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Reversible and non-reversible thermal denaturation of lysozyme with varying pH at low ionic strength

Alice Blumlein, Jennifer J. McManus *

Department of Chemistry, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

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ABSTRACT

DSC analysis has been used to quantify the reversibility of unfolding following thermal denaturation of lysozyme. Since the temperature at which protein unfolding occurs, T_m, varies with different solution conditions, the effect on the melting temperature and the degree of refolding after thermal denaturation in low ionic strength sodium phosphate buffers (5–1000 mM) over a range of pH (5–9) in the presence/absence of disaccharides is examined. This study compares the enthalpies of unfolding during successive heating cycles to quantify reversibility following thermal denaturation. The disaccharides, trehalose and maltose were used to assess if the disaccharide induced increase in T_m is reflected in the reversibility of thermally induced denaturation. There was extensive overlap between the T_m values where non-reversible and reversible thermal denaturation occurred. Indeed, for pH 6, at the highest and lowest T_m, no refolding was observed whereas refolding was observed for intermediate values, but with similar T_m values having different proportions of refolded protein. We established a method to measure the degree of reversible unfolding following thermal denaturation and hence indirectly, the degree to which protein is lost to irreversible aggregation, and show that solution conditions which increase melt transition temperatures do not automatically confer an increase in reversibility. This type of analysis may prove useful in assessing the stability of proteins in both the biopharmaceutical and food industries.

1. Introduction

The transformation of proteins from native to non-native conformations is an acute issue clinically [1] and in pharmaceutical research and development [2]. Aggregates can be formed from non-native folding intermediates and this has been shown both in-vitro [3] and in computational studies [4]. Non-native protein conformations in solution may lead to protein aggregation and the shelf life of a product may be reduced due to decreased therapeutic capacity in biopharmaceutical formulations [5]. Additionally, aggregated proteins may produce an immunogenic response resulting in compromised product safety [6]. The physiochemical conditions required to purify and concentrate proteins is often not conducive to maximal protein structural stability [7]. The structural stability of a protein is sometimes assessed by measuring the temperature at which thermal denaturation occurs [8], and is dependent upon solution conditions such as protein concentration, pH, ionic strength, presence of co-solutes and ion type [9]. Aggregation associated with unfolding may occur following thermal denaturation which may be unavoidable, or occur inadvertently, during the processing of proteins [10,11]. Conversely, thermal denaturation may be the desired outcome of some processes [12]. The melt transition temperature, the temperature at which the change in apparent heat capacity reaches a maximum during differential scanning calorimetry (DSC), T_m, is widely used as a measure of protein stability [8,13]. This study uses DSC analysis to quantify the reversibility of unfolding following thermal denaturation, at moderately high protein concentration (100 mg/ml) and investigates if T_m is an adequate thermodynamic parameter to describe the structural stability of the protein or if both T_m and the reversibility of unfolding might yield a more complete analysis.

Protein aggregation occurs in a concentration dependent manner [14] and a critical concentration may be necessary for aggregation initiation [15]. For this reason the lysozyme concentration was fixed at 100 mg/ml. From a practical perspective, conducting measurements at these concentrations is useful since it is consistent with the total protein level in serum [16] and also the final protein concentration in many biopharmaceutical formulations [7].

Changes in pH alter protein stability by multiple mechanisms, including, but not limited to; hydrogen bond interactions, salt-bridge and like-charge repulsion effects [17]. Ion type and ionic strength have a complex effect on the stability of proteins. The process by which ions affect the stability of proteins is not fully understood, but is thought to be mediated by both specific and non-specific interactions between the ions and proteins and between the ions and water [18,19]. The buffer type and pH range (pH 5 to pH 9) were chosen for a number of reasons. In previous work we have shown that for lysozyme, T_m is pH dependent [20]. In that study, different buffer salts

* Corresponding author. Tel.: +353 1 7086926.
E-mail address: jennifer.mcmanus@nuim.ie (J.J. McManus).

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were used to obtain the desired pH (i.e. sodium phosphate for pH 7.0 and sodium acetate for pH 4.5). Co-solutes such as disaccharides are known to increase T_m and this is thought to occur as a result of preferential exclusion of disaccharides from the surface of proteins [21,22]. Trehalose and maltose are used here to assess whether a disaccharide induced increase in T_m has an impact on the reversibility of thermally induced lysozyme denaturation. Thus, we have used a single buffer salt across a range of pHs and ionic strengths and in the presence and absence of co-solutes to methodically adjust T_m and generate a set of data to assess the degree of aggregation following thermal denaturation. While lysozyme is not of biopharmaceutical interest, it is used here to demonstrate the utility of the approach.

