Chapter 8

Determination of Folding Reversibility of Lysozyme Crystals Using Microcalorimetry

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http://dx.doi.org/10.5772/51131

1. Introduction

An important aspect in the preparation of proteins as pharmaceutical products is stabilisation of the native protein conformation (folded, three-dimensional, tertiary state), which is required for biological activity. Moreover, it is not enough for this conformation to be stable, but the protein must be able to find the state or folding pathway in a short time from a denatured, unfolded conformation [1]. Folding minimises exposure of non-polar groups and maximises exposure of polar groups to the solvent [2].

Lysozyme, a globular protein, molecular weight 14,300 Da, was chosen as a model protein; it consists of a single 129 amino acid chain divided into two domains cross linked by four disulfide bridges. The hydrophilic groups tend to concentrate on the surface and the hydrophobic groups in the core [3]. The goal of this study was to investigate the influences of crystallisation on folding reversibility of lysozyme in solution as assessed calorimetrically.

Many literature reports cited the value of High Sensitivity Differential Scanning Calorimetry (HSDSC) for determining thermodynamic parameters (transition temperature, T_m, and enthalpies, ΔH) that describe the folded and unfolded states [4-6]. Furthermore, HSDSC was used to measure thermal transition reversibility that is no less important than T_m and ΔH [7-8]; a protein transition is considered reversible if the molecule renatures upon cooling after heat treatment. Thermodynamic or conformational stability is defined as the difference in free energy (ΔG) between the folded and unfolded state. This stability is the sum of weak non-covalent interactions including hydrogen bonds, van der Waal interactions, salt bridges and hydrophobic forces [9]. Thermodynamic stability is divided into biophysical, which includes the study of thermodynamics, protein denaturation and renaturation (as is discussed in this Chapter) and biochemical, which involves comparative studies of protein conformation and stability of two or more proteins to establish various structural features that deduce stability within a given biomolecule [10].

Differential scanning calorimetry has the ability to provide detailed information about both the physical and energetic properties of a substance [11]. The HSDSC technique was described in detail by Cooper and Johnson (1994) [12]. In summary, all equilibrium processes involving molecules are governed by free energy changes (ΔG), made up of enthalpy (ΔH) and entropy (ΔS). The relationship between (ΔH) and (ΔS) is given by the Gibbs free energy equation:

$$\Delta G = \Delta H - T.\Delta S \quad (1)$$

where T is the temperature. Differential scanning calorimeters measure enthalpies, which provide the basis for determining the thermodynamic properties of a system. Both enthalpy and entropy are related to the heat capacity of the system. The enthalpy is the total energy (at constant pressure) required to heat the system from absolute zero to the required temperature:

$$H = \int_0^T C_p(T).dT \quad (2)$$

where C_p (T) is the temperature-dependent heat capacity at constant pressure. The total entropy of the system can be expressed as:

$$S = \int_0^T [C_p(T)/T].dT \quad (3)$$

Accordingly, differences in H and S can be expressed as:

$$\Delta H = \int_0^T \Delta C_p(T).dT \quad (4)$$

and

$$\Delta S = \int_0^T \Delta[C_p(T)/T].dT \quad (5)$$

Thermodynamic parameters depend on conditions, such as temperature, pressure, concentration and composition. Thus, it is necessary to correct experimental results to standard conditions (standard states) denoted by the superscript 0 (*e.g.* ΔG^0) for simplicity of comparison of data from different situations. The standard state of solutes in dilute solutions is a concentration of 1 M. The standard temperature and pressure are usually 25°C and 1 atm, respectively. The standard free energy change (ΔG^0), which is the free energy change during the reaction where all components are in their standard states, can be measured from:

$$\Delta G^0 = - RT.\ln K \quad (6)$$

where R is the gas constant and K is the equilibrium constant for the process and is related to (ΔH^0) and (ΔS^0) and to the absolute temperature by:

$$\ln K = - (\Delta H^0/RT) + (\Delta S^0/R) \quad (7)$$

assuming that (ΔH^0) and (ΔS^0) do not vary significantly with temperature over the range of interest.

