Journal of Dairy Research (1977), 44, 509–520

A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate

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(*Received 8 January 1977*)

Summary. Differential scanning calorimetry (DSC) was used to study thermal transitions of the following whey proteins and enzymes in milk ultrafiltrate solution: β-lactoglobulin, α-lactalbumin, serum albumin, γ-globulin, apo- and Fe-lactoferrin, lysozyme, ribonuclease, α-chymotrypsin and xanthine oxidase. Denaturation enthalpies (ΔH_D), denaturation temperatures (T_D) and the half width of the denaturation peaks in DSC thermograms ($\Delta T_D^{\frac{1}{2}}$) were determined and the degree of renaturation was estimated by rescanning previously denatured samples. A fair correlation between the results obtained by DSC and other more classical methods was found in general. However, for some proteins (α-lactalbumin, lysozyme, ribonuclease and xanthine oxidase), which have so far been considered relatively thermostable, calorimetry reveals conformational changes starting at temperatures as low as about 45 °C. In these cases thermostability observed after heat treatment of milk should be interpreted in terms of renaturation and not of high temperatures of denaturation.

Calorimetric techniques have been found useful for studying the effect of heat on proteins in aqueous solutions (Privalov, 1974; Sturtevant, 1974). The published calorimetric investigations of protein denaturation, however, either do not include milk proteins or were not carried out in solutions resembling the natural milk medium. It was thus considered useful to examine the thermal transitions of milk proteins in milk ultrafiltrate by differential scanning calorimetry (DSC). This should give some insight into the structure and stability of proteins in milk as well as the changes in the properties of milk and milk products during heat treatment.

The present communication summarizes the results of a calorimetric investigation of the heat-induced transitions and reactions observed in milk ultrafiltrate of the following proteins: β-lactoglobulin (β-lg), α-lactalbumin (α-la), serum albumin, γ-globulin, lactoferrin, lysozyme, ribonuclease-A, α-chymotrypsin and xanthine oxidase.

EXPERIMENTAL

Materials

α-Lactalbumin was separated by ion exchange chromatography (Thompson, 1965, cited by Gordon, 1971) from material purchased from Sigma (Sigma Chemical Co., St Louis, Mo., U.S.A.). Iron saturated bovine lactoferrin and iron free lactoferrin (apo-lactoferrin) were prepared from colostrum of Simmenthal cows according to the method of Baer, Oroz & Blanc (1976*a*). Xanthine oxidase, which is available as a suspension in 3·2 M-ammonium sulphate solution (Boehringer Mannheim GmbH, West Germany), was diafiltered and concentrated with simulated milk ultrafiltrate (SMUF) (Jenness & Koops, 1962), using a continuous Amicon ultrafiltration system and Diaflo type PM-10 membranes (Amicon Corp., Lexington, Mass., U.S.A.). The following commercial protein preparations were used without further purification: β-lg (B.D.H. Chemicals Ltd, Poole, Dorset, England), bovine serum albumin (Serva Feinbiochemica, Heidelberg, West Germany), lysozyme (Sigma), ribonuclease-A (Sigma), α-chymotrypsin (Sigma), and bovine serum γ-globulin