In this study, solution conditions were adjusted to generate a set of data where the T_m and degree of aggregation following thermal denaturation were expected to vary.

Protein aggregation is a collective term which includes reversible [23] and irreversible aggregate formation [24]. Aggregates may be formed from proteins which are either essentially native in structure [14] or non-native, unfolded or partially folded moieties [4,7,25]. For small globular proteins the amino acid sequence encodes sufficient information to allow the spontaneous reformation of the native structure from the denatured state in-vitro [26].

It has long been known that DSC can be used to assess reversibility, but there is a lack of published studies for proteins. In a recent perspective, Pethica noted a shortage of data on second (and subsequent) DSC scans in the literature [27]. Lysozyme has been chosen as our model protein since many of the biophysical properties of this 14.7 kDa, globular protein are well understood [28]. DSC is a classical method used to evaluate protein thermodynamic parameters including the enthalpy of unfolding, ΔH_{unf}, and heat capacity, C_p. Recently, Sasahara et al. studied amyloid fibril formation for lysozyme during a heating cycle by DSC in a sample that had previously been incubated with agitation [29]. The DSC thermograms showed a sharp decrease in heat capacity which was ascribed to the heat flow associated with the conversion from an 'aggregation-competent' lysozyme species, formed through agitation, to heat-induced amyloid fibrils. In a series of interruption–incubation DSC experiments, Luo et al. established a method to distinguish between two-state and non-two-state (lysozyme and bovine serum albumin respectively) denaturation processes [30]. A combination of Raman spectroscopy and temperature-modulated scanning calorimetry shows support for a two-stage process during the thermal denaturation of lysozyme [31]. An initial change in the tertiary structure without concurrent unfolding of secondary structure, followed by unfolding of the secondary structure was observed. Temperature-modulated scanning calorimetry has been used to decouple reversible and irreversible enthalpy changes during the thermal denaturation of lysozyme [32].

The aim of this study is to assess whether T_m is related to the degree of reversibility following thermal denaturation. By comparing DSC thermograms after successive heating and cooling cycles, we determine the extent of reversibility of thermal denaturation. We do not find any direct correlation between solution condition induced changes in T_m and the degree of reversibility. Indirectly, the proportion of protein lost to aggregation during the heating and cooling process can also be determined from these measurements. As expected, the degree of reversibility decreases as the pI of the protein is approached and also with increasing ionic strength. While sugars increase the T_m for the protein, little significant increase in reversibility is observed.

2. Experimental methods

2.1. Sample preparation

Chicken egg white lysozyme was purchased from Calbiochem and used without further purification. Sodium phosphate buffers of 5 mM–1000 mM concentrations were prepared using analytical grade reagents. The lysozyme was dissolved in buffer at ≈4 mg/ml. Co-precipitated acetate salts were removed by concentrating the sample and reconstituting the sample repeatedly by ultrafiltration for 5 min using Amicon Ultra-4 MWCO 10 kDa centrifugal devices at 4000 ×*g*. When the pH of the lysozyme solution matched the buffer pH a final spin at 7000 ×*g* for 10 min was used to concentrate the sample. Lysozyme concentrations were determined by UV absorbance using an extinction coefficient of 2.64 ml mg^{-1} cm^{-1} [33]. The final concentration was adjusted, using appropriate buffer, to 200 mg/ml. Fresh solutions were prepared for each DSC cycle.

Maltose monohydrate and trehalose dehydrate were purchased from Calbiochem and used without further purification. Each sugar was prepared in the appropriate buffer to a final concentration of 40% (v/v). Samples were prepared for DSC mixing the lysozyme 50/50 with either appropriate buffer or sugar solution. The final concentration of lysozyme was therefore 100 mg/ml protein and the final concentration of sugar was 20% (v/v) where present.

2.2. DSC measurement

A Perkin Elmer Pyris-6 Differential Scanning Calorimeter (Perkin Elmer, Ireland) was used to conduct the DSC measurements [34,35]. A 55 μl sample was prepared and weighed into a large volume stainless steel capsule (Perkin Elmer, Ireland) which was then sealed and analyzed. The sample was equilibrated at 25 °C for 5 min before the temperature was increased from 25 °C to 95 °C at a scan rate of 1 °C/min. The scan rate was maintained throughout subsequent scans and the sample was cooled from 95 °C to 40 °C whereupon heating to 95 °C and cooling to 40 °C was repeated twice more. Thus, three heating and cooling cycles were completed. Baseline subtractions and data analysis were executed using OriginPro version 9.0 software (Origin-Lab Corporation, Northampton, MA).