The HSDSC provides an insight into the thermal stability and instability (e.g. formation of soluble and insoluble aggregates) of solutions of different formulations. HSDSC was used to assess the thermal stability of lysozyme solutions after storage at stressed conditions [13]. Consecutive heating scans indicated the folding reversibility of thermal transitions [8, 14] and the validity of calorimetrically measured protein folding reversibility [15-17]. Creighton (1994) [18] reported mechanisms and thermodynamic factors controlling protein folding-and-unfolding.

In the present study, HSDSC investigated thermal changes; in particular protein refolding performance, of crystallised samples (in low and high protein concentrations) upon heat treatment. The thermal structural transition of lysozyme involves two thermodynamic states, native and denatured [19] as for other globular proteins [20]. However, Hirai et al. (1999) [21] indicated that folding-and-unfolding kinetics of proteins depend on the number of amino acid residues. Proteins with residues above ~100 do not follow simple two state kinetics in a folding-and-unfolding process as a single cooperative unit. Accordingly during HSDSC analysis of lysozyme, there might be formation of intermediates between native and denatured states. The thermodynamic stability of proteins not only requires that the transition temperature (T_m) and other thermodynamic parameters remain constant but also implies reversibility of protein from unfolded (denatured) to folded (native) state after removing the effect of an external condition such as heat. Anfinsen (1973) [22] reported that denaturation of Ribonuclease A, by heat or urea, was reversible when denatured molecules returned to a normal environment of temperature and solvent. Hence, both structure and enzymatic activity were regained.

Consequently, formation of the native state is a global property of the protein as described [1]. This state is necessary for stability and activity; proteins are marginally stable and achieve stability only within narrow ranges of conditions of solvent and temperature. The free energy of stabilization of proteins under ordinary conditions is ~ 5-15 kcal mol^{-1} [1].

Proteins undergo various structural changes if physiological conditions alter. Accordingly, they may denature and the denatured protein tends to adsorb to surfaces and aggregate with other protein molecules. Katakam et al. (1995) [23] proposed that denaturation of recombinant human growth hormone involves unfolding of the molecule; the unfolded part adsorbs to surfaces and aggregates with neighbouring molecules. Shaking and exposure to an air/water interface, heating, lyophilisation or reconstitution of lyophilised protein may aggregate protein with subsequent loss of stability and activity.

The combination of HSDSC and enzymatic activity determined if refolding of denatured crystallised lysozyme after thermal denaturation in HSDSC arises from the nativeness, three-dimensional folded state, of the initial lysozyme structure. This means that enzymatic activity was employed to investigate if folding reversibility of the thermal transition reflects the renaturation of the unfolded protein to folded native structure.

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme (purity 95%, 5% sodium chloride and sodium acetate), sodium chloride (99.5%), sodium phosphate (99.3%) and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Company (St. Louis, Mo). Sodium acetate anhydrous (98%), potassium dihydrogen orthophosphate (> 99%) were obtained from BDH Chemicals Ltd. Poole, UK. Water was deionised, double distilled.

2.2. Preparation of crystallized lysozyme

Lysozyme was crystallised using a published method [24]. Crystals formed were filtered, dried and kept in a freezer (-15°C) until tested.

2.3. High Sensitivity Differential Scanning Calorimetry (HSDSC)

Solution samples of crystallised lysozyme were analysed with a Microcal MCS differential scanning calorimeter (Microcal Inc., MA, USA). Degassed samples (5 and 20 mg product / 1 mL 0.1M sodium acetate buffer, pH 4.6) and reference (0.1M sodium acetate buffer, pH 4.6) were loaded into cells using a gas tight Hamilton 2.5 mL glass syringe. The folding reversibility of lysozyme denaturation was assessed by temperature cycling using two scan calorimetric methods. The upscan-upscan method (UU) employed two consecutive upscans from 20-90°C at 1°C/min. After the first upscan, the sample was immediately cooled in the calorimeter (downscan) to 20°C at 0.75°C /min (the fastest cooling rate allowed by the instrument) and the heating cycle was immediately repeated. Transition reversibility was measured as ratio (%) of enthalpy change of second upscan (ΔH_2) over that of first upscan (ΔH_1). The upscan-downscan method (UD) involved heating of protein solution from 20-90°C at 1°C/min immediately followed by downscan (cooling) from 90-20°C at a cooling rate of 0.75°C/min. Enthalpies were measured and downscan (ΔH_3) / upscan (ΔH_1) enthalpy ratios were calculated as a measure of folding reversibility. The calorimeter was temperature- and heat capacity-calibrated using sealed hydrocarbon standards of known melting points and electrical pulses of known power, respectively.

