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The suitability of scanning calorimetry to investigate slow irreversible protein denaturation

M.A.M. Hoffmann[a], J.C. van Miltenburg[b], P.J.J.M. van Mil[a,*]

[a] *Netherlands Institute for Dairy Research (NIZO), P.O. Box 20, 6710 BA Ede, The Netherlands*

[b] *Debye Institute, Department of Interfaces and Thermodynamics, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*

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Abstract

The denaturation and aggregation of bovine β-lactoglobulin (50 g/l) at neutral pH was investigated by scanning calorimetry. To cover a wide range of scan rates (2–1200°C/h), we used three different calorimeters: two scanning calorimeters (PE DSC7 and Hart Scientific DSC 4207) and an adiabatic calorimeter. The temperature corresponding to maximum heat capacity, T_p, varied with scan rate. Especially with scan rates below 90°C/h, T_p became strongly dependent on scan-rate, indicating that the reactions involved in the denaturation/aggregation of β-lactoglobulin are strongly kinetically controlled. With DSC, only one peak was seen at all scan rates, but with decreasing scan rate the peak became more asymmetrical. With the low scan rate of the adiabatic experiment (≈2°C/h) a shoulder at ca. 60°C was observed, clearly indicating that more than one process is occurring. Furthermore, the adiabatic experiment showed that the denaturation/aggregation of β-lactoglobulin may occur within an infinitely small temperature interval, due to very slow irreversible processes. © 1997 Elsevier Science B.V.

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1. Introduction

β-Lactoglobulin (β-lg) is the most abundant globular protein of milk and the major protein component of whey, a by-product of cheese manufacturing. Because of their various functional properties (i.e. nutritive value, and their gelling, water-retention, emulsification, foaming and adhesion properties) whey proteins are used in a wide range of food applications [1,2]. The effect of heat treatment on these functional properties is essential to a variety of applications of these proteins [3]. Greater knowledge and control of the effect of heat on the whey proteins would stimulate further utilisation of these high-quality food proteins.

The thermal behaviour of whey proteins is dominated by β-lg and, therefore, this protein has been extensively studied [3]. A very commonly used method in studies of the thermal denaturation of β-lg is differential scanning calorimetry (DSC) [4–10]. Thermodynamic analysis of DSC curves representing protein unfolding relies on the assumption that (chemical) equilibrium exists throughout the temperature-induced unfolding process [11,12]. Denaturation of β-lg is followed by aggregation of the denatured molecules at neutral pH and the overall reaction becomes totally or partially irreversible, the degree

*Corresponding author. Tel.: +31 318 659585; fax: +31 318 650400; e-mail: pvanmil@nizo.nl

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of irreversibility depending on experimental conditions such as scan rate and protein concentration [8,13]. Especially in the case of high levels of irreversibility, the DSC curves cannot be interpreted in terms of equilibrium thermodynamics; only kinetic data can be obtained [14–16]. To obtain accurate kinetic data, it is necessary to split up the DSC signal into the separate contributions of the denaturation and aggregation process [17]. However, denaturation is an endothermic process, whereas aggregation can be exothermic; nevertheless, the two processes may give rise to only one peak in the thermogram which is, thus, difficult to analyse. With the high scan rates used in most DSC studies (rates above ≈60°C/h), only one peak is seen. By using lower scan rates the processes may become separated in time, leading to more accurate and reliable kinetic parameters.

To cover a wide range of scan rates, we used two different calorimeters: a Perkin–Elmer DSC 7, which is convenient for high scan rates because of low thermal inertia, and a Hart Scientific DSC 4207, which is preferable with lower scan rates, due to the high stability, higher sensitivity (0.2 vs. 1 μW) and the larger size of its calorimetric ampoules (1000 vs. 60 μl). The PE DSC 7 is of the heat compensation type, whereas the Hart Scientific DSC 4207 is of the heat conduction type. Furthermore, an experiment was made with an adiabatic calorimeter, which enabled us to heat the β-lg solution at a rate as low as 2°C/h.

2. Experimental

2.1. Materials

The β-lg used in the experiments was a purified β-lg sample, containing the genetic variants A and B (in a nearly 1 : 1 ratio), which was prepared at NIZO from whey, basically following the procedure of Maubois et al. [18]. The sample contained ca. 92% β-lg, 2% α-lactalbumin, 2% non-protein nitrogen material and 2.1% ash (including 0.73% Na^+, 0.02% K^+, 0.12% Ca^{2+}, 0.008% Mg^{2+}) on a dry mass basis. It contained 4% moisture [19].

The β-lg powder was dissolved in double-distilled water in a concentration of 50 g dry matter/l (pH 6.85) and stirred for 2 h at room temperature. After preparation, the solution was filtered (0.1 μm Millipore low-protein-binding filter) and directly used in an experiment.

