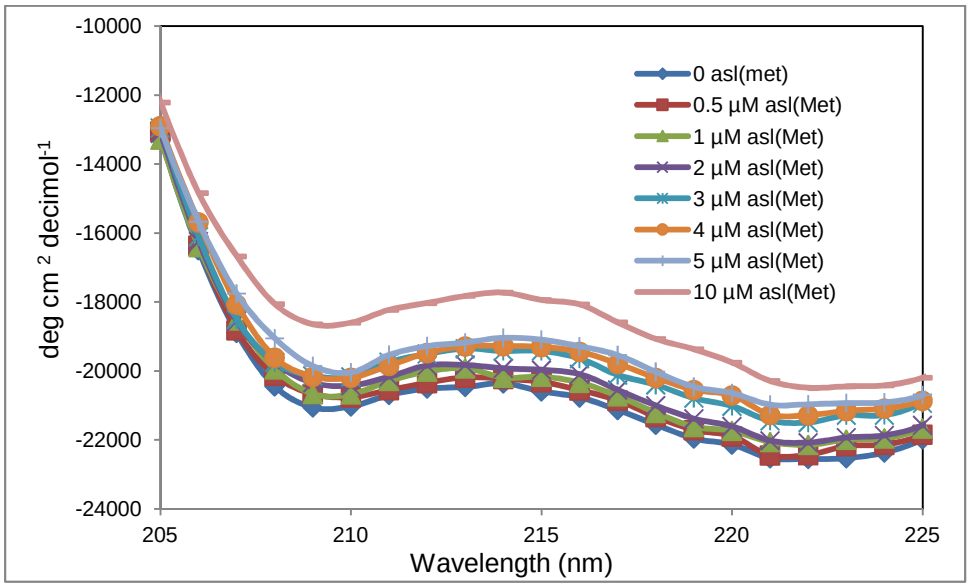


Figure 3.7 CD Spectra of MiaE with asl(Met). MiaE (5 µM) was titrated with increasing amounts of asl(Met) directly in the cuvette. The final concentrations of asl(Met) in the cuvette were 0.5, 1, 2, 3, 4, 5 and 10 µM. The CD spectra were normalized by subtracting the asl baseline spectra for each concentration and the data converted to MRE.

The apparent binding constant, K_d, was determined by plotting the fractional change in MRE for the baseline-subtracted spectra at a variety of wavelengths. The goodness of fit varied depending on the wavelength selected so multiple wavelengths were evaluated. The fractional change for asl(Leu) was more challenging to fit. A complete list of K_d values and RMSE is shown in Table 3.1. The average K_d was determined based on the best curve fits for each substrate and were 0.75, 1.0 and 0.6 µM for asl(Met), asl(Leu) and asl(Trp), respectively. Since the binding constants are not significantly different, this indicates that binding is less specific to the tRNA composition but rather binds based on the local hairpin conformation.

Table 3.1 Binding Constants Based on Fractional Change in CD

	asl (Met)		asl (Leu)		asl (Trp)	
Wavelength (nm)	K_d	*RMSE	K_d	RMSE	K_d	RMSE
195	0.63	0.08	1.25	0.099	0.156	0.085
200	0.72	0.09	0.89	0.081	-	-
208	1.518	0.12	1.28	0.137	0.923	0.071
215	1.096	0.08	0.81	0.08	0.556	0.072
218	0.87	0.07	0.81	0.11	-	-
222	0.44	0.06	0.61	0.123	-	-
233	0.377	0.123	0.443	0.174	0.51	0.078
255	-	-	1.27	0.113	2.31	0.24
210	-	-	-	-	0.86	0.065
Average	0.75		1.01		0.60	
Standard Deviation	0.25		0.23		0.31	

*RMSE (root mean square error) was reported to show the varying goodness of fit for each wavelength presented. For asl(Met) and asl (trp) the fits with RMSE < 0.9 were used for the calculation of the average. For asl (leu) the fits with RMSE <0.11 were used for the calculation of the average