Experiments were performed under 2 bar nitrogen pressure. A base line was run before each measurement by loading the reference in both the sample and reference cells; this base line was subtracted from the protein thermal data and the excess heat capacity was normalized for lysozyme concentration. Data analysis and deconvolution employed ORIGIN DSC data analysis software. The T_m (mid point of the transition peak) values for all transitions were calculated.

2.4. Enzymatic assay

Biological activities of thermally denatured crystallised lysozyme were determined after cooling (in HSDSC) to determine whether the renaturation is due to the nativeness of the

protein structure i.e. to correlate the folding reversibility with biological activity. In this assay, a bacterial suspension was prepared by adding 20 mg of *Micrococcus lysodeikticus* to 90 mL of phosphate buffer 0.067 M, pH 6.6, and 10 mL of 1% NaCl. The biological reaction was initiated by addition of 0.5 mL of each enzyme solution to 5 mL of the bacterial suspension. The activity unit of lysozyme is defined as the amount of enzyme decreasing the absorption rate at 450 nm at 0.001 /min at 25°C and pH 6.6. Rates were monitored using a UV/Vis. spectrophotometer (Pu 8700, Philips, UK) at 25°C.

All data were presented as mean of three determinations ± standard deviation. The Student's *t*-test assessed significance.

3. Results and discussion

3.1. High Sensitivity Differential Scanning Calorimetry (HSDSC)

Differential scanning microcalorimetry experiments can thermodynamically characterise the unfolding transition by determing heat capacities, enthalpies and melting temperatures of native and denatured protein [25]. HSDSC monitored thermal stability and folding reversibility of reconstituted lysozyme preparations. For samples, traces for thermal denaturation and folding reversibilities, using (UU) method, of reconstituted crystallised lysozyme are illustrated in Figure 1(a) and (b) for 5mg/mL and 20mg/mL protein concentrations, respectively. Thermodynamic parameters and enzymatic activities are in Table 1. Figure 2 shows an example for folding reversibility of unprocessed lysozyme using (UD) method. As is evident in Figures 1 and 2, HSDSC profiles of all samples showed a single endothermic peak (first upscan). Lysozyme crystals started to unfold at ~65°C with a mean T_m of 76.1°C (T_{m1}).

It is noticeable that rescan profiles, whether endothermic (second heating cycle, Figure 1a and b dotted lines) or exothermic (downscan upon cooling, Figure 2 lower trace) showed two peaks, a main one and a small peak or shoulder. Deconvolution of the data (using ORIGIN DSC data analysis software) revealed two transition regions characterised by T_m at ~76.1°C (T_{m2}) for the main peak and at ~66°C for the shoulder, indicating that the lysozyme transition is not a two state transition. This may be explained on the basis that lysozyme consists of a single polypeptide chain divided into two structural domains (α-helix and β-sheet). During refolding each domain may be refolded separately with different pathways. Consequently, two peaks appear instead of one; this explanation agrees with Remmele et al. (1998) [14] who attributed the three T_m peaks to the three domains of immunoglobulin-type domains that make up the Interleukin-1 receptor.

The other reasonable explanation is that lysozyme, when its folding process is analysed using circular dichroism, does not obey a single co-operative transition, but the process involves several parallel folding pathways. Each of the two domains stabilises with different kinetics [26]. In particular, the amides in the α-helix are involved in the formation of stable helical structure and assembly of the hydrophobic core. Then a stable hydrogen bonded structure in the β- domain forms. Accordingly, partially structured intermediates develop

during the folding of lysozyme. This explanation is supported by Buck et al. (1993) [27] who reported that lysozyme consists of two structural domains that are stabilised by different pathways.