2.2. PE DSC7

Large-volume stainless steel capsules containing 50 μl of protein solution were heated (25–120°C) using scan rates in the 60–1200°C/h range. As reference, the same amount of previously heat denatured β-lg solution was used. The calorimeter was calibrated at each scan rate using pure indium to correct for temperature lag. The peak temperature T_p (i.e. the temperature corresponding to maximum excess heat capacity (C_p^{ex} in J $°C^{-1}$ $g_{protein}^{-1}$)) was determined from three replicate runs and varied by not more than 0.5°C.

2.3. Hart Scientific DSC 4207

This DSC has four measuring cells; one is used for a reference and the other three for samples. Thereafter, 60 or 700 μl β-lg solution was put into the stainless steel ampoules and the reference contained the same amount of double-distilled water. The temperature was scanned from 25 to 100°C, using scan rates in the 5–90°C/h range. Baselines were recorded at each scan rate by filling the sample ampoules with 60 or 700 μl double-distilled water. Afterwards, these baselines were subtracted from the β-lg thermograms. T_p values were determined from three replicate runs and varied by not more than 0.3°C.

The Hart Scientific DSC software was used for data collection and analysis. This software provides an automatic correction for fluctuations in scan rate and for differences between the monitored temperature and the temperature in an (almost) empty sample vessel. This thermal lag correction will suffice only with small sample volumes. With larger volumes (which are needed for more sensitive measurements) an additional correction has to be made for the temperature lag in the sample itself. To correct for the effect of sample volume on the measured temperatures we applied the following procedure. About 1 mg of pure naphthalene was placed in glass capillaries of ca. 1 cm in length which were closed at both ends. The capillaries were introduced into a sample ampoule containing 60 or 700 μl of double-distilled water and the melting point at the different scan rates was determined. With a sample volume of 60 μl, the

melting temperature was within 0.5°C of the literature value and was found to be independent of scan rate. With 700 µl, the melting temperature measured at the lowest scan rate was identical to the value obtained with 60 µl whereas for higher scan rates the melting temperature increased linearly with scan rate (slope 0.011 h). These results were used to correct the DSC thermograms obtained with the β-lg solutions. After applying this thermal lag correction procedure to the excess heat capacity curves of 50 g/l β-lg, T_p became independent of sample volume at all scan rates.

2.4. Adiabatic calorimeter

The adiabatic calorimeter we used has been described previously [20]. The internal precision is ca. 0.02% and the absolute accuracy is estimated to be 0.2%.

The sample (50 g/l β-lg solution) weighing 8.02025 g was enclosed in a gold-plated vessel. This vessel is surrounded by shields which are controlled at the temperature of the vessel. High vacuum ensures further isolation. Measurements (in the 20–95°C range) were made using the intermittent method: energy was supplied to the sample for 900 s (input period), then the temperature was followed for 1500 s (stabilisation period). After each stabilisation period the following were recorded:

1. the temperature in the middle of the stabilising period;
2. the slope of the second half of the temperature-against-time function of the stabilising period (the "drift"); and
3. the total electrical energy (Q, in J) supplied to the calorimeter during the input period.

The temperatures in the stabilising periods were extrapolated forwards and backwards to the middle of the input period, resulting in a temperature difference ΔT. The heat capacity of the sample + sample container ($C_{p,tot}$) was calculated as: $C_{p,tot} = Q/\Delta T$. The heat capacity of the β-lg solution was obtained by subtracting the value of the empty container. In the temperature range where denaturation occurred (55–75°C) the above-mentioned calculation method could not be used as the "drifts" observed were caused by the reaction. In this region the heat capacities were calculated using a linear extrapolation of the drifts measured in the stabilising periods before and after the denaturation process.

3. Results and discussion

3.1. Typical DSC thermograms

Fig. 1 shows typical thermograms obtained with the Hart Scientific DSC (corrected for thermal lag) at various scan rates and the thermogram obtained with the adiabatic calorimeter. As is evident in this figure, the temperature corresponding to maximum heat capacity, T_p, and the shape of the excess heat capacity curve are highly dependent on scan rate. With decreasing scan rate, T_p decreases and the curve becomes more asymmetrical. With the very low scan rate of the adiabatic calorimeter the peak becomes even more asymmetrical and a shoulder at 60°C can be seen. These results clearly demonstrate that more than one process must be taking place and that, by lowering the scan rate, these processes can be, at least partially, separated in time. With the adiabatic experiments we used relatively long stabilisation periods, but even within this time (1500 s) no equilibrium was reached. At temperatures above ≈60°C, the temperature in this stabilising period was found to decrease almost