3.4 Conclusion

This brief study characterizing MiaE by CD spectroscopy provided important information about the stability of the enzyme (Figure 3.5), estimates of the secondary structure content (Table 2.2) and confirmed that conformational changes occur when bound to the asl substrate (Figure 3.7). Initial results indicated that MiaE may be unstable once thawed as the MRE varied depending on the day or age of the solution. It would be interesting to test additional conditions such as pH, ionic strength and temperature to evaluate the secondary structural content as a function of these parameters. Because the full structural composition of MiaE is still unknown, this information could be a novel addition to published information. The CD data presented in this report suggests that MiaE could have approximately 65-80% alpha helix under the conditions tested. Correspondingly, obtaining the CD spectra after determining the most appropriate solution conditions (pH, ionic strength and temperature) for native folding would likely provide valuable structural information. In addition, a correlation of structure to activity would be another future study that could yield novel information about MiaE's interaction with

the anticondon stem loop of tRNA. Although the presented data confirmed previous EPR studies that asl substrates induce structural changes in MiaE, specific changes in structure such as which residues are affected are still unknown and would require additional spectroscopic techniques[19]. An attempt to quantify the binding kinetics using the fractional change in ellipticity provided apparent K_d values (Table 3.1). K_d values were similar for the three asl substrates tested. This suggests binding may not be specific to the stem loop nucleotide sequence but rather to the general conformation of the hairpin structure. CD spectroscopy is clearly a complementary tool for studying proteins and other bio-macromolecules. The use of CD spectroscopy has advantages including its speed, low sample requirements, range of allowable conditions and quantity of information that can be derived from the spectra. However, diligence must be applied in terms of proper calibration, baseline subtraction, minimizing signal/noise and proper data analysis to ensure reliable results. Following these guidelines and collecting numerous control spectra, a package of qualitative and quantitative structural data has been generated for MiaE. Overall, this study was a successful starting point for future work on MiaE and demonstrated the utility of CD spectroscopy for protein research.

APPENDIX A

REFERENCE WEBSITES CONTAINING CD ANALYSIS PROGRAMS

A) CDPro http:lamar.colostate.edu/~sreeram/CDPro/. NOTE: Superior fits of globular proteins

B) CONTIN http://s-provencher.com/index.shtml NOTE: Programs are compiled by the user

C) DICROPROT http://dicroprot-pbil.ibcp.fr NOTE: Easy to use but wavelength ranges must be 260-178 nm

D) Dicroweb http://dichroweb.cryst.bbk.ac.uk/html/home.shtml NOTE: Tutorials available

E) K2D3 Server http://www.ogic.ca/projects/k2d3//info/about.html NOTE: Easy to use

.

APPENDIX B

CD SPECTROSCOPY LAB

CIRCULAR DICHROISM OF PROTEINS

LAB PREPARATION

Read the attached publications [*J. Chem. Ed*. 87, 891-893 (2010), and *Biochimica et Biophysica Acta*, 1751, 119-139 (2005)]. The following papers will may also be useful in your discussion [*Biochemistry* 8(10), 4108-16 (1968); and *Macromolecules* 2(6) 624-628(1969)]. Use these as a guide to determine the relative secondary structure of an unknown protein and follow the denaturation of lysozyme.

EXPERIMENT

The CD spectra of four protein samples will be collected and processed into mean residue ellipticity (deg* cm^2 $decimol^{-1}$). The observed ellipticity, [θ_λ], in the 190-240 nm range is diagnostic of the macromolecular chirality due to a proteins secondary structure (alpha helix, beta sheet, or random coil) as described by equation 1. For any protein, the observed CD spectra within this region can be used to determine the relative fraction of secondary structure [alpha helix (X_a), beta sheet (X_a) and random coil X_r)] by comparing the mean residue ellipticity for a specific wavelength to samples of known secondary structure. In addition to the polypeptide poly-L-lysine, the proteins lysozyme and myoglobin are frequently used as protein standards for CD spectroscopy.

Table 1. Secondary structure of selected proteins and polypeptides.