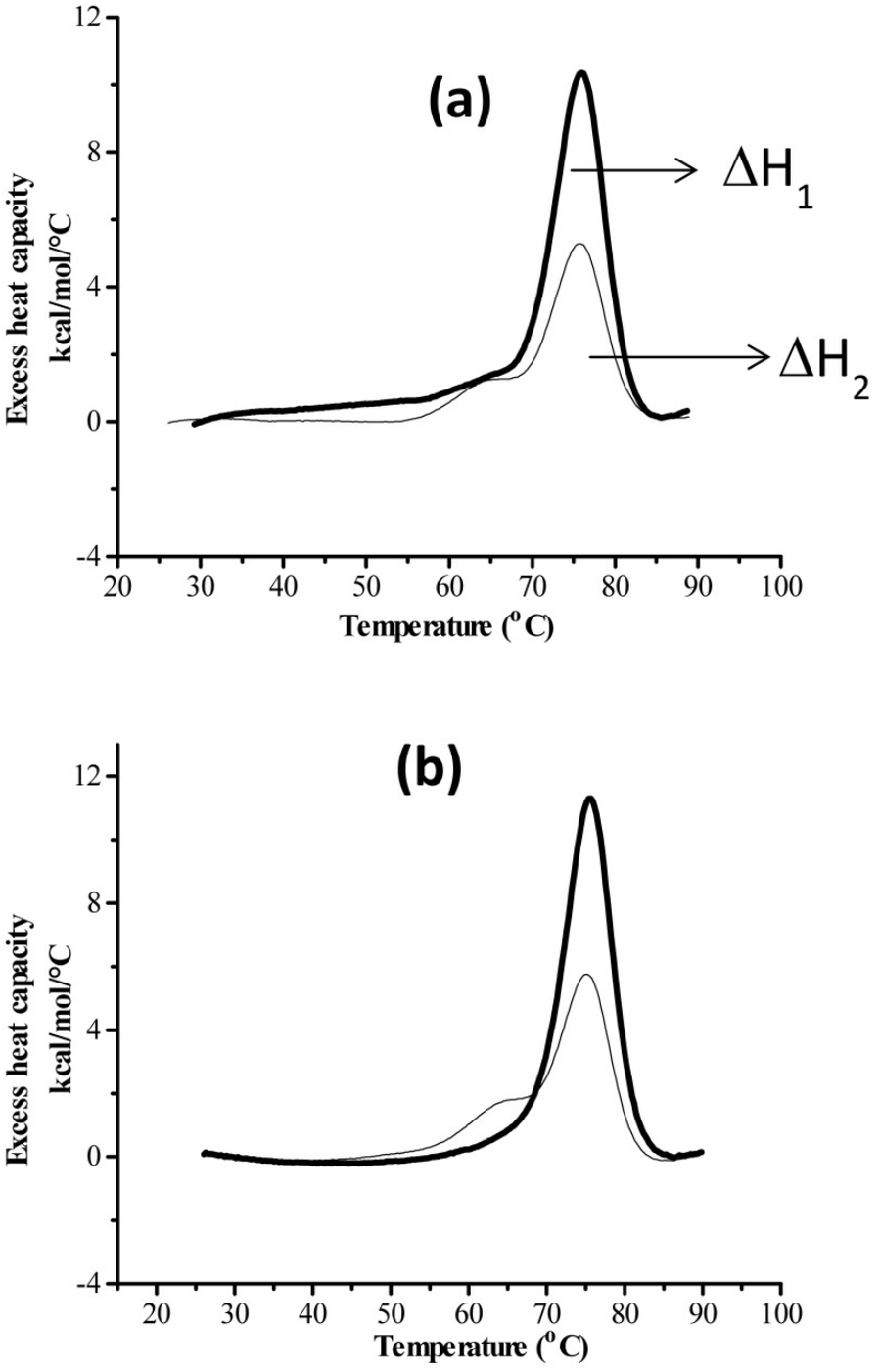

Figure 1. Normalised consecutive calorimetric upscans, first upscan (solid line) and second upscan (dotted line) of crystallised lysozyme. Conditions: (a) 5mg/mL protein, 0.1 M sodium acetate buffer, pH 4.6, heating rate 1°C/min and (b) 20mg/mL protein, 0.1 M sodium acetate buffer, pH 4.6, heating rate 1°C/min.