2.3. SDS PAGE

Lysozyme samples were prepared as for DSC. Two 1 ml aliquots were pipetted into 1.5 ml Eppendorf tubes which were sealed. The samples were then heated to 95 °C and allowed to cool to 23 °C. One of the samples was heated for a second cycle. Thus, the DSC conditions were simulated. The sample was centrifuged at 13,000 ×g using a VWR Galaxy D, microcentrifuge to separate the aggregate from the supernatant. The protein concentration in the supernatant was determined using UV absorbance. The samples were diluted to 1 mg/ml and resolved on a 12.6% SDS PAGE. A Thermo Scientific PageRuler Plus prestained protein ladder was used to determine the molecular weight of the lysozyme bands. The gel was run at 200 mV for 50 min followed by Coomassie Brilliant Blue staining.

2.4. Intrinsic fluorescence

The samples were prepared in the same manner as for SDS page. A Molecular Devices SpectraMax M2^e (Molecular Devices, USA) was used to conduct intrinsic fluorescence measurements. Fluorescence intensity (F. I.) and maximum emission wavelength (λ_{max}) were measured following excitation at 280 nm and 295 nm for samples at 1 mg/ml lysozyme concentration.

2.5. Reversibility index

The enthalpy of unfolding per mole of native lysozyme is constant during a given DSC experiment since the system is closed after preparation in the initial solution conditions and an apparent decrease in ΔH during successive heating cycles is a consequence of fewer natively folded lysozyme molecules undergoing thermal denaturation. The decrease in ΔH, therefore, indicates the extent of the depletion of the native conformation, or the extent of reversible

thermal denaturation. Thus, a refolding index (*RI*) was calculated, using Eq. (1):

$$RI = \frac{\Delta H_{me}(n+1)}{\Delta H_{me}(n)} \quad (1)$$

where, ΔH_{me} is the integrated area under the unfolding peak from the melt transition temperature, T_m to the end of the endothermic transition, T_e, during an unfolding event, n.

3. Results

3.1. Typical thermogram and baseline subtraction

This study investigates the degree of reversibility following thermal denaturation at varying pH and low ionic strengths (5 mM–1000 mM) for lysozyme. DSC analysis was used to measure the change in a sample's apparent heat capacity, C_p, with respect to temperature. During the heating cycle, the endothermic, denaturation phase transition is recorded in the DSC thermogram as an increase in C_p whereas the signature for refolding is exothermic and appears as a decrease in C_p during the cooling cycle.

The sample heat capacity did not return to the original value following cooling to 40 °C, due to the difference in C_p between native and irreversibly denatured protein [32]. In order to compare the area under the curve between heat cycles it was therefore necessary to perform a sample baseline subtraction. Although irreversible denaturation and aggregation of protein following thermal denaturation may deform the DSC transition [36], these thermograms (Fig. S1) were qualitatively consistent with the thermograms of Salvetti et al., inferring that the irreversible process of thermal denaturation did not contribute significantly to the apparent heat capacity measurement during DSC. The data was fitted using a cubic baseline subtraction, performed in OriginPro 9.0. Any baseline correction unavoidably introduces minor distortion to the DSC trace [36], nevertheless any bias introduced by baseline subtraction was later eliminated during the calculation of the proportion of refolded lysozyme. A representative, baseline subtracted DSC thermogram is shown in Fig. 1. This thermogram charts a typical thermal unfolding and refolding for lysozyme over three heating and cooling cycles, H1–H3 and C1–C3, respectively.

3.2. Confirmation of aggregation and return to natively refolded protein after heating and cooling

As the DSC capsules are sealed, the heated samples were not available for further analysis. Therefore the heating process was replicated with the samples heated in sealed 1.5 mL eppendorf tubes. The samples were visibly turbid after an initial heating and cooling cycle, consistent with the formation of protein aggregates. The aggregated material could be separated by centrifugation and the soluble protein was removed by aspiration for further analysis.

Since high-temperature-induced chemical changes may prevent the protein refolding to a native conformation [37], the presence of non-fragmented, monomeric lysozyme was confirmed in the supernatant by SDS PAGE as shown in Fig. S2. Using the replicated DSC heating methods, supernatant samples were resolved using 12.6% SDS PAGE. For all three samples; not heated, heated for a single cycle and heated for two cycles, a single band is present at ~14.7 kDa, consistent with monomeric, non-fragmented lysozyme.

Intrinsic fluorescence was used to determine whether the soluble lysozyme monomer found in the supernatant had refolded to the native conformation upon cooling. The fluorescence emission spectra for unheated lysozyme and the monomeric lysozyme recovered after a single heating and cooling cycle are shown in Fig. 2 and indicate that soluble protein which was isolated following heat treatment had refolded to a native form.