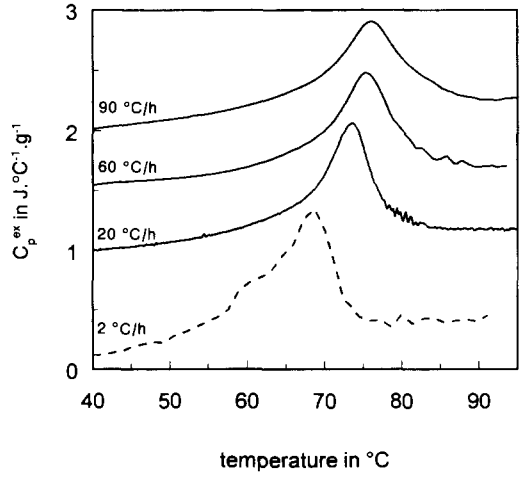

Fig. 1. Thermograms of 50 g/l β-lg obtained with the Hart Scientific DSC 4207 (——— sample volume 700 µl) at three different scan rates and with the adiabatic calorimeter (- - - at a scan rate of ≈2°C/h). The thermograms obtained with the Hart Scientific DSC 4207 have been corrected for thermal lag.

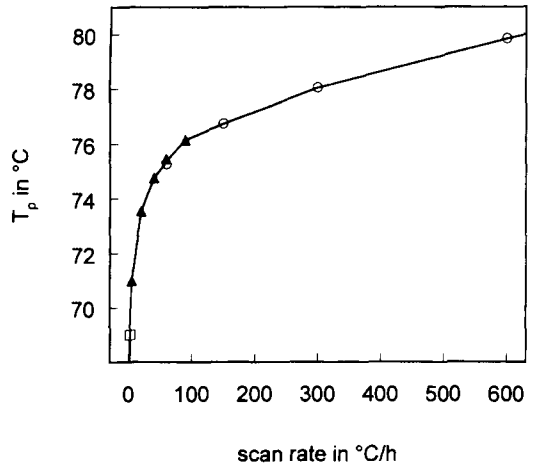

Fig. 2. T_p values determined with the three different calorimeters for 50 g/l β-lg as a function of scan rate: (○) – Perkin–Elmer DSC7; (▲) – Hart Scientific DSC 4207; and (□) – the adiabatic calorimeter. With the Perkin–Elmer DSC 7 and the Hart Scientific DSC 4207, T_p was determined from three replicate runs. For further experimental details see text.

linearly with increasing time. As we are supplying no energy to the vessel in the stabilisation period and the shield regulation system is working, the total energy of the vessel and its contents is constant. In the sample, energy is consumed by the occurrence of the endothermic denaturation/aggregation processes, so the temperature has to decrease. The almost constant drift in the stabilising period indicates that the denaturation/aggregation process remains far from reaching equilibrium. These results show that with β-lg the denaturation/aggregation process may occur within a small temperature interval.

3.2. Dependence of T_p on scan rate

Fig. 2 shows T_p values determined with the three different calorimeters. The excellent agreement of results obtained with the PE DSC7 and the Hart Scientific DSC in the overlapping range of scan rates corroborates the internal reliability of the T_p values. The values obtained with the PE DSC7 increased approximately linearly with scan rate, with a slope which was independent of protein concentration (results not shown). The values obtained are comparable to those found in other investigations with this calorimeter [8,10]. More surprising is the much higher scan-rate dependence observed with the low scan rates of the Hart Scientific DSC and the adiabatic calorimeter. Qi et al. [10] also reported that T_p became strongly scan-rate-dependent for scan rates ≤ 90°C/h, but they ascribed this to the low protein concentration used (8 g/l β-lg). Our results show that in this range of scan rates T_p becomes strongly dependent on scan rate with higher β-lg concentrations also. Here, it has to be remarked that the time-response of the calorimeter may also produce a scan-rate dependence of the traces, especially when one is dealing with sharp transitions. However, with the slow transitions of β-lg, no correction for the finite-time response of the calorimeter has to be made and the observed scan-rate dependence is due to kinetic factors.

Frequently, a denaturation temperature (T_d) is determined by extrapolation of T_p to zero scan rate [4,5,9]. But in these studies, only scan rates above ≈60°C/h were used, resulting in T_d values in the 70–73°C range. As a matter of principle, the concept of T_d ceases to exist in the case of irreversible denaturation. Furthermore, our results show that it will be virtually impossible to determine T_d.

4. Conclusions

The processes involved in the denaturation and aggregation of β-lg take place on a much larger time-scale than a DSC experiment, even when very low scan rates (≤ 5°C/h) are used. The information is smeared out over the temperature trajectory and, therefore, we think that it will be virtually impossible to derive correct kinetic parameters from DSC thermograms. However, due to the occurrence of very slow irreversible processes the protein can perform isothermal unfolding, which implies that the heat of the denaturation/aggregation process may be realised within an infinitely small temperature interval. This offers the possibility to measure the heat exchange of the sample with the surroundings in an isothermal calorimeter at a fixed temperature, which will simplify the interpretation of data [21]. In a further investigation we will show that, compared with conventional DSC, isothermal calorimetry is more useful for unravelling the processes involved in the denaturation and aggregation of β-lactoglobulin [22].

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