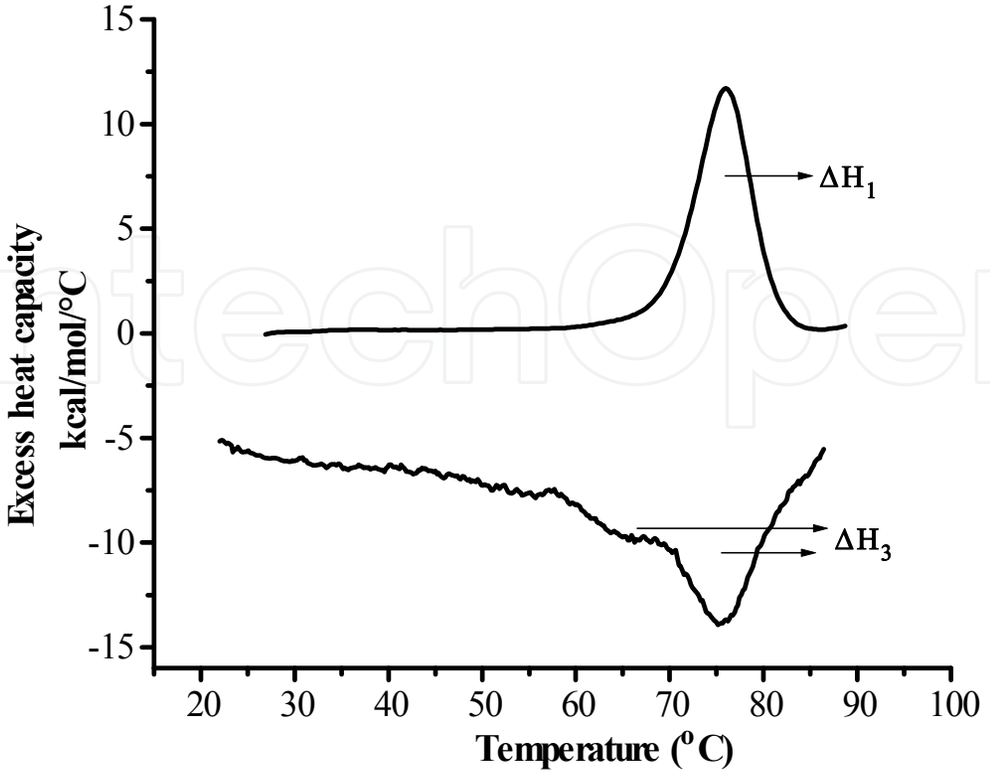

Figure 2. Normalised calorimetric upscan (upper trace) and downscan (lower trace) of lysozyme, as received. Conditions: 5mg/mL protein, 0.1 M sodium acetate buffer, pH 4.6, heating rate 1°C/min, cooling rate 0.75°C/min.

Lysozyme Sample	T_{m1} (°C)	T_{m2} (°C)	% folding reversibility ($\Delta H_2/\Delta H_1$)	% enzymatic activity
Crystallised	76.1±0.21	75.9±0.15	66.5±1.4	65.7±1.4
5 mg mL^{-1}	75.6±0.07	75.2±0.02	52.4±1.6	52.7±2.2
20 mg mL^{-1}				

[a] T_{m1}, T_{m2} are mid-point peak transition temperatures of first and second upscans; ΔH_1, ΔH_2 are calorimetric enthalpies of transitions of first and second upscans; % enzymatic activity is the activity of each sample relative to fresh material of that sample, ±S.D., n= 3.

Table 1. Thermodynamic parameters for the thermal denaturation, folding reversibilities using consecutive upscan method (UU) and enzymatic activities of crystallised lysozyme samples[a].

Lysozyme Sample	% folding reversibility ($\Delta H_3/\Delta H_1$)
Crystallised	
5 mg mL^{-1}	43.6 (1.6)
20 mg mL^{-1}	48.6 (3.8)

[a] Values between brackets are S.D., n= 3.

Table 2. Folding reversibilities using upscan-downscan (UD) method of crystallised lysozyme samples [a].

