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Application of T_{ZERO} calibrated modulated temperature differential scanning calorimetry to characterize model protein formulations

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Abstract

The objective of this study was to evaluate the feasibility of using T_{ZERO} modulated temperature differential scanning calorimetry (MDSC) as a novel technique to characterize protein solutions using lysozyme as a model protein and IgG as a model monoclonal antibody. MDSC involves the application of modulated heating program, along with the standard heating program that enables the separation of overlapping thermal transitions. Although characterization of unfolding transitions for protein solutions requires the application of high sensitive DSC, separation of overlapping transitions like aggregation and other exothermic events may be possible only by use of MDSC. A newer T_{ZERO} calibrated MDSC model from TA instruments that has improved sensitivity than previous models was used. MDSC analysis showed total, reversing and non-reversing heat flow signals. Total heat flow signals showed a combination of melting endotherms and overlapping exothermic events. Under the operating conditions used, the melting endotherms were seen in reversing heat flow signal while the exothermic events were seen in non-reversing heat flow signal. This enabled the separation of overlapping thermal transitions, improved data analysis and decreased baseline noise. MDSC was used here for characterization of lysozyme solutions, but its feasibility for characterizing therapeutic protein solutions needs further assessment.

Keywords: T_{ZERO} modulated DSC; Circular dichroism; Thermal stability; Thermal analysis; Lysozyme; Protein unfolding

1. Introduction

A critical aspect in the development of protein formulations is the stabilization of its native, biologically active conformation. Proteins, unlike conventional drug molecules, possess a complex 3-dimensional structure that makes them physically and chemically unstable. Protein stability arises generally from a combination of different forces such as hydrogen bonding, hydrophobic interactions, and electrostatic interactions. Factors that displace the balance among these forces tend to affect the stability of proteins (Wang, 1999; Branchu et al., 1999). This necessitates the need to characterize protein formulations at every stage of their development as therapeutically efficacious pharmaceuticals.

Differential scanning calorimetry (DSC) is a thermal analysis technique that is well established and widely used in the characterization of polymers and pharmaceuticals. However, to characterize protein solutions, one needs to use a high-sensitivity differential scanning calorimeter (HSDSC) that is capable of detecting small changes in enthalpy or heat capacity that arise when proteins unfold. But the cost of such an instrument is very expensive.

Modulated temperature differential scanning calorimetry (MDSC), is an extension of conventional DSC, in which a sinusoidal wave modulation is applied to the standard linear temperature program. A discrete Fourier Transform algorithm is then applied to the resultant data to deconvolute the sample response to the underlying (linear) and modulated temperature programs. The response to the underlying temperature program is similar to that obtained by a conventional DSC. MDSC is thus, a software development rather than a change in the basic DSC equipment. The use of the modulated temperature program improves the quality and quantity of information that may be

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obtained by conventional DSC (Reading et al., 1994; Coleman and Craig, 1996).

In case of a conventional DSC, the heat flow signal is a combination of 'kinetic' and 'heat capacity' responses. During a linear heating program, in the absence of any physical or chemical reaction, the heat flow signal is governed by the heat capacity of the sample. When temperature attains a certain value such that a kinetically controlled event occurs (e.g.: polymer cure, crystal reorganization or aggregation) the resultant heat flow signal is a combination of the heat capacity of the sample and that associated with the kinetically controlled event. MDSC is capable of separating these two processes, while conventional DSC heat flow signal represents the sum of the two types of process. The basis of the separation of the two types of heat flow signals is their difference in response to the underlying and modulated temperature programs. MDSC thus, represents a significant advance over conventional DSC. The application of large modulation amplitudes produces large instantaneous heating rates that result in increased heat flow sensitivity compared to conventional DSC. Also, the use of low underlying heating rates provides better resolution as compared to conventional DSC (Reading et al., 1994; Coleman and Craig, 1996). High resolution would be necessary if two thermal events such as aggregation (exothermic event) and unfolding of protein (endothermic event) occur simultaneously. Unfolding may be overwhelmed by aggregation, such as the single exothermic peak observed for rhKGF unfolding and aggregation (Chen et al., 1994). MDSC would be able to differentiate overlapping thermal events due to its higher resolution (Craig and Royall, 1998). A combination of large modulation amplitude and a low underlying heating rate enables high sensitivity without compromising on resolution (Reading et al., 1994; Coleman and Craig, 1996; Craig and Royall, 1998). This may not be possible in case of either conventional DSC or high sensitive DSC. Furthermore, a four-term heat flow equation used in the newer T_{ZERO} calibrated models (TA instruments, Q-100 & 1000 series) makes the instrument more sensitive due to flatter baselines and improved signal-to-noise ratios. Although, the sensitivity achieved by this instrument may not compare to that of a high sensitivity DSC (HSDSC), it is a relatively cheaper instrument. The use of such an instrument for characterization of protein solutions was investigated. The objective of our study was to investigate the applicability of MDSC technology in achieving sensitivity comparable to a HSDSC at a cost comparable to a conventional DSC.

Lysozyme, an enzyme with a molecular weight of 14,400 Da and a p*I* of 10.7, was used as a model protein. Lysozyme has been studied extensively by DSC and is known to exhibit reversibility upon unfolding (Velicelebi and Sturtevant, 1979; Sturtevant and Velicelebi, 1981; Takano et al., 1999; Branchu et al., 1999). It was used here to investigate effect of formulation factors on such behavior as characterized by MDSC. Immunoglobulin G (IgG) is the most abundant of the immunoglobulin with an average molecular weight of 150,000 Da. Most of the FDA-approved monoclonal antibodies available in the market are one of the subclasses of IgG and are currently being used to treat various types of cancers such as prostate adenocarcinoma, non-hodgkin lymphoma, colorectal, and ovarian cancers. Human IgG is a multi-domain protein and was used as a model antibody to study the feasibility of MDSC technique for thermal characterization of monoclonal antibodies. Circular dichroism is a widely used and well-established technique to study protein unfolding and probe changes in higher order structure of proteins (Vermeer et al., 2000; Vermeer and Norde, 2000). It was used here to validate the results obtained by the T_{ZERO} calibrated MDSC.