Where no second unfolding event was observed by DSC, samples were found to be gel-like and no soluble lysozyme was present which could be separated from the aggregated phase by centrifugation. Where refolding was observed, a second, broad, exothermic, overlapping transition appeared in the thermogram during the cooling cycle. This broad transition also appeared as a second endotherm during second and subsequent heating cycles. A similar low-temperature shoulder was also observed in previous studies; however no characterization of this peak was attempted [32,38]. Here a modified DSC procedure was used to determine whether the additional broad transition observed upon cooling and in subsequent heating cycles was associated with the soluble lysozyme or the aggregate. Samples were prepared and heated for one cycle under the same conditions as for the DSC measurements. The sample was centrifuged to remove the aggregated material and the supernatant placed in a DSC pan which was then subjected to a second heating cycle. The broad overlapping transition previously observed during second and third heating cycles was not present in the absence of pre-aggregated material, Fig. S3. The melt transition temperature observed for lysozyme during this experiment was consistent with the T_m observed (74.6 °C), for the same solution conditions (100 mM sodium phosphate, pH 6), during a standard DSC experiment (74.1 °C), providing further confirmation that the soluble fraction consisted of lysozyme that had refolded to the native conformation.

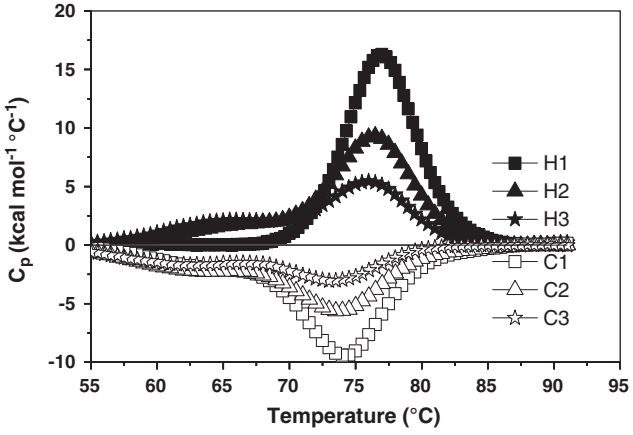

Fig. 1. The normalized and baseline subtracted DSC thermogram for lysozyme (100 mg/ml) in 0.01 M sodium phosphate buffer, pH 5 in the absence of sugar during three heating (H1–H3) and three cooling cycles (C1–C3).

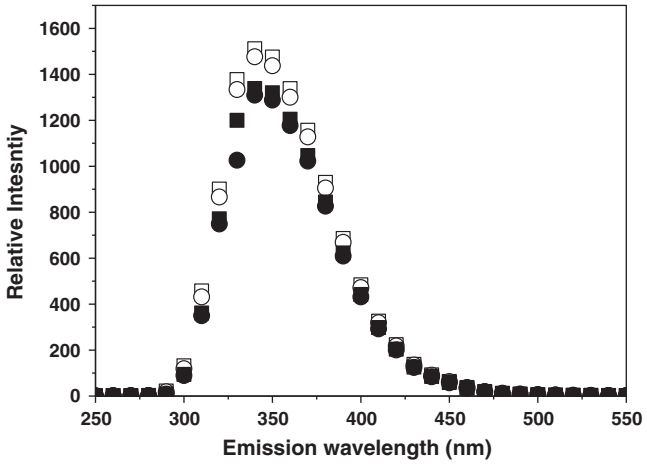

Fig. 2. Intrinsic fluorescence spectra for soluble lysozyme in 50 mM sodium phosphate buffer pH6, before and after heating. Open symbols — excitation at 295 nm; closed symbols — excitation at 280 nm. ■ no heat treatment and ● one heating cycle.

Table 1
Area associated with lysozyme unfolding at different protein concentrations. Where the initial protein concentration of 100 mg/ml is thermally denatured and an R.I. value of 0.20 is measured this is consistent with the area of an equivalent 20 mg/ml solution.

Lysozyme concentration (mg/ml)	Area $T_m - T_e$
100	55.5
50	29.1
25	18.6
20	12.4
$RI = 0.20$	11.7

3.3. Quantifying reversibility

Eq. (1) was used to quantify reversibility of the heat induced unfolding process, and indirectly, the degree to which protein is lost to aggregation after successive heating cycles. Table 2 shows the *RI* for all solution conditions following a single heat/cool cycle. To verify the accuracy of the *RI* value, further DSC experiments were performed at lysozyme concentrations of 20, 25 and 50 mg/ml in 50 mM sodium phosphate buffer at pH 6. The ΔH_{cal} values at each concentration are shown in Table 1. For comparison, the ΔH for an *RI* value of 0.20 is shown and is consistent with the ΔH value for an unheated protein solution at that concentration. Where $RI > 0$ following the first heat treatment, a second *RI* value was determined to quantify the degree of reversibility following a second heat treatment. These data are shown in Table 2.