2°Structure	Myoglobin	Lysozyme	Poly-L-Lysine
alpha Helix (X_a)	68	29	*See TA*
beta Sheet (X_b)	5	11	
Random Coil (X_r)	27	60	

Equation 1 indicates that the observed CD spectra for any protein can be described as the sum of molar ellipticities at a fixed wavelength (□) for alpha helix, beta sheet and random coil ($\theta_{□\ (\lambda)}$, $\theta_{□\ (\lambda)}$, and $\theta_{r\ (\lambda)}$) weighted by the relative fraction of each secondary structure (□□, □□, and □$_r$). For accurate structural prediction, it is important to select wavelenghths appropriate for each secondary structure basis set. For example, the observed molar ellipticity at 222 nm is predominately due to alpha helix. Using this approach, the secondary structure of an unknown protein can be determined by simultaneously solving a series of 4 linear equations based on the three standard proteins and one unknown.

Equation 1 $$[\theta_{(\lambda)}] = X_a \cdot [\theta_{a\ (\lambda)}] + X_b \cdot [\theta_{b\ (\lambda)}] + X_r \cdot [\theta_{r\ (\lambda)}]$$

CD spectroscopy can also be utilized to observe changes in protein conformation due to denaturating conditions. In these experiments, the change in the mean residue ellipticity is monitored as secondary structure is lost by protein unfolding. In this experiment, lysozyme is denatured with 5 M guanidine hydrochloride and subsequently refolded by serial dilution into an appropriate buffer. The calculated secondary structure composition should return to reported values.

PROCEDURE

Instrumentation. Jasco 715 UV-visible circular dichroism spectrometer. The high-voltage UV-lamp is capable of producing ozone and thus the lamp is purged by a continuous flow of N_2 gas. Prior to igniting the lamp, make sure to purge the lamps for at least 10-minutes. Note: The lamp will also need approximately 10-minutes to warm up after ignition. To ensure accurate measurements, make sure that the HT[V] is below 700 for the entire spectrum.

Sample considerations. Stock solutions of protein are prepared to 1 mg/mL in 10 mM sodium phosphate buffer, pH 6.7 (with or without 20% glycerol). Make sure the buffer is filtered through a 0.2 or 0.45 μm membrane to remove contaminants and avoid light scattering or noise. To accurately determine concentration the absorbance at 280 nm can be recorded. For the most accurate measurement the stock solution should be diluted 1/10 in 6 M guanidine-HCL for the measurement. The A_{280} for a 1mg/mL solution can be estimated using the following equation:

A_{280} (1mg/ml; 1 cm) = (5690 nW + 1280 nY + 60 nC)/ M

where nW, nY and nC are the number of Trp, Tyr and Cys residues per polypeptide chain and M is the molecular mass, Daltons. The nC term can be omitted if no disulphide bonds exist. Alternatively, the native protein can be measured at a 1/10 dilution and the concentration determined using reported molar extinction coefficients.

Materials:

Buffer:10 mM sodium Phosphate at pH 6.9 prepared from di and monobasic sodium phosphate (minimal pH adjustment) in ultrapure water. Filtered to remove any particles.

Guanidine HCL- A 6M solution was diluted to 5 M in ultrapure water and filtered.

Quartz cuvette- 0.1 cm circular, holds ~0.3 mL and can be filled using pipettes and possibly transfer pipettes.

Standards

A. Myoglobin: Prepare a ~ 0.5 mg/mL solution in phosphate buffer.
B. Lysozyme: Prepare samples as described in Table 2. Prepare control samples in phosphate buffer.
C. Poly-L-Lysine: This standard will be prepared and run by your TA and made available to each group. Ask your TA what the sample conditions are for this sample as well as the corresponding secondary structure.

Table 2

Sample	Control Series		Test Series	
	Lysozyme (mg/mL)	Gdn-HCl (Mol/L)	Lysozyme (mg/mL)	Gdn-HCl (Mol/L)
1	0.4	-	0.4	5
2	0.2	-	0.2	2.5
3	0.13	-	0.13	1.67

a. Dilutions from 5 M Gdn-HCl were given at least 10 minutes for folding to occur.
b. Baseline Corrections were performed using buffer only for the control series. For the test series dilutions of 5M Gdn-HCl were prepared in equivalent manner but without lysozyme and baseline corrections were made using the appropriate dilution.