2. Materials and methods

2.1. Chemicals

Chicken-egg lysozyme, human immunoglobulin G (IgG), sucrose and mannitol were purchased from Sigma. Citric acid anhydrous, sodium citrate, Tris and acetic acid were purchased from Fisher Scientific and sodium acetate and PEG 8000 was purchased from Mallinckrodt Chemical Works. HPLC grade water was used for preparation of all solutions.

2.2. Sample preparation

Lysozyme solutions (20 mg/ml for MDSC and 0.25 mg/ml for CD) were prepared in different buffers (100 mM acetate, citrate, citrate–phosphate, phosphate and Tris) at pH 4, 7.4 and 9.0 with various concentrations of sucrose (0–50%, w/v), mannitol (0–25%, w/v) and PEG 8000 (0–50%, w/v). IgG solutions (20–50 mg/ml) were prepared in 10 mM phosphate buffer (pH 5.8 and 7.4).

2.3. Modulated temperature differential scanning calorimetry

Thermal analysis was performed using a modulated differential scanning calorimeter (DSC Q100, TA instruments, DE). Indium (m.p. = 156.6 °C) was used for temperature calibration. Aqueous solutions (25 μl) were placed in aluminum pans and sealed hermetically. DSC analysis was carried out in the modulated heat only mode, by scanning the samples from a temperature of 30–100 °C at an underlying heating rate of 2 °C/min and a modulation amplitude of +0.5 °C every 100 s to determine unfolding temperature (T_m). The data obtained was analyzed using the TA Universal Analysis 2000 software. The baseline extrapolation method using the peak sigmoidal horizontal option was used to determine T_m.

2.4. Circular dichroism

CD analysis (Jasco J-810 spectropolarimeter) was carried out in the spectrum measurement mode in the far UV region (260–190 nm) at a scanning speed of 100 nm/min at standard sensitivity for three accumulations with a response of 1 s and in the variable temperature scan mode by scanning the samples from a temperature range of 30–100 °C at 222 nm at a heating rate of 5 °C/min. Samples (300 μl) were placed into 0.1 cm path length quartz cells. For the CD studies, use of 100 mM concen-

tration buffer gave a lower signal-to-noise ratio. The $H[T]V$ value was very high at 100 mM buffer concentration giving noisy CD spectra. It was therefore, necessary to use a lower concentration of buffer to decrease the $H[T]V$ signal thereby improving the signal-to-noise ratio. A lid covered the quartz cell during each temperature scan. Data obtained was plotted using an Excel spreadsheet.

3. Results and discussion

3.1. MDSC analysis of lysozyme solutions

A typical MDSC graph for lysozyme solution at pH 4 in 10 mM acetate buffer is shown in Fig. 1. The graph comprises of three signals, namely, reversing heat flow signal, non-reversing heat flow signal and total heat flow signal. The total heat flow signal is essentially similar to heat flow signal obtained when using a conventional DSC. Fig. 2 shows a comparison of a MDSC temperature profile and constant heating rate ramp. The application of a modulated temperature program in MDSC separates the total heat flow signal into reversing and non-reversing signals. As seen in Fig. 1, the total heat flow signal which is the sum of the reversing and non-reversing heat flow signals (Coleman and Craig, 1996), shows an endothermic event around 74 °C that is followed closely by an exothermic event. The endothermic event may be attributed to protein unfolding because proteins absorb energy during unfolding. Since unfolding exposes hydrophobic residues to solvent, these exposed hydrophobic residues associate with each other, thereby releasing energy. Thus the exothermic event may be due to aggregation. By looking at the total heat flow signal alone, it is not possible to resolve the two different events as separate transitions. Under the operating conditions selected the endothermic event around 74 °C, which reflects the unfolding of lysozyme, appears in the revers-

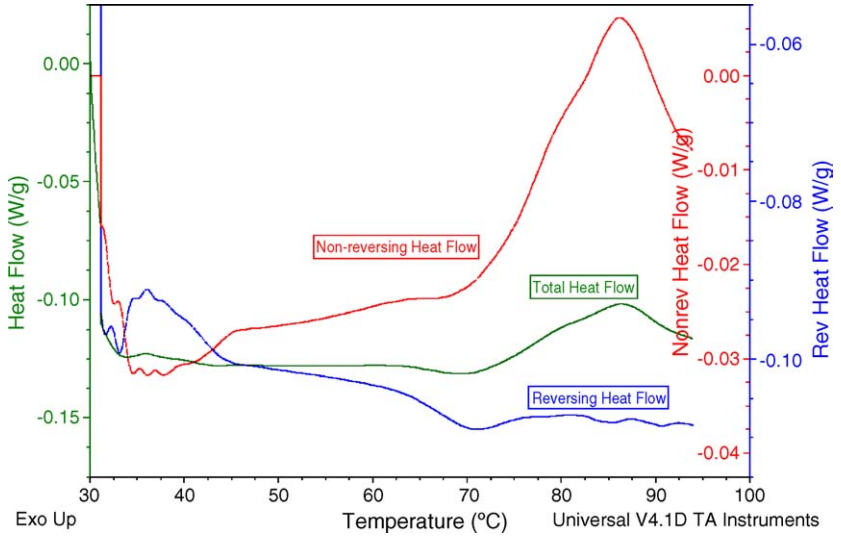

Fig. 1. MDSC analysis showing total, reversing and non-reversing heat flow signals at pH 4 in 10 mM acetate buffer.