3.4. Effect of solution conditions

The pH and ionic strength dependence of the melt transition temperature during the first DSC heating cycle, T_{m1}, was measured. During sample preparation, it was necessary to repeatedly wash the lysozyme by ultrafiltration as the co-precipitated acetate salts present in the lyophilized protein caused a pH change in the low ionic strength buffers. This extensive desalting process ensured that both the ionic strength and the sample pH were explicitly controlled. Fig. 3 shows a decrease in T_{m1} as pH was increased from pH 5 to pH 9. A sharp decrease in T_m was observed between pH 5 and pH 6 for 50 mM and 100 mM buffers ($\Delta T_m = 2.9$ °C and 2.6 °C, respectively). The decrease was somewhat more gradual for 10 mM buffer, $\Delta T_m = 1.7$ °C. For all three buffers, there was a sharp decrease in T_m between pH 6 and pH 7 ($\Delta T_m = 3.1$ °C, 2.9 °C and 2.8 °C, respectively). A more gradual decrease in T_m was observed between pH 7 and pH 9 for all three buffers ($\Delta T_m = 1.3$ °C, 0.0 °C and 0.8 °C, respectively). The T_m values converge at pH 5 and pH 9, regardless of the ionic strength. Since T_m was observed to be a function of both pH and ionic strength, a set of samples was prepared, where the pH was fixed at pH 6 and additional DSC measurements were taken for an extended range of ionic strengths (5–1000 mM). In the absence of added sodium phosphate the pH of the lysozyme solution was measured in-situ and found to be pH 6 following multiple desalting washes with deionized MilliQ water. T_m in the absence of sodium phosphate was 75.2 °C. As the ionic strength was increased, T_m decreased for ionic strength values ≤ 200 mM. However, when the ionic strength was increased above 200 mM, T_m began to increase and exceeded the T_m value for water at 1000 mM sodium phosphate.

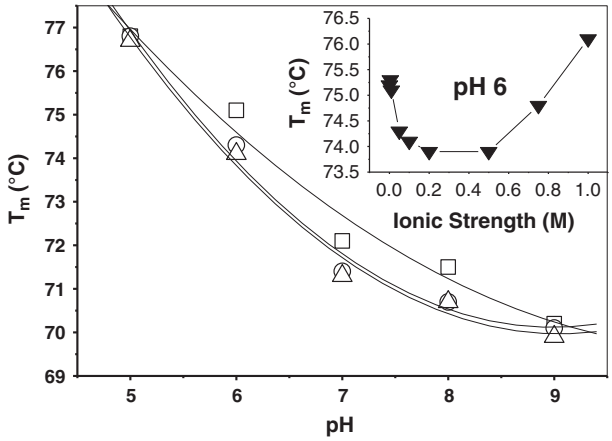

Fig. 3. The pH and ionic strength dependence of the melt transition temperature, T_{m1}, during the first DSC heating cycle. □ 10 mM, ○ 50 mM, and △ 100 mM.

Fig. 4 shows the dependence of the refolding index, *RI* on pH and ionic strength. *RI* decreased as pH increased and no refolding was observed at pH ≥ 7. At pH 5 the refolding index was greater at 10 mM than at either 50 mM or 100 mM — both of which had the same value. At pH 6, where an extended range of ionic strengths were measured, refolding of thermally denatured lysozyme was greatest in the absence of sodium phosphate (i.e. in deionized MilliQ water). As ionic strength increased, reversibility decreased and was absent for concentrations ≥ 200 mM.

The relationship between the thermal stability of lysozyme and its refolding capability following thermal denaturation is shown in Figs. 5 & 6. As reported above, refolding was observed only at pH 5 and pH 6 and T_m values for this pH range were higher than T_m values for pH ≥ 7. The refolding behavior is complex. Where no refolding was observed, T_m values ranged from 69.9 °C to 79.7 °C. Where refolding was observed T_m varied between 74.1 °C and 80.7 °C. Thus, there was extensive overlap between the T_m values where non-reversible and reversible thermal denaturation occurred. Furthermore, for a given pH value e.g. pH 6, at the highest and lowest T_m, no refolding was observed whereas refolding was observed for

Table 2
Reversibility index data for each of the solution conditions probed ($RI = \Delta H_{me2} / \Delta H_{me1}$). Symbols are used in Fig. 6.