CD measurements. Prior to measuring samples baseline measurements should be made using the buffer solution. If samples are prepared from solutions with glycerol, appropriate reference solutions containing glycerol should be used for the baseline scan. Stock solutions should be diluted carefully to concentrations between 0.1-0.5 mg/mL in buffer. Carefully load the cuvette using a transfer pipette (~0.3 mL), avoiding air bubbles. Prior to running sample ensure the baseline correction is selected and parameters are correct. CD scans are collected in θ (milli-degrees) and should be converted to mean residue ellipticity. The conversion requires the exact concentration of the sample, molecular weight, the number of peptide bonds (# of amino acids-1) and the pathlength of the cuvette (0.1 cm). Export data as a text file with listed wavelengths and measured mean residue ellipticities. Thoroughly clean the cuvette with deionized water between measurements.

Table 3. CD Instrumental settings for determination of protein secondary structure.

Parameter	
Scanning range	190-250 nm
Bandwidth	2 nm
Time constant	2 sec
Scanning Speed	50 nm/min
Sensitivity	Standard 100 mdeg
Accumulation	2

Conversion to mean residue ellipticity. In the Processing menu select optical constant. Calculate molecular ellipticity. Input path length in the appropriate box but in the molar concentration box input the mean residue weight shown below:

MRW= (number of peptide bonds) * Concentration (mol/L)

APPENDIX C

ADDITIONAL DATA

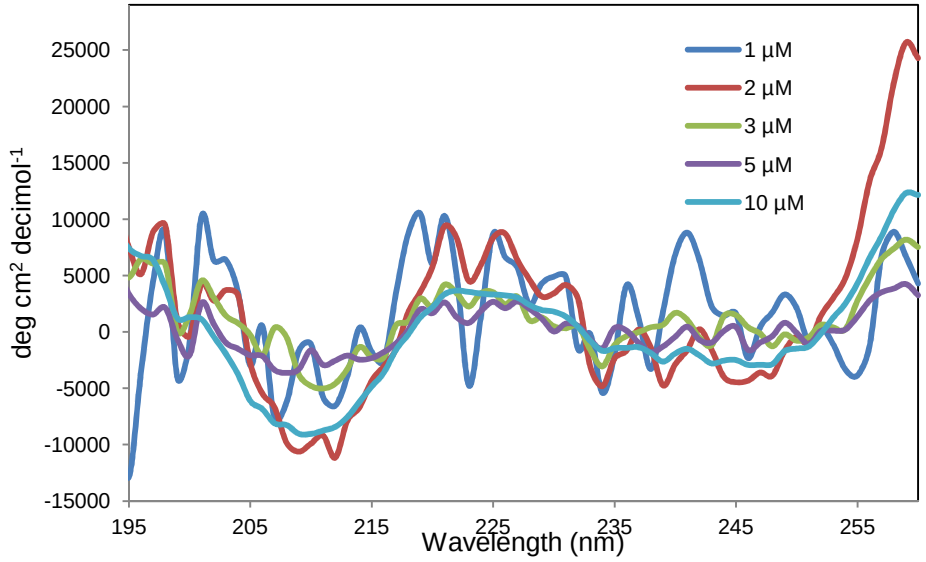


Figure C1 CD Spectra of asl(Met). Molecular Ellipticity of asl(Met) at the concentrations used in the MiaE binding assay.