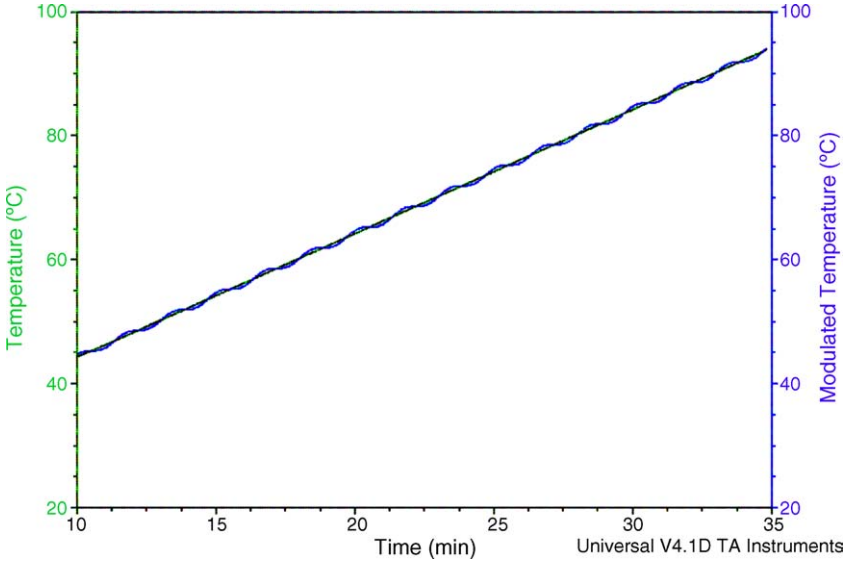

Fig. 2. Comparison of MDSC temperature profile and standard heating rate ramp. Heating rate 2 °C/min, modulation amplitude 0.5 °C and modulation period 100 s.

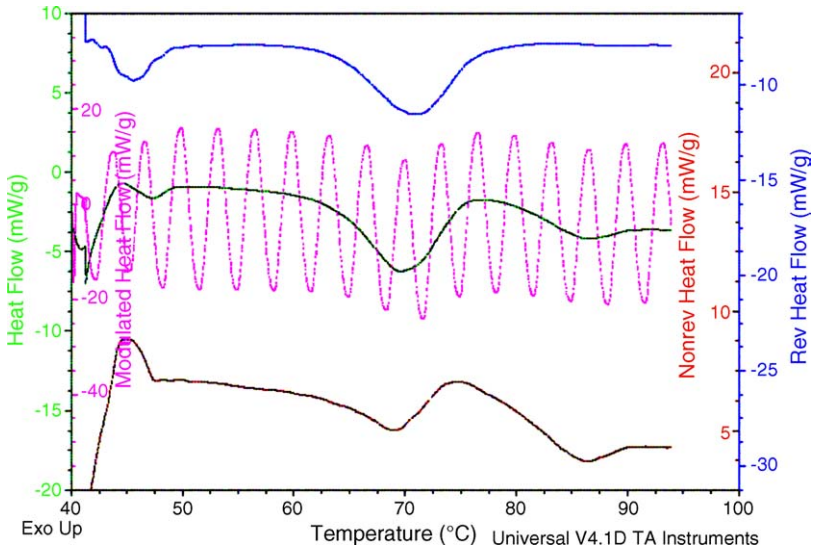

Fig. 3. Showing occurrence of four to six modulations each during the unfolding and aggregation of lysozyme as seen in the total heat flow signal.

ing heat flow signal whereas the exothermic event that could be attributed to protein aggregation is seen in the non-reversing signal. The separation of the total heat flow signal into its components (reversing and non-reversing heat flow signals) by MDSC depends immensely upon the choice of operating parameters. Optimization of these parameters, namely, underlying heating rate, modulation amplitude and modulation period, is important. These parameters determine the quality of separation afforded by the instrument, which in turn governs the reliability of the data obtained. Low underlying heating rates improve the resolution of analysis (properly separate total heat flow into its components) where as, large modulation amplitudes provide large instantaneous heating rates and hence increase sensitivity. Long modulation periods are necessary for allowing the sample to follow the applied temperature modulation. In order to enable the complete separation of underlying and cyclic responses, the combination of parameters used should be such that at least four to six modulations occur throughout the duration of each thermal event. This is depicted in Fig. 3. If this requirement is not satisfied, the data obtained cannot be viewed with confidence. As a generalization, low underlying heating rates (0.1–5 °C), large modulation amplitudes (±0.1–1 °C) and long modulation periods (40–100 s) must be applied to suffice the above requirement.

Before performing an MDSC experiment to determine unfolding temperatures, it is necessary to run the sample using the conventional DSC mode. This gives an approximate temperature range within which the thermal transition can be expected to occur. Once this is known the next step would be to use the MDSC mode. While in the MDSC mode, one has the option of

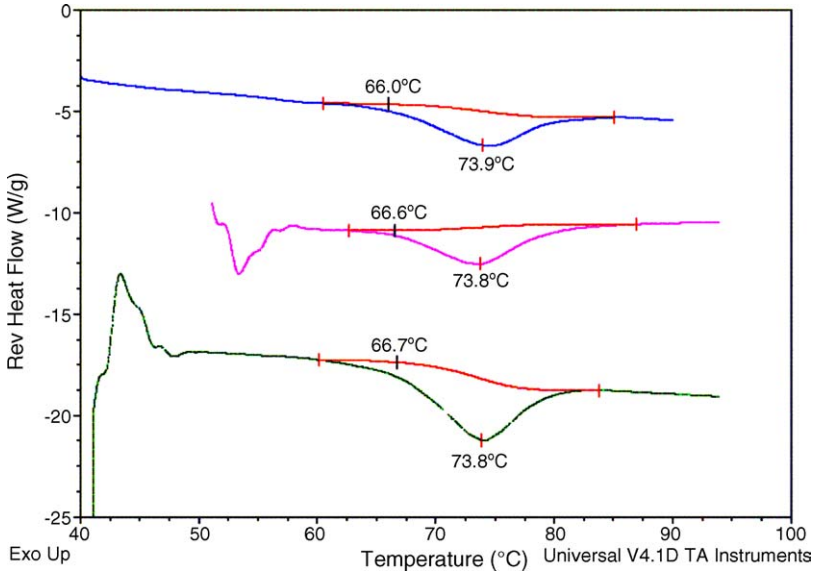

Fig. 4. Showing three independent calorimetric determinations for lysozyme unfolding at pH 4 in 10 mM acetate buffer.

