CD APPLICATION NOTE 01-03

Thermal Denaturation of Hen-egg Lysozyme with Concurrent CD and Fluorescence Detection

Protein folding is one of the most important processes studied because when the linear polypeptide chains of a protein are converted into a three-dimensional structure that gives the proteins with their vital activity. Studies of protein folding are often plagued with competing aggregation processes. Denaturation and aggregation of proteins is also a problem with serious medical implications, *e.g.* in human diseases like Alzheimer's, Parkinson's, and monoclonal immunoglobulin amyloidosis. Furthermore, protein denaturation during production, shipping, storage, and delivery of therapeutic proteins is a problem of significant economic importance. Through the combination of fluorescence and circular dichroism this phenomenon can be readily investigated.

Introduction

Studies on the mechanisms of protein folding are currently focusing on the role of partially folded states. A number of biologically and functionally important proteins have marginal stability in solution, and are easily denatured in high stress situations (*e.g.*, extreme salt concentration, high temperature). Lysozyme is a common model protein used to investigate the reversible inactivation of proteins at high temperature because it is inexpensive, easy to handle, easy to denature, and has just enough complexity in the fluorescence spectra to make it interesting.

Lysozyme, a small globular protein found in chicken egg white, has 129 amino acids in the primary sequence and 4 intrachain disulfide bridges between sulfhydryl containing amino acid residues. It is composed of a predominantly α-helical part (the α - domain) and a part with predominantly ß-sheet structure (the ß-domain). As the name implies, it is an enzyme (biological catalyst). Its substrate is a specific sequence in the bacterial cell wall of Micrococcus, a potential invading organism of eggs. Lysozyme has 2 tryptophans - 1 solvent exposed Trp and 1 buried Trp.

Experimental

Hen egg-white lysozyme (1mg) was dissolved in 15mL of deionized water. The thermal denaturation of the protein was evaluated using a JASCO J-810 CD spectropolarimeter equipped with a PFD-425S Peltier temperature controller and an FMO-427 emission monochromator for detection of fluorescence. The sample was contained in a 1cm quartz cuvette. Lysozyme CD and fluorescence spectra were automatically measured at 5° intervals from 20-95°C using the Macro Command Program JWMCR-482 with the protein denaturation package. After the final measurement at 95°, the sample was cooled back to 20°C and a final set of spectra collected. The totally automated study was completed in under 1.5 hours.

CD spectra were collected from 275-195nm with a data pitch of 0.1nm. A band width of 1nm was used with a detector response time of 4sec. and scanning speed of 50nm/min. The fluorescence spectra were collected from 290 - 400 nm using an excitation of 288nm. Excitation band width was set at 2nm and emission band width set to 10nm. A detector response of 1sec. was used with a 1nm data pitch.

Results and Discussion

Circular Dichroism (CD)

Thermal denaturation of the lysozyme resulted in changes in the CD spectra indicating a coincident denaturation of both tertiary and secondary structures with T_m values of 74.8 +/- 0.4 degrees C and 74.3 +/- 0.7 degrees C, respectively. (Lysozyme becomes completely denatured at 100°C).

Figure 1 illustrates the alteration of the CD spectra that occurs during thermal denaturation of the lysozyme. As the temperature increases, the intensity of the CD spectra decrease and the peak maxima shift to shorter wavelengths. The peak initially found at 207 nm gradually shifts to approximately 202 nm with the largest shift occurring between 75 and 80°C.

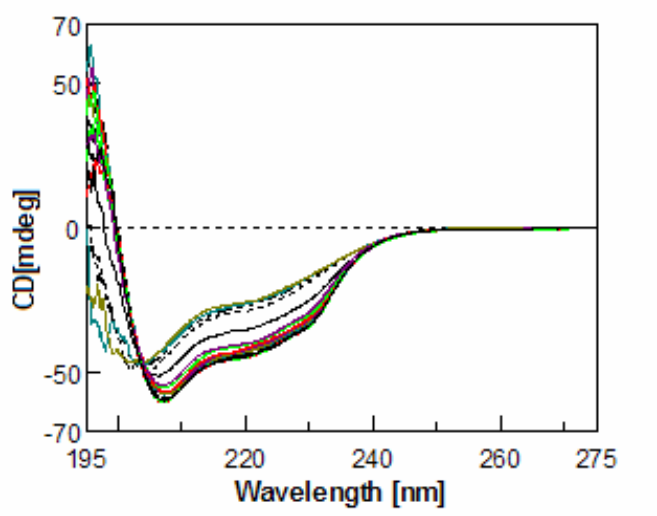

Figure 1. CD spectra demonstrating the thermal denaturation of lysozyme from 20 to 95°C.

Once the initial melt was complete, the lysozyme was brought back to 20°C, to verify the folding was reversible. Figure 2 shows the CD spectrum of the initial 20°C scan compared with that at 95°C, and at

20°C after the melt. The spectra before and after are very similar indicating that the protein does refold once the temperature is reduced however, it does not completely refold. This inability of the protein to completely reconstitute may be a result of taking it so near its point of complete denaturation at 100°C.