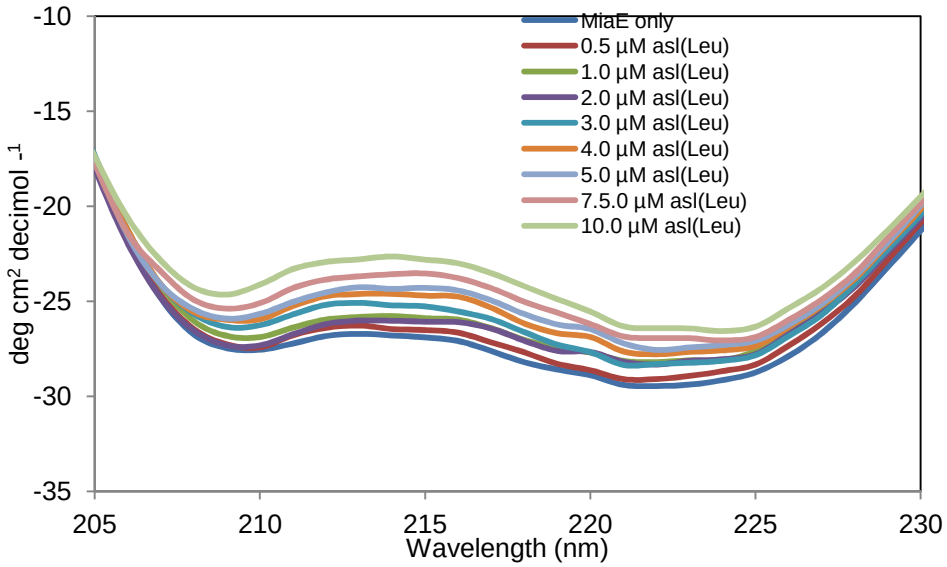


Figure C2 CD Spectra of MiaE with asl(Leu). Change in MRE x $^{10\text{-}3}$ for MiaE (5 µM) as a function of asl(Leu) concentration.

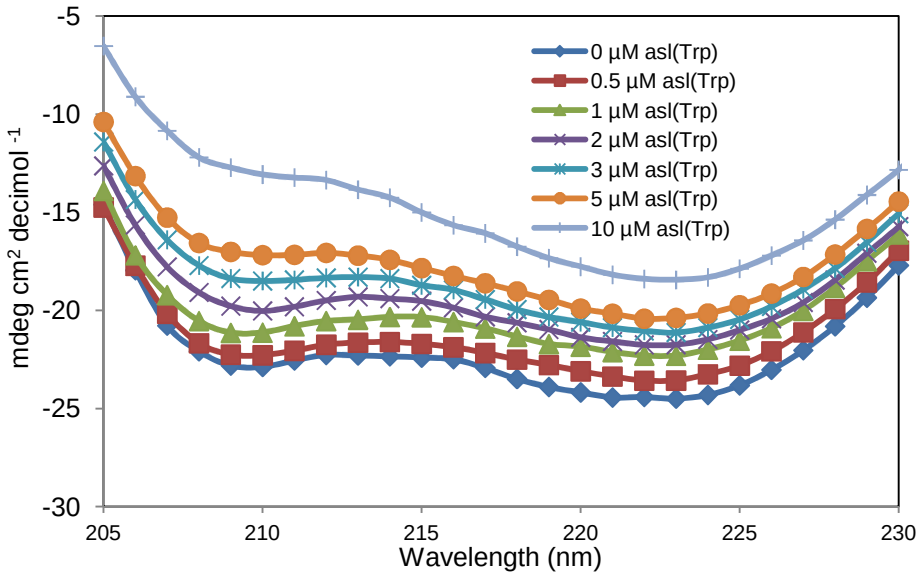


Figure C3 CD Spectra of MiaE with asl (Trp). Change in MRE x 10^{-3} for MiaE (5µM) as a function of asl(Trp) concentration.

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BIOGRAPHICAL INFORMATION

Catherine Van Der Kar attended Trinity University, San Antonio, for her undergraduate studies and earned a B.S. in Biochemistry. She worked under Dr. Christopher Pursell and Dr. Peter Kelly-Zion for her undergraduate research project studying the evaporation of mixed hydrocarbons using FTIR in the application of understanding the heating and burning of fuel components. After graduation in 2006, Catherine began working at Healthpoint Biotherapeutics, a bioactive wound care company. Initially in formulations, Catherine has progressed to microbiology and biochemistry positions in the Research and Development Department and has been working for Healthpoint for over 6 years. She predominately focuses on developing advanced treatments for wound healing including antimicrobials, enzymes and cell based products. At University of Texas at Arlington, Catherine worked with Dr. Brad Pierce to develop and validate a Circular Dichroism laboratory for undergraduates as well as performed characterization studies of a diiron monooxygenase MiaE by CD spectroscopy. In the future she hopes to continue working in the wound healing industry and would like to advance within this industry by continuing to research the potential applications of bioactives for wound care and by studying the complex dynamics of wound healing.