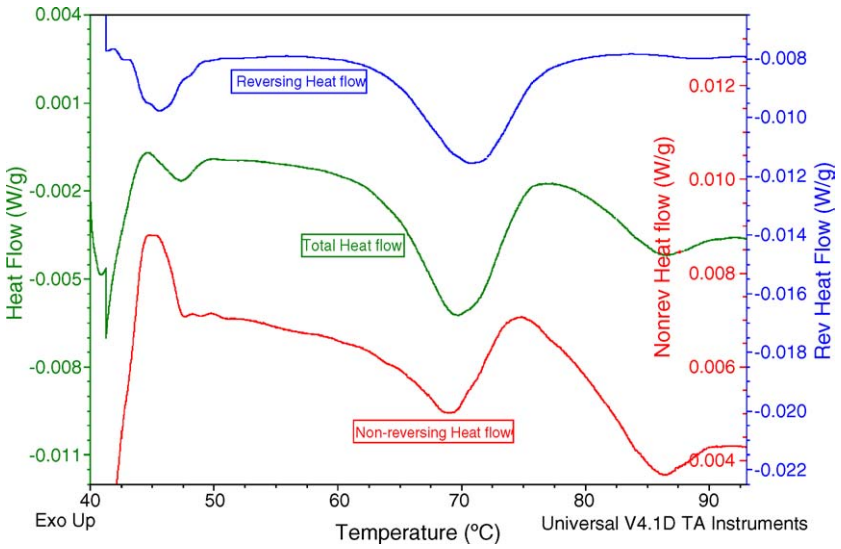

Fig. 5. MDSC analysis for lysozyme in 500 mM citrate buffer, pH 4 depicting improved resolution compared to conventional DSC.

using the general MDSC mode or the MDSC heat only mode. In the general MDSC mode, all three parameters, namely, underlying heating rate, modulation amplitude and modulation period need to be specified by the operator. The modulation amplitude in this mode oscillates from positive to negative. The modulation amplitude is the most important parameter from the viewpoint of interpreting the data with confidence. In the MDSC heat only mode, the underlying heating rate and modulation period are specified, while the modulation amplitude is automatically adjusted by the software (based on the other two parameters) such that no cooling of the sample occurs during temperature modulation, i.e., the modulation amplitude is always positive and never negative. The modulation amplitude selected in the MDSC heat only mode will be such that at least four to six cycles occur during the thermal transition, resulting in better resolution.

3.2. Effect of pH, buffers and excipients on lysozyme unfolding

Fig. 4 shows typical calorimetric recordings of lysozyme unfolding in acetate buffer (pH 4) for three independent samples. The values for T_m seem to be reproducible and precise with very less standard deviation. Having optimized the parameters for MDSC analysis of lysozyme solution at pH 4 in 10 mM acetate buffer, it was now necessary to determine the feasibility of using MDSC as a novel technique to investigate the effect of different formulation variables on lysozyme stability. Although it is possible to perform a complete thermodynamic analysis using the MDSC instrument, determination of the free energy of unfolding (ΔG_{unf}) is a complex process. Determination of changes in T_m rather than ΔG_{unf} is a much simpler and faster method to determine changes in protein stability. Liter-

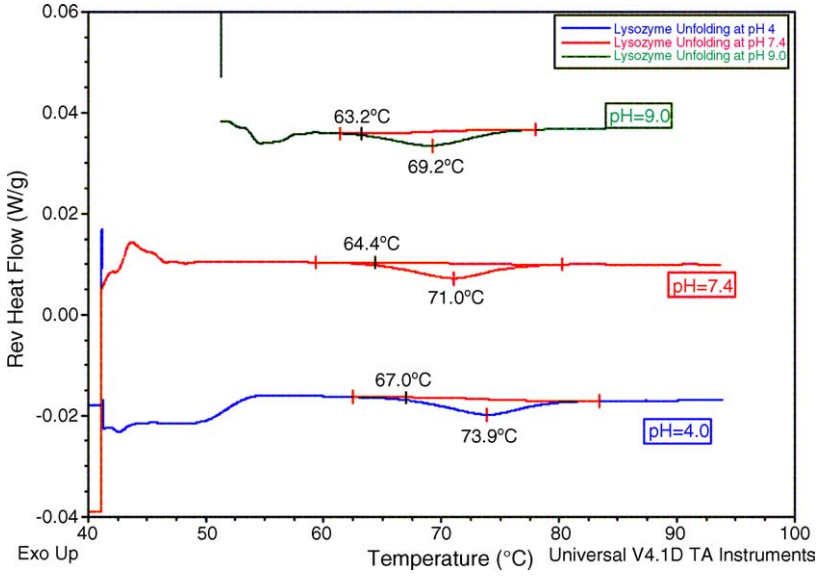

Fig. 6. Effect of pH on unfolding temperature of lysozyme. T_m was found to increase as pH decreased.