Solution conditions	pH 5		pH 6		pH 7		pH 8		pH 9	
	T_m	*RI*	T_m	*RI*	T_m	*RI*	T_m	*RI*	T_m	*RI*
0 mM phosphate (MilliQ)	–	–	75.2✱	0.64	–	–	–	–	–	–
5 mM phosphate	–	–	75.3■	0.33		–	–	–	–	–
10 mM phosphate	76.8○	0.58	75.1●	0.30	72⇦	0.00	71.5🡐	0.00	70.2🡔	0.00
10 mM 20% v/v maltose	81.9△	0.59	82.3▲	0.66	79.7⇨	0.21	77.7🡒	0.00	–	–
10 mM 20% v/v trehalose	83.8▽	0.64	80.1▼	0.42	80.7⇧	0.07	79.7🡑	0.00	–	–
50 mM phosphate	76.8▷	0.55	74.3◆	0.20	71.4⇩	0.00	70.1🡓	0.00	70.1🡒	–
50 mM 20% v/v maltose	83.5□	0.56	79.7◀	0.35	77.4⇖	0.00	74.3🡖	0.00	–	–
50 mM 20% v/v trehalose	85.1◇	0.56	81.0▶	0.27	77.6⇗	0.00	76.4🡕	0.00	–	–
100 mM phosphate	76.7▽	0.51	74.1⬢	0.17	71.3⇗	0.00	70.7🡗	0.00	69.9🡕	0.00
200 mM phosphate	–	–	73.9⬟	0.00	–	–	–	–	–	–
500 mM phosphate	–	–	73.9◎	0.00	–	–	–	–	–	–
750 mM phosphate	–	–	74.8✦	0.00	–	–	–	–	–	–
1 M phosphate	–	–	76.1✪	0.00	–	–	–	–	–	–

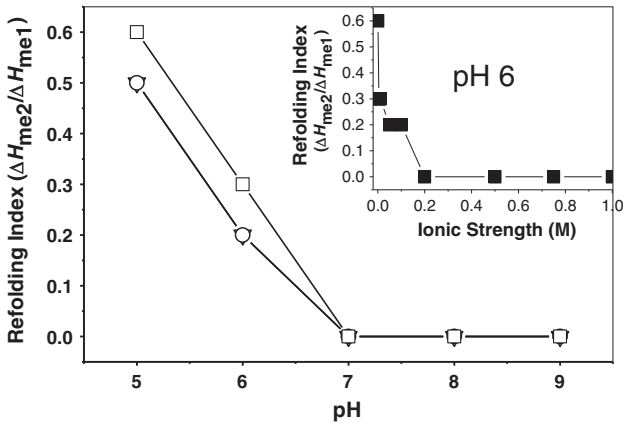

Fig. 4. The pH and ionic strength dependence of the refolding index, $\Delta H_{me2}/\Delta H_{me1}$, after the first DSC heating cycle. □ 10 mM, ○ 50 mM, and △ 100 mM.

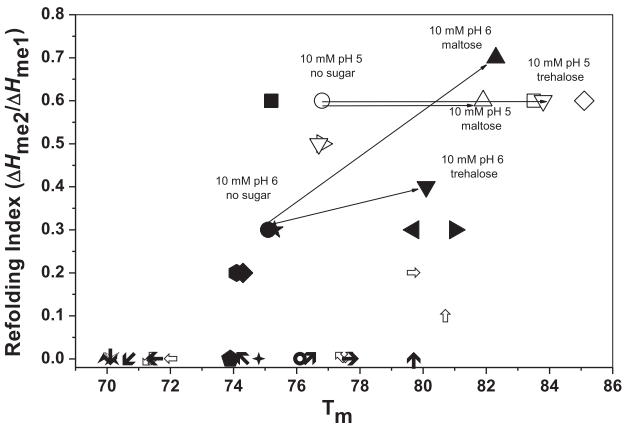

Fig. 6. Comparing the T_m to the refolding index (*RI*) over all solution conditions probed. Solution conditions that result in an increase in T_m do not generally correlate with an increase in refolding following thermal denaturation. For key see Table 2.

intermediate values, but with similar T_m values having different proportions of refolded protein (Fig. 5).

3.5. Addition of sugar

Co-solutes such as disaccharides are known to increase T_m [21]. To assess whether a disaccharide induced increase in T_m had an impact on the reversibility of thermally induced denaturation, measurements were repeated, in the presence of 20% v/v trehalose and maltose (Table 2). Under all solution conditions studied, T_m was increased in the presence of disaccharide. At pH 5 and pH 8, the proportion of refolded protein was not increased. At pH 6, the proportion of refolding was increased for all solution conditions studied. For pH 7, an increase in refolding was only observed at 10 mM. No direct correlation between T_m and *RI* (see Fig. 6) was observed nor was any correlation observed between ΔT_m and ΔRI (data not shown).