A comparison of calorimetric enthalpy (ΔH_{cal}) and the theoretical enthalpy (ΔH_{VH}, van't Hoff enthalpy) changes, to judge the validity of a two-state mechanism for the unfolding of lysozyme, reveals the presence of intermediates [25]. It was reported that, in the unfolded state, proteins aggregate and react chemically with amino acid residues exposed to the solvent; this can lead to misfolding or irreversible denaturation [28]. Also, the formation of any irreversible component alters the shape of a HSDSC thermodynamic peak over the temperature range at which it forms [29].

For low protein concentration (5mg/mL, Figure 1a), Table 1 shows no significant difference between T_{m1} (transition temperature of first upscan) and T_{m2} (transition temperature of second upscan) for protein samples.

It was reported that determination of the mechanism and pathway of unfolding and refolding depends on the identification of the intermediates that may not be stable at the equilibrium [18]. Thus, detection and characterisation of kinetic folding intermediates is complex. This intricacy can arise from accumulation of intermediates or from subpopulations of the unfolded state refolding at different rates. Also, events in folding are obscure [1]. With respect to samples with high protein concentration (20mg/mL, Figure 1b), Table 1 demonstrates that T_{m2} decreased compared to its corresponding T_{m1} .

On comparing low and high protein concentrations, thermal stabilities (T_{m1} and T_{m2}) of lysozyme crystals at high concentration significantly decreased ($p < 0.05$). Accordingly, high protein concentration influences thermal stability, this is in agreement with previously published data for dried proteins [17]. Moreover, folding reversibilities and enthalpies of first upscan of all samples (Figure 1 and Table 1) decreased with increasing concentration. Enthalpy values correlated with the content of ordered secondary structure of protein [30]. The decrease in enthalpy of protein may be attributed to denaturation at high protein concentration because a partially unfolded protein needs less heat energy to denature than a native form [17]. In general, crystals are chemically and physically pure substances (atoms or molecules within crystals are arranged in highly ordered patterns in three dimensional structures); the other possible reason for the observed reversibility of lysozyme at high protein concentration is that the water in lysozyme crystal lattices inhibits protein-protein interactions and aggregation, to some extent, which may take place at high lysozyme concentration after denaturation by heat in the HSDSC. Consequently, the crystallisation maintains the three-dimensional folded structure of lysozyme and enhances the renaturation of the protein. Water molecules play an important role in the function of proteins through maintaining their tertiary structure. The structure of biological macromolecules in an aqueous solution is similar to that in a crystalline state [31]. There are two kinds of hydration shell of biomolecules in aqueous solution; the primary and secondary hydration shells. Water molecules in the primary hydration shell are directly bound to molecules. The water molecules in the secondary hydration shell have a character intermediate between those of the primary hydration shell and bulk water. On the other hand, crystal water is classified into groups that correspond to the hydration shells in solutions. These water molecules correspond mainly to those in the primary hydration shell and partly to those in the secondary hydration shell [31]. Consequently, there is strong

interaction between water and protein molecules in crystalline states. Takano et al. (1999) [6] used differential scanning calorimetry to examine the contribution of hydrogen bonds to the conformational stability of mutant human lysozyme. The authors commented that hydrogen bonding between human lysozyme atoms and water bound with the protein molecules in crystals contributes to the protein conformational stability. The net contribution of one intramolecular hydrogen bond to protein stability in terms of Gibbs energy was ~8.9 kJ/mol. On the basis of Takano et al. (1999) [6] study, hydrogen bonds are one of the important factors stabilising the folded conformations of proteins. From these results crystals were capable of refolding and hence lysozyme crystals not only maintained thermal stability and conformational integrity as suggested previously [24], but also improved refolding ability, which is necessary in protein formulation and processing. Refer to a study by Elkordy et al. [17] for folding reversibility of lyophilised and spray dried lysozyme. Also, a study by Forbes et al. [16] reported the folding reversibility of spray dried and crystallised trypsin.

For folding reversibility calculated by (UD) method, Table 2 above summarises the results of folding reversibilities of crystallised lysozyme at low and high protein concentrations. From Table 2, it is apparent that the percentage folding reversibility calculated by (UU) method (Table 1) was significantly higher ($p < 0.05$) than that derived from the (UD) method (Table 2). This implies that the latter method underestimates the apparent folding reversibility of samples.

