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

Protein folding is one of the most important processes studied in biophysics and structural biology because it converts linear polypeptide chains into three-dimensional structures that endow proteins with all their vital activities. 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 its fluorescence 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 a-helical part (the a-domain) and a part with predominantly b-sheet structure (the b-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 FMO-427 Emission monochromator for 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. After the final measurement at 95 degrees, 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 275-195 nm with a data pitch of 0.1 nm. The band width used was 1 nm with a response time of 4 sec. and scanning speed of 50 nm/min. The fluorescence was collected from 290 - 400 nm at an excitation of 288nm. The band width was 2 nm (Ex) and 10 nm (Em). The response was set at 1 sec. with a 1nm data pitch. The (Em) sensitivity was 600V.

Results and Discussion

Circular Dichroism (CD)

Thermal melting of lysozyme in water followed by CD spectral changes indicated a coincident denaturation of both tertiary and secondary structures with Tm values of 74.8°C and 74.3 °C degrees respectively. (Lysozyme is completely denatured at 100°C). Figure 1 illustrates the changes in the CD spectra that occur during the thermal denaturation of lysozyme. As the temperature increases, the intensity of the cd signal decreases and the peak maxima shifts to shorter wavelengths. The peak initially found at 207nm gradually shifts to approximately 202nm. The largest shift occurs between 75 and 80°C.

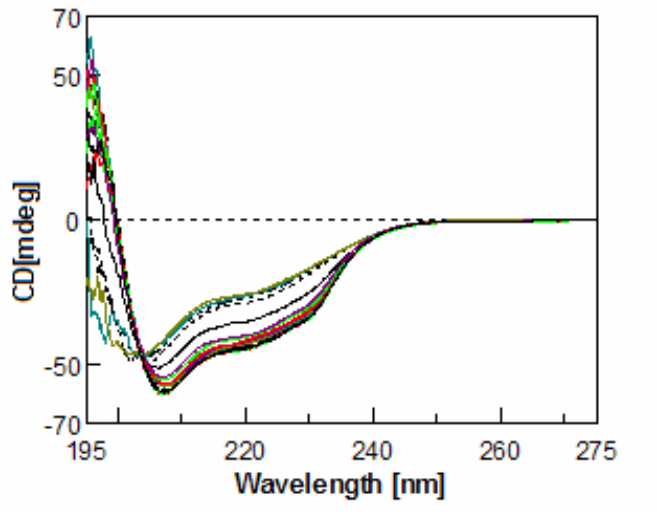

Figure 1. CD spectra demonstrating the thermal denaturation of lysozyme from 20 to 95°C.

After the spectra were collected at 95°C, the lysozyme was brought back to 20°C, to verify the reversibility. 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 is not complete. The inability of the protein to completely reconstitute may be a result of taking it so near its point of complete denaturation at 100°C.

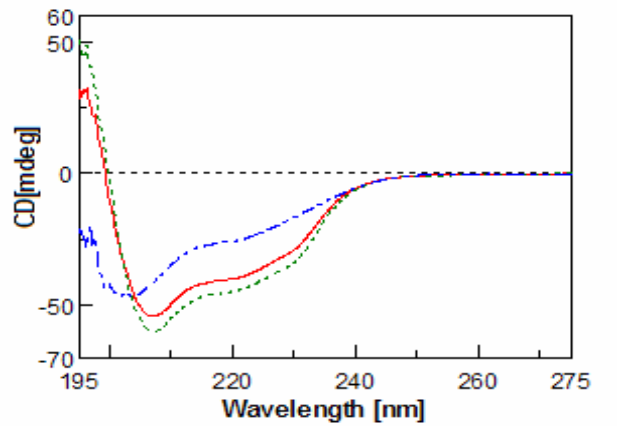

Figure 2. The CD of the lysozyme. (Green- 20°C initial, Blue 95°C, Red 20°C final)

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 incorporated into 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. Since ionic species must remain in the polar solvent 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 lies at 338 nm however as the protein denatures the fluorescence decreases and the emission maxima shifts to 347nm. The largest shift occurs between 75-80°C as it does in the CD spectra.

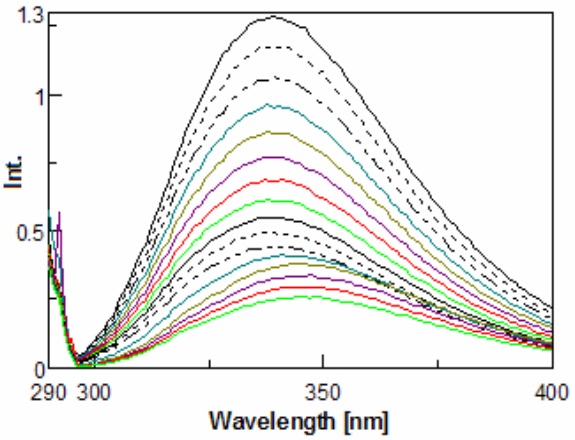

Figure 3. The fluorescence changes of lysozyme as it thermally denatures.

The ability of the lysozyme to refold was evaluated by allowing the sample to cool back to 20°C and collecting a final set of spectra. Figure 4 illustrates the fluorescence changes that take place before and after the thermal ramp. As you can see 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 its complete denaturation at 100°C.

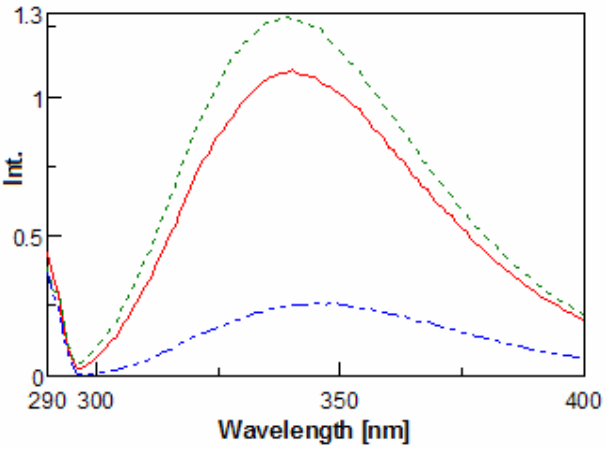

Figure 4. The fluorescence changes of lysozyme. (Green- 20°C initial, Blue 95°C, Red- 20°C final)

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