4. Discussion

DSC measurements have been used to quantify the degree of reversibility following thermal denaturation. Between the initial heating ramp and each subsequent heating cycle, progressively smaller changes in apparent heat capacity were observed. Because of irreversible aggregate formation the quantity of unfolded protein which is available to refold to the native conformation during the cooling cycles is depleted. Two distinct protein phases were present after heating; visibly aggregated material, which could be separated by centrifugation and soluble protein, which we have shown is monomeric and in a natively folded state. Where no exothermic refolding was detected during the DSC cooling cycle the replicate sample was observed to be gel-like and no natively folded lysozyme was detected in the supernatant following centrifugation. Thus, the diminution or absence of the unfolding endotherm during successive heating cycles was due to the reduced quantity of lysozyme which successfully refolded during the previous cooling cycle [32].

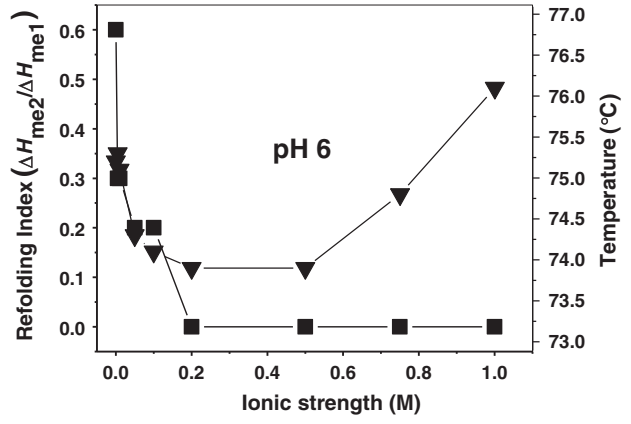

Fig. 5. The ionic strength dependence of the refolding index and melt transition temperature at pH 6. ▲ T_m and ■ refolding index.

During the DSC heating cycle, aggregation is recorded as an exothermic process. It was therefore necessary to consider any impact this competing energy flow might have on the unfolding endotherm. The endothermic, unfolding transition during the heating cycle, was neither followed by, nor appreciably foreshortened by the competing energy flow associated with aggregate formation. Indeed, the area under the peak was integrated to calculate the calorimetric enthalpy, $\Delta H_{cal} = 111$ kcal mol^{-1} (50 mM, pH 6), a value which was in agreement with previously reported values [32,39]. Furthermore, calculation of enthalpy using the van't Hoff method, which relies on the two state model, yields $\Delta H_{VH} = 118$ kcal mol^{-1}, giving $\Delta H_{cal} / \Delta H_{VH} \approx 1$, Fig. S4. Thus, even though ΔH could not be unambiguously calculated, both the ΔH_{cal} and the cooperativity values ($\Delta H_{cal} / \Delta H_{VH}$) indicate that the unfolding signature is not radically altered, under these solution conditions, by aggregate formation.

A scan rate of 1 °C/min is commonly used for lysozyme DSC measurements and slower scan rates do not affect T_m [40,41]; thus kinetic effects which effect the unfolding are not registered in these thermograms. However, some hysteresis ($T_m > T_f$) was observed upon cooling, due to the kinetics of refolding, therefore the *RI* values calculated are based on the second unfolding rather than the first refold, thereby allowing the refolding to reach an equilibrium state. It should be noted that, despite this hysteresis, when the cooling thermogram was correspondingly integrated, there was no significant difference in the *RI* values (data not shown). In addition, since the DSC scans were performed at a constant rate of 1 °C/min, and for solutions with lower T_m values, the length of time for which the protein was unfolded was greater than for those with higher T_m values. It was observed that very similar T_m values had very different *RI* values, thus a kinetic component to the aggregation process was ruled out

The effects of pH, anion type and ionic strength on melt transition temperature have been much studied, particularly for lysozyme [18,33,42]. Thus, by taking advantage of these effects we have methodically generated a set of solution conditions whereby the T_m of the protein varies. To the best of our knowledge, such a comprehensive set of data for lysozyme is unavailable in the literature. Thus, these data may be considered in their own right. The decrease in T_m was not linear (Fig. 3), due to the deprotonation of key stabilizing amino acid residues (His 15 and Glu 35, pKa 5.4 and 6.2, respectively)

between pH 5 and pH 7 [43,44]. In addition to this residue specific effect, varying pH also alters the hydrogen bonding network within the protein's secondary structural units in a non-specific manner, which, in turn, alters thermal stability [45]. Between 10 mM and 100 mM the thermal stability of lysozyme decreases and the values converge at pH 5 and pH 9 regardless of ionic strength. At pH 6 measurements were extended up to 1000 mM. The thermal stability decreased with ionic strength for concentrations ≤ 200 mM, after which T_m begins to increase. Therefore, it is possible to tune T_m by varying ionic strength between pH 5 and pH 9 but beyond these pH values, T_m is not sensitive to sodium phosphate ionic strength at these low salt concentrations.