ature reports (Wang, 1999; Cueto et al., 2003) show that T_m values have been used as a stability indicator, fundamentally for comparative purposes. Hence we used T_m as a parameter to study changes in lysozyme stability as a function of various formulation parameters. Unfolding temperature is known to be a function of various formulation conditions such as pH, buffer concentration, type of buffer and excipients. Changes in any of these factors could cause changes in T_m suggesting a change in formulation (solution) stability. Changes in formulation conditions caused a change in the T_m of lysozyme. Surprisingly, formulation changes also influenced the onset of the exothermic event that was seen in Fig. 1. This made it difficult to properly analyze the thermal scans by looking at the total heat flow signal alone. As seen from the total heat flow signal in Fig. 5, it is difficult to distinguish the end point of unfolding and the onset of exothermic peak. As formulation conditions varied, the temperature for onset of this exothermic event changed, thereby overwhelming the unfolding transitions in some cases. It was not possible to correctly assign integration limits for many of the thermal scans by looking at the total heat flow signal alone. The use of MDSC resolved this issue as the exothermic event was now seen in the non-reversing heat flow signal. By looking at the reversing heat flow signal; it was now possible to properly integrate the unfolding transition without any interference from aggregation and other kinetically controlled events. Also the post-transition baseline for the reversing heat flow signal was flat, with a good signal-to-noise ratio. Literature reports (Vidanovic et al., 2003) show that the use of conventional DSC may sometimes generate thermal scans that may not have well separated transitions and hence lead to problems in data interpretation. The use of MDSC precludes this possibility. Hence for all further analyses, the unfolding transition seen in the reversing heat flow signal was used to characterize changes in the T_m as a function of formulation parameters. In all of the scans, there is a baseline noise within a temperature range of 40–50 °C, which is due to instrument start up.

Table 1
Effect of pH and buffers on lysozyme T_m as determined by MDSC

Buffer system	T_m
100 mM Acetate buffer, pH 4	73.9
100 mM Citrate buffer, pH 4	73.2
500 mM Citrate buffer, pH 4	70.9
100 mM Citrate–phosphate buffer, pH 4	73
100 mM Citrate–phosphate buffer, pH 7.4	70.2
100 mM Phosphate buffer, pH 7.4	71
100 mM Tris buffer, pH 9.0	69.2

Fig. 6 and Table 1 summarize the effects of pH and buffer on the unfolding of lysozyme. T_m was found to be dependant on pH and buffer concentration. An increase in pH led to a decrease in T_m suggesting higher stability at lower pH values in commonly used buffers. Repetitive scans with MDSC show that unfolding was reversible only at lower pH (pH 4) and irreversible at higher pH (pH 7.4, 9.0) as seen in Fig. 7. Addition of 50% sucrose to lysozyme solutions at higher pH (9.0) also was unable to show reversibility in unfolding (figure not shown). Thus reversibility of lysozyme unfolding seems to be a function of pH rather than excipients. These changes could be attributed to a combination of pH and/or buffer effects. To probe dependence of T_m on type of buffer species, a study was conducted wherein pH was kept constant and the type of buffer varied. Since a minimum change of 1 °C in T_m is considered as the threshold of thermostability change (Querol et al., 1996) it can be inferred from Table 1 that lysozyme exhibited no significant change in T_m, and hence stability, when formulated in 100 mM acetate buffer, 100 mM citrate buffer or 100 mM citrate–phosphate buffer, pH 4. Similarly, at higher pH (pH 7.4), lysozyme T_m in 100 mM phosphate buffer was not significantly different from 100 mM citrate–phosphate buffer. Thus, change in T_m as seen in Fig. 6 was due to change in pH, rather than a change in the type of buffer used. Interestingly, T_m was also found to be a function of the strength of the buffer used. It was observed that a low buffer concentration (100 mM

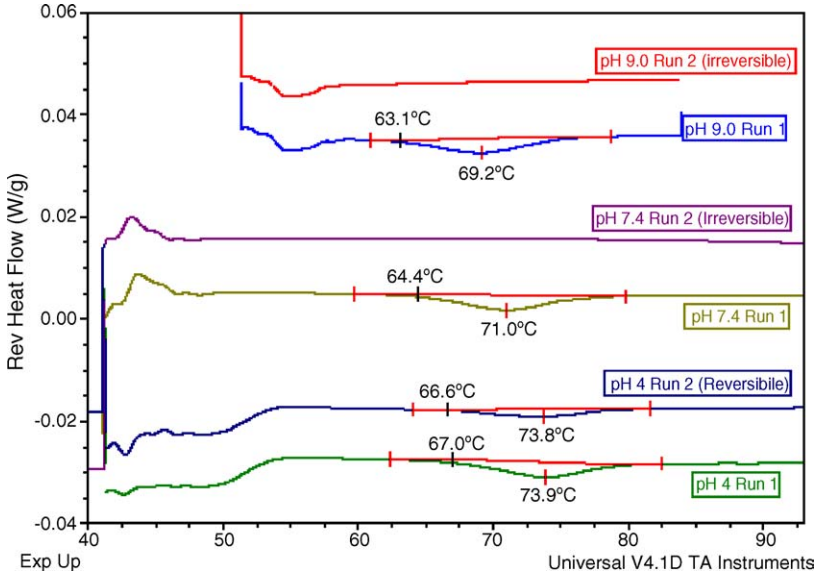

Fig. 7. Reversibility of lysozyme unfolding as a function of pH.

citrate buffer, pH 4) stabilized the protein better than high concentration (500 mM citrate buffer, pH 4) (Table 1). pH affects the formulation stability of proteins. Solution pH determines the type (positive or negative) and total charge on the protein, thereby affecting electrostatic interactions. Changes in pH can cause changes in the electrostatic environment of the protein leading to charge effects and protonation changes during thermal unfolding. Moreover, lysozyme is known to associate within a pH range of 5–9, especially above pH 6.5 and at concentrations above 10 mg/ml. In contrast, association is insignificant at lower pH and at lower concentrations. This association has shown to cause a decrease in T_m (Branchu et al., 1999; Chi et al., 2003a,b). Thus, decrease in T_m as observed by us at high pH may be attributed to changes in the electrostatic environment, resulting in dimerization of lysozyme. At low buffer concentrations, charge-shielding effects result in reduced electrostatic interactions. However, at higher concentrations, in addition to charge-shielding effects, preferential binding of ions to protein surface can further decrease the thermodynamic stability of the native conformation (Chi et al., 2003a,b). Unfolding temperature of lysozyme was also found to be a function of excipient type and concentration. The effect of increasing concentration of various excipients studied is seen in Table 2. Sucrose and mannitol had a positive effect on lysozyme stability as shown by an increase in T_m. Addition of sucrose and mannitol to lysozyme in acetate buffer increased the T_m from 74.0 to 82.4 and 80.7 °C, respectively, for the highest concentration used. Sugars and polyols have been commonly employed as protein stabilizing excipients. Their non-specific stabilizing effect is usually attributed to the mechanism of preferential exclusion (Wang, 1999). Preferential interaction, preferential hydration and preferential exclusion are terms used to explain the effects of additives on protein stability. Preferential interaction means that a protein prefers to interact with either water or an excipient. In the presence of a stabilizing excipient, a protein prefers to interact with water i.e. preferential hydration and the excipient is preferentially excluded from the protein domain i.e. preferential exclusion. This preferential hydration of protein leads to increased hydrogen bonding between protein and solvent molecules, thereby stabilizing the folded state.