3.2. Enzymatic assay

Lysozyme solutions upon cooling in the HSDSC after thermal denaturation were assayed for biological activity towards *Micrococcus lysodeikticus.* Based on the HSDSC results, all samples renatured to some extent after thermal stress. Thus, enzymatic assay should answer an important question. Is this renaturation or folding reversibility related to regain of the native structure of lysozyme (which is essential for biological activity), or does it result from misfolding, i.e. folding of the protein in a manner different from the original natured structure which subsequently leads to loss of activity?

Table 1 presents percentage enzymatic activities of preheated solutions, in HSDSC, of crystallised lysozyme (relative to an aqueous solution of a fresh sample). It is evident that the biological activity of lysozyme was maintained by crystals (5mg/mL and 20mg/mL). The results were consistent with data of folding reversibilities. This answers the question posed previously in that folding reversibility was related to the native structure of lysozyme that is required for its activity, as the greater the folding reversibility, the higher the enzymatic activity. The results illustrated that lysozyme crystals maintained structural integrity even after heating in the HSDSC. A review by Jen and Merkle (2001) [32] showed that hydrated protein within crystals is present in a folded, native form.

From the HSDSC and enzymatic activity results, the folding reversibility, calculated by consecutive upscans (UU, Tables 1), correlated with enzymatic activity of lysozyme, confirming that the upscan-downscan method (UD, Table 2) underestimates the magnitude of folding reversibility. However, proteins are diverse molecules and the presence of

correlation between folding reversibility and biological activity of lysozyme, as demonstrated in this study, may not be applicable to other proteins.

4. Conclusions

The overall results suggested that reconstituted lysozyme crystals were able to refold after heating. The folding reversibility arises from the nativeness of the initial lysozyme structure as demonstrated by biological activity data. The results indicated that the upscan-downscan method underestimated the extent of folding reversibility. Consequently, it is preferable to calculate this reversibility, employing high sensitivity differential scanning calorimetry, by the consecutive heating upscan method.

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5. References

[1] Lesk A M 2001. In vivo, in vitro, in silicio. In: Lesk, A.M. (Ed.), Introduction to protein architecture. Oxford University Press, Oxford, New York, pp.: 15-35, 143.
[2] Rupley J A, Gratton E, Careri G 1983. Water and globular proteins. Trends in Biochem Sci. 8: 18-22.
[3] Rosenberger F 1996. Protein crystallization. J Cryst Growth. 166: 40-54.
[4] Pikal M J, Lukes A L, Lang J E, Gaines K 1978. Quantitative crystallinity determination for b-lactam antibiotics by solution calorimetry: correlations with stability. J Pharm Sci. 67: 767-772.
[5] Freire, E. (1995). Thermal denaturation methods in the study of protein folding. In *Methods in Enzymology*, Vol. 259 (Johnson, M. L. and Ackers, G. K., Eds.) Academic Press, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto, pp. 144-168.
[6] Takano K, Yamagata Y, Kubota M, Funahashi J, Fujii S, Yutani K 1999. Contribution of hydrogen bonds to the conformational stability of human lysozyme: Calorimetry and x-ray analysis of six ser →ala mutants. Biochem. 38: 6623-6629.
[7] Maneri L R, Farid A R, Smialkowski P J, Seaman M B, Baldoni J M, Sokoloski T D 1991. Preformulation of proteins using high sensitivity differential scanning calorimetry (DSC). Pharm Res. 8: S-48.
[8] Boye J I, Alli I, Ismail A A 1997. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of α-lactalbumin. J Agric Food Chem. 45: 1116-1125.