The reversibility data indicates that no refolding occurs above pH 7, indicating that maximum aggregation occurs beyond this pH. The aggregation behavior of proteins is characterized by the opposing forces of electrostatic repulsion and the attraction of both van der Waal's and hydrophobic effects. As the pH of the solution approaches the isoelectric point, the electrostatic repulsions decrease and the protein–protein attractive forces dominate, leading to the formation of aggregates at pH ≥ 7.

At pH 6, where an extended range of ionic strengths were tested, refolding of thermally denatured lysozyme was greatest in MilliQ water (in the absence of added salt) and was absent for concentrations ≥ 200 mM. Ion specific effects on the aggregation behavior of proteins are more complex and are mediated by both specific and non-specific interactions between the ions and proteins and between the ions and water [46,47]. At low salt concentrations the anions bind to the positive side chains and neutralize the charge, thus reducing repulsion. When the electrostatic binding has reached saturation point, the polarizability of the anion determines its degree of interaction with hydrophobic binding sites [38]. Our reversibility index as shown in Fig. 5 is consistent with these studies. At low ionic strength the phosphate ions initially bind to the positive charges on lysozyme, neutralizing those charges, decreasing repulsions and hence increasing aggregation. Above the saturation point, the phosphate ions are excluded from the hydrophobic binding sites, increasing the interfacial surface tension, which is subsequently minimized by aggregation. The melt transition temperature is expected to decrease initially as the phosphate anions are accumulated at the protein surface relative to the bulk water. This partitioning favors the salting-in process with a concomitant decrease in T_m. As the positive charges on the lysozyme become neutralized, the phosphate anions accumulate preferentially in the bulk. Thus, the salting-out process becomes favored with an accompanying increase in T_m.

Melt transition temperature is widely used as a measure of protein stability [8,13,48]. Depending upon the circumstances, thermal denaturation may occur, deliberately [11,12] or inadvertently [10], during protein purification, processing or handling. Our results show that T_m provides no indication of the reversibility of that thermal denaturation (Fig. 6). There was extensive overlap between the T_m values where non-reversible and reversible thermal denaturation occurred. The results for pH 6 are a clear case in point, where, at the highest and lowest T_m, no refolding was observed whereas refolding was observed for intermediate values, but with similar T_m values having different proportions of refolded protein.

Co-solutes, such as disaccharides are known to increase T_m [21] and are a common excipient in protein formulations [10]. In previous work, we have shown that while saccharides consistently increase T_m, an increase in colloidal stability is only observed when the interaction potential is not dominated by electrostatic repulsion [20]. The presence of 20% v/v trehalose or maltose resulted in an increase in T_m values; however, this increased thermal stability was not always accompanied by an increase in the proportion of refolded lysozyme. No quantifiable relationship between the increase in T_m due to the presence of disaccharide excipients and the extent of refolding could be determined. For example, where melt transition temperature increased by ≈7 °C, the protein refolding increased by 40% for 10 mM pH 6 but no increase in refolding was observed for 10 mM pH 5.

Thus, using DSC, we designed a method to measure the magnitude of reversible unfolding following thermal denaturation and show that where melt transition temperatures are exceeded, deliberately or inadvertently, during protein purification, processing or handling, T_m provides no indication of the potential reversibility of that thermal denaturation.

5. Conclusion

In summary, we have methodically generated a set of solution conditions, by varying pH, ionic strength and the addition of disaccharides, whereby the T_m for lysozyme varies. The proportion of reversible thermal denaturation was then measured and compared with T_m. Where solution conditions resulted in the same thermal transition temperature, large differences in the degree of reversibility were observed. We found that reversibility is both pH and ionic strength dependent with the greatest degree of reversibility shown at pHs furthest from the isoelectric point and at lower ionic strengths. Hence, repulsive electrostatic interactions are important in determining the degree of reversibility. Disaccharide excipients increase the temperature at which unfolding occurs without necessarily providing increased reversibility. These results are consistent with our previous work, where increased colloidal stability for natively folded lysozyme in the presence of disaccharides was limited to solution conditions where the interaction potential was not dominated by electrostatic repulsion [20]. Likewise, disaccharides provide limited colloidal stability to the thermally unfolded lysozyme. We established a method to measure the degree of reversible unfolding following thermal denaturation using DSC. We show that the melt transition temperature does not provide a suitable measure of degree of refolding following thermal denaturation of lysozyme. This type of analysis may prove useful in assessing the stability of proteins in both the biopharmaceutical and food industries. It would be interesting to see if similar effects are observed with other proteins.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2013.06.001.

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