Addition of increasing concentrations of PEG 8000 resulted in a decrease in T_m as seen in Table 2. T_m decreased from 74.0 to 69.0 °C for the highest concentration used. PEG's are commonly used as cryoprotectants and precipitating/crystallizing agents in aqueous media at high concentrations. Also, PEG 8000 has been used as a cryoprotectant in the lyophilization of LDH (Corveleyn and Remon, 1996; Wang, 1999). Being hydrophobic in nature PEG 8000 may interact, especially at higher concentrations, with hydrophobic side chains in proteins and interfere with hydrogen-bonding between water molecules and proteins, promoting unfolding, especially at high temperatures. Extensive binding of an excipient to a protein molecule decreases preferential hydration and destabilizes its conformation (Farruggia et al., 1997; Wang, 1999).

Table 2
Effect of varying concentrations of different excipients on unfolding temperature of lysozyme in 100 mM acetate buffer

Excipients	T_m
Lysozyme alone	74.1
10% Sucrose	75.1
25% Sucrose	77.7
40% Sucrose	80.6
50% Sucrose	82.4
5% Mannitol	75.8
10% Mannitol	76.4
25% Mannitol	80.6
15% PEG 8000	71.2
50% PEG 8000	69.1

From MDSC analysis, it was not only possible to determine the unfolding temperature of lysozyme, but also possible to improve the resolution of analysis. Protein aggregation, in some cases, may occur simultaneously as unfolding occurs. The onset of aggregation may depend on various formulation conditions such as pH, buffer concentration and excipient. In such cases the aggregation peak could obliterate the proper analysis of the unfolding peak. By the selection of optimal MDSC operating parameters, such interferences as aggregation and instrument noise may be separated from the unfolding transitions.

3.3. Heat-induced changes in the secondary structure

The main aim of our study was to determine the feasibility of MDSC as a tool for the biophysical characterization of protein solutions. For this, it was necessary to confirm the validity of the data obtained by MDSC using a second biophysical technique. The second technique used would have to be complimentary to MDSC. Circular dichroism (CD) is one such technique. Data obtained by calorimetric or MDSC technique provides information regarding protein's structural stability on a macroscopic level. Complimentary information, on a molecular level may be obtained by CD spectroscopy (Vermeer et al., 2000; Vermeer and Norde, 2000). CD measures heat induced secondary and tertiary structural changes that can be correlated to the unfolding of a protein. This is possible by measuring the ellipticity, either as a function of the wavelength at a given temperature, or as a function of temperature at a given wavelength (Vermeer et al., 2000; Vermeer and Norde, 2000). The % loss in mean residual ellipticity as a function of temperature can then be correlated to the unfolding temperature of the protein.

CD analysis for lysozyme in the far UV region showed two minima's, one at 222 nm and the other at 208 nm (Fig. 8). Presence of both these minima's in the CD scan suggests a predominance of alpha helical content in the secondary structure of lysozyme. Presence of minima at 222 nm is unique for α-helical structures. The ellipticity at 208 nm is more negative as compared to that at 222 nm, suggesting presence of other secondary structural elements such as random coil and β-sheets along with α-helical structure. By measuring the ellipticity at 222 nm it is possible to measure changes occurring in the helical content of lysozyme. Hence, changes in mean residual ellipticity at 222 nm were used to study changes in the alpha helical content of lysozyme. The data obtained by CD correlated well with that obtained by MDSC. Table 3 summarizes the data obtained

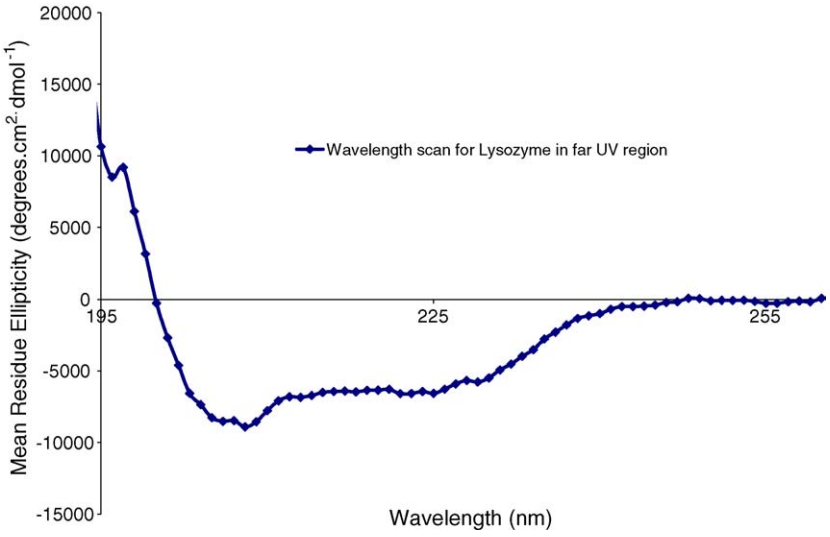

Fig. 8. Wavelength scan for lysozyme by circular dichroism in the Far UV region showing predominance of α-helical structure.