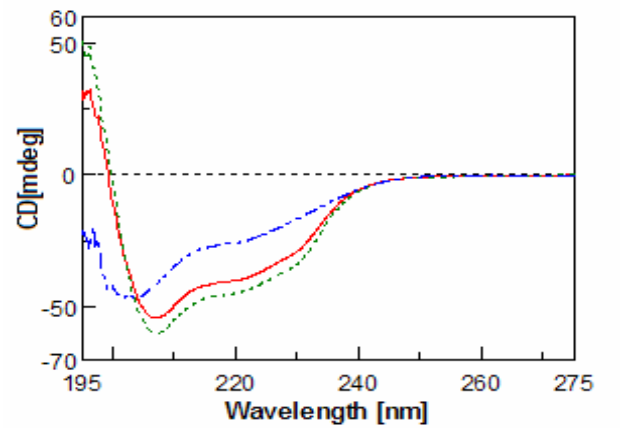

Figure 2. The CD of the lysozyme. (Green- 20°C initial, Blue-95°C, Red-20°C final)

Fluorescence

The dominant fluorophore in most proteins is the amino acid tryptophan (Trp), which is characterized by the indole moeity - a planar conjugated bicyclic system. Tryptophan has an absorbance maximum near 280 nm and an emission maximum which is very sensitive to the polarity of the environment. Tryptophan in a nonpolar environment (free Trp dissolved in an organic solvent or a Trp buried deep inside a protein) has an emission maximum near 320 nm while Trp in a polar environment (free Trp dissolved in aqueous solvent or a solvent-exposed Trp in a protein) has an emission maximum near 350 nm.

Frequently in proteins, spectral shifts are observed as a result of several phenomena, such as binding, protein-protein association, and denaturation. The fluorescence of Trp can also be quenched by small molecules. Ionic species prefer a polar environment and neutral species can penetrate into the hydrophobic core of a protein; a neutral and an ionic quencher allow one to distinguish between buried and exposed tryptophans, and provide data concerning the proximity of the Trp to (+) or (-) charged groups.

Figure 3 shows the change in fluorescence with respect to temperature increase. Initially, the peak maxima lie at 338 nm however as the protein denatures, the fluorescence decreases and the emission maximum shifts to 347nm. The largest shift occurs between 75-80°C as it does in the CD spectra.

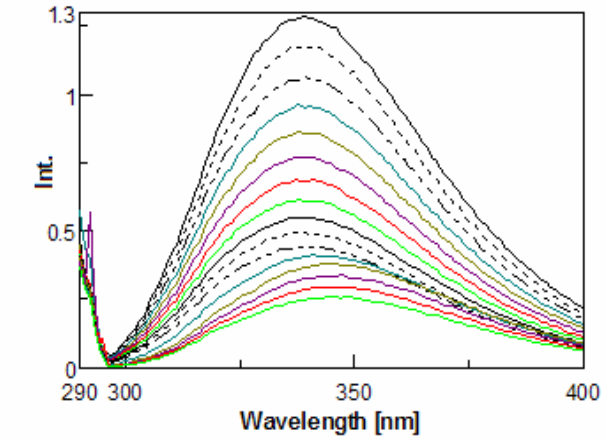

Figure 3. The fluorescence spectral change as lysozyme thermally denatures.

The ability of the lysozyme to refold was evaluated by allowing the sample to cool back to 20°C and collecting a final spectrum. Figure 4 illustrates changes in the fluorescence spectrum that take place before and after the thermal ramp. The spectra demonstrate that the lysozyme structure almost completely returns to its initial state. The inability to completely return may be a result of the temperature ramp to 95°C, only a few degrees from complete denaturation at 100°C.

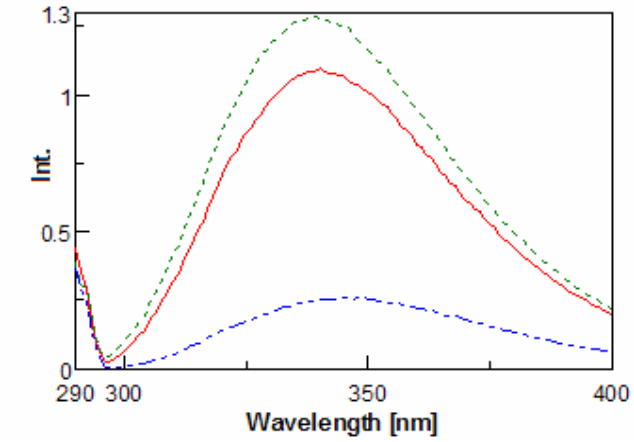

Figure 4. The fluorescence changes of lysozyme. (Green- 20°C initial, Blue-95°C, Red-20°C final)

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