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[9] Daniel RM 1996. The upper limits of enzyme thermal stability. Enzyme Microb. Technol. 19: 74-79.
[10] Quinn ÉÁ 2000. The stability of proteins in hydrofluoroalkane propellants. Ph. D. Thesis, University of Bradford, UK.
[11] Clas S.-D, Dalton CR, Hancock BC 1999. Differential scanning calorimetry: applications in drug development. PSTT 2: 311-320.
[12] Cooper A, Johnson CM (1994). Introduction to microcalorimetry and biomolecular energetics. In Methods in Molecular Biology: Microscopy, Optical Spectroscopy, and Macroscopic Techniques, Vol. 22 (Jones, C., Mulloy, B. and Thomas, A. H., Eds.) Humana Press Inc., Totowa, NJ, pp. 109-124.
[13] Elkordy A A, Forbes R T, Barry B W 2004. Stability of crystallised and spray-dried lysozyme. Int J Pharm. 278: 209-219.
[14] Remmele R L, Nightlinger N S, Srinivasan S, Gombotz W R 1998. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. Pharm Res. 15: 200-208.
[15] Branchu S, Forbes R T, Nyqvist H, York P 1998. The relationship between the folding reversibility and enzymatic activity of trypsin. J Pharm Sci. 1 (suppl.): 541.
[16] Forbes R T, Barry B W, Elkordy AA 2007. Preparation and characterisation of spray-dried and crystallised trypsin: FT-Raman study to detect protein denaturation after thermal stress. European J Pharm Sci. 30: 315-323.
[17] Elkordy A A, Forbes R T, Barry B W 2008. Study of protein conformational stability and integrity using calorimetry and FT-Raman spectroscopy correlated with enzymatic activity. European J Pharm Sci. 33: 177-190.
[18] Creighton T E 1994. The protein folding problem. In: Pain, R.H. (Ed.),. Mechanisms of protein folding. Oxford University Press, Oxford, New York, Tokyo, pp. 1-25.
[19] Schmid F X 1992. Kinetics of unfolding and refolding of single-domain proteins. In: Creighton, T.E. (Ed.), Protein folding. Freeman, W.H. and Company, New York, pp. 197-241.
[20] Catanzano F, Giancola C, Graziano G, Barone G 1996. Temperature-induced denaturation of ribonuclease S: A thermodynamic study. Biochem. 35: 13378-13385.
[21] Hirai M, Arai S, Iwase H 1999. Complementary analysis of thermal transition multiplicity of hen egg-white lysozyme at low pH using X-ray scattering and scanning calorimetry. J Phys Chem B. 103: 549-556.
[22] Anfinsen C B 1973. Principles that govern the folding of protein chains. Sci. 181: 223-230.
[23] Katakam M, Bell L N, Banaga A K 1995. Effect of surfactants on the physical stability of recombinant human growth hormone. J Pharm Sci. 84: 713-716.
[24] Elkordy A A, Forbes R T, Barry B W 2002. Integrity of crystalline lysozyme exceeds that of a spray dried form. Int J Pharm. 247: 79-90.
[25] Matouschek A, Serrano L, Fersht A R 1994. Analysis of protein folding by protein engineering. In: Pain, R.H. (Ed.), Mechanisms of protein folding. Oxford University Press, Oxford, New York, Tokyo, pp. 137-159.

[26] Radford S E, Dobson C M, Evans P A 1992. The folding of hen lysozyme involves partially structured intermediates and multiple pathways. Nature. 358: 302-307.
[27] Buck M, Radford S E, Dobson C M 1993. A partially folded state of hen egg white lysozyme in trifluoroethanol: Structural characterization and implications for protein folding. Biochem. 32: 669-678.
[28] Creighton T E 1993. Proteins in solution and in membranes. In: Creighton, T. E. (Ed.),. Proteins: Structures and molecular properties, 2nd Ed., Freeman, W.H. and Company, New York, pp. 287-323.
[29] Lepock J R, Ritchie K P, Kolios M C, Rodahl A M, Heinz K A, Kruuv J 1992. Influence of transition rates and scan rate on kinetic simulations of differential scanning calorimetry profiles of reversible and irreversible protein denaturation. Biochem. 31: 12706-12712.
[30] Koshiyama I, Hamano M, Fukushima D 1981. A heat denaturation study of the 11 S globulin in soybean seeds. Food Chem. 6: 309-322.
[31] Urabe H, Sugawara Y, Ataka M, Rupprecht A 1998. Low-frequency Raman spectra of lysozyme crystals and oriented DNA films: dynamics of crystal water. Biophys J. 74: 1533-1540.
[32] Jen A, Merkle H P 2001. Diamonds in the rough: Protein crystals from a formulation perspective. Pharm Res. 18: 1483-1488.