Table 3
Unfolding temperature of lysozyme as determined by CD (as measured by 20% loss in α-helical content)

Buffer system	T_m
10 mM Acetate buffer, pH 4	74.5
10 mM Phosphate buffer, pH 7.4	71.5
10 mM Tris buffer, pH 9.0	69.5
10 mM Acetate buffer, pH 4 + Sucrose	79

by CD analysis. Temperature scans from 30–100 °C at 222 nm showed that unfolding (as defined by a 20% loss in alpha helical content) of the protein occurred in the vicinity of 74.5 °C in acetate buffer (pH 4), 71.5 °C in phosphate buffer (pH 7.4) and 69.5 °C in Tris buffer (pH 9.0). Fig. 9 shows this for lysozyme in pH 7.4 Wavelength scans taken before and after heating to 100 °C (Fig. 10) showed that the loss of alpha helical content was reversible in acetate buffer (pH 4) but irreversible in phosphate buffer (pH 7.4) and Tris buffer (pH 9.0) which suggests that even at lower concentrations (0.25 mg/ml for CD studies as compared to 20 mg/ml for MDSC), there is a decrease in the thermal stability of lysozyme with an increase in pH. Thus changes in pH may have a dominant effect on the association of lysozyme resulting in subsequent decrease in protein stability. Addition of sucrose to lysozyme in acetate buffer, pH 4 increased the unfolding temperature to around 79 °C (Table 3). The effect of pH and sucrose on lysozyme stability as inferred by CD spectroscopy therefore supports the data obtained by MDSC.

3.4. Characterization of human IgG solutions by MDSC

After determining the feasibility of using MDSC technique for characterizing lysozyme solutions, we wanted to see if this

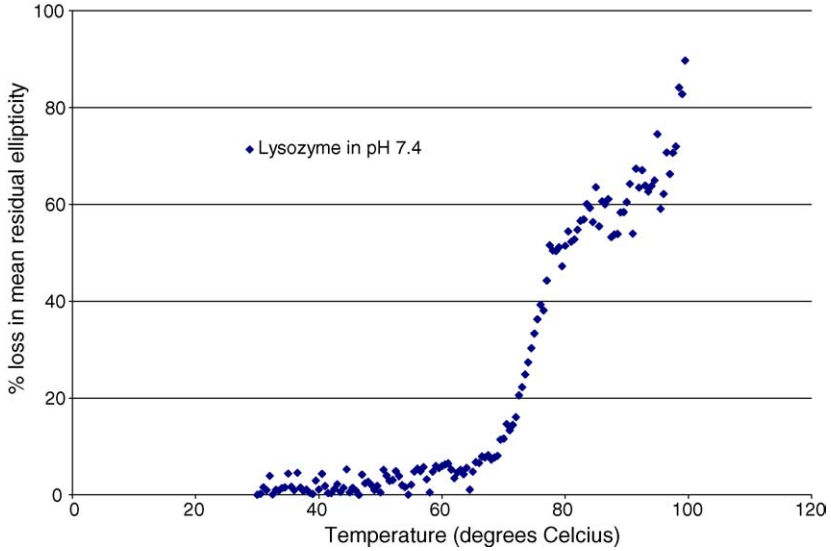

Fig. 9. Loss in secondary structure of lysozyme, as determined by loss in ellipticity at 222 nm, in pH 7.4 (phosphate buffer) as a function of increasing temperatures.

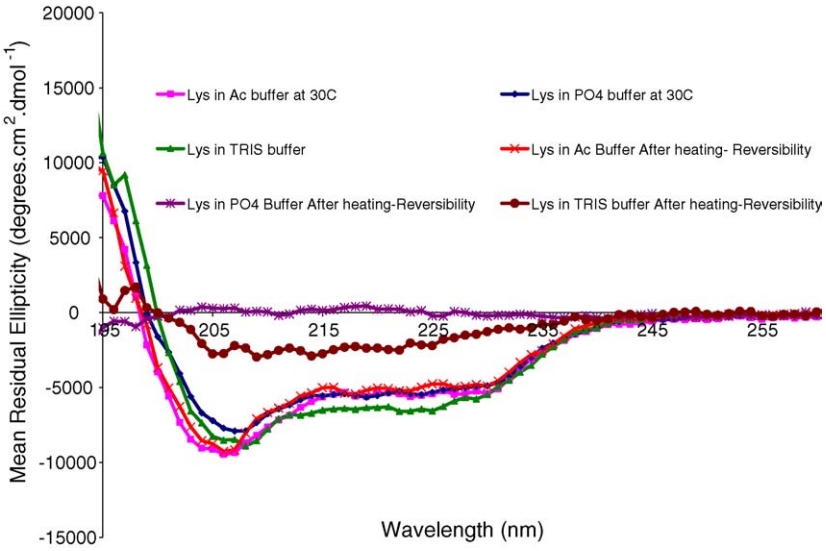

Fig. 10. Reversibility of α-helical content of lysozyme as a function of pH.

technique could be extended to other therapeutically active proteins, such as human IgG. Human IgG is a relatively complex protein as compared to lysozyme and is known to exhibit multiple values of T_m, which may be related to its multi-domain nature (Vermeer et al., 2000; Vermeer and Norde, 2000). Due to the complex nature of IgG, we thought it would be prudent to start with the conventional DSC mode initially so as to guesstimate a thermal range within which thermal transitions could be expected and then move on to the MDSC mode. This was necessary to expedite the optimization of MDSC parameters. Based on the lysozyme data we started with a concentration of 20 mg/ml and a heating rate of 2 °C/min. The use of the conventional DSC mode for IgG solutions showed no perceptible transition at 20 mg/ml. Upon inspection of the sample pans, the solution had completely gelled suggesting that aggregation may have occurred. This was consistent irrespective of either changing the magnitude of heating rate used (0.1–10 °C/min) or increasing the concentration of IgG solutions to 50 mg/ml. Due to the absence of any perceptible transition, it was difficult to apply the modulated temperature program. Increasing the sample volume from 25 to 75 μl by using high volume pans and keeping the concentration of IgG solutions at 50 mg/ml enabled detection of a thermal transition. The heating rate used was 0.5 °C/min. This can be seen in Fig. 11. The figure shows an endothermic event around 70 °C that may be attributed to IgG unfolding, and an exothermic event around 80 °C attributed to aggregation. The sample volume was increased by using stainless steel high volume pans. Based on this result an underlying heating rate of 0.5 °C/min was chosen and various combinations of modulation amplitudes (±0.1–1 °C) and modulation periods (30–100 s)

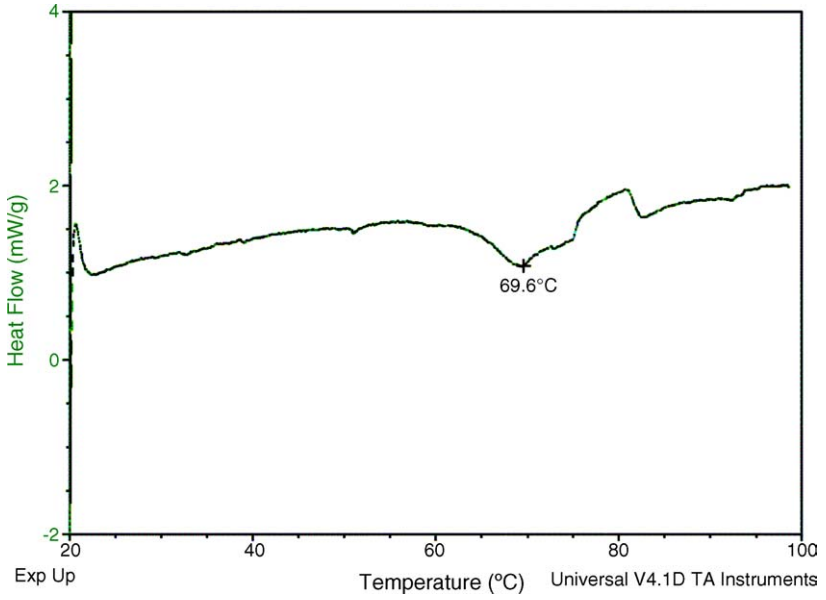

Fig. 11. DSC analysis for IgG solution at 50 mg/ml using the conventional DSC mode at 0.5 °C/min showing a T_m at 69 °C followed by aggregation.

were selected so as to achieve four to six modulations throughout the duration of thermal transition. This resulted in thermal scans that were not reproducible and with increased baseline noise, therefore difficult to interpret (data not shown). This could be explained as the application of high sample volumes, used to improve the sensitivity could increase the possibility of thermal gradients when using MDSC analysis. Increasing the sample size could make it difficult for the sample to follow the applied modulation program, which in turn could result in thermal gradients resulting in transitions that are difficult to interpret.

4. Caveats in characterization of therapeutically active protein solutions using MDSC

MDSC, in the past, has been extensively used for the characterization of lyophilized protein formulations, frozen protein solutions and polymers (Coleman and Craig, 1996; Craig and Royall, 1998; Liao et al., 2002). The goal of our study was to investigate the feasibility of using T_{ZERO} modulated DSC as an alternative biophysical technique to evaluate the stability of protein solutions using lysozyme as a model protein and IgG as a model antibody. Although, we were able to use MDSC for thermal characterization of lysozyme solution as a model protein, there are some caveats that need to be considered before using MDSC as an alternative technique for therapeutically active proteins. Firstly, lysozyme and IgG used in this study are not therapeutic proteins. Lysozyme, especially, is a relatively stable enzyme that exhibits reversibility in unfolding and hence considered to be a 'model or ideal protein'. Most of the therapeutically active proteins are not known to be 'ideal proteins'. Many of them exhibit much lower thermodynamic stability and often show irreversibility in unfolding. Secondly, the concentration of lysozyme used for MDSC analysis was 20 mg/ml. At concentrations lower than 20 mg/ml, the thermal transition is imperceptible from the baseline noise. Changes in heat capacity or enthalpy of therapeutic protein solutions are usually very small. Characterization of such changes requires the use of a HSDSC. Thus, although the cost of an MDSC instrument may be cheaper than an HSDSC, sensitivity is still an issue that needs improvement. Thirdly, during DSC analysis of therapeutic proteins at such high concentrations of 20 mg/ml or higher, self-association or aggregation of these proteins much before the unfolding temperature could occur resulting in no unfolding transition per se. Finally, the reliability of data obtained by MDSC depends on the proper selection of operating parameters. Since more than one parameter needs to be optimized, MDSC, initially, is a more complex technique compared to conventional DSC or HSDSC. Operating parameters for different therapeutic proteins need to be individually optimized. Also it would be prudent to first scan the protein using the conventional DSC mode before trying the MDSC modes. The applicability of MDSC technique for characterization of model protein solutions was investigated here. The feasibility of using this technique for characterizing therapeutic protein solutions needs further assessment.

5. Conclusions

Stability of lysozyme, as investigated by T_{ZERO} modulated DSC and confirmed by circular dichroism, was found to be a function of pH, buffer concentration and excipients and independent of buffer type. Reversibility of lysozyme T_m was found to be dependent on pH. Lower pH stabilized lysozyme in contrast to neutral or alkaline pH. Sucrose and mannitol, in contrast to PEG 8000, increased the thermal stability of lysozyme. Under the experimental conditions studied, MDSC analysis of IgG solutions gave increased baseline noise and data that was not reproducible. This could be due to increased thermal gradients during MDSC analysis of IgG solutions using high volume pans. As sample size increases, the sample may not follow the modulation applied by the instrument giving rise to increased thermal gradients. MDSC, because of its ability to separate overlapping transitions, has improved resolution as compared to conventional DSC or HSDSC but its sensitivity does not compare to that of a HSDSC. Although it was possible to use MDSC for characterizing lysozyme formulations as model protein solutions, its applicability for characterization of therapeutic protein solutions needs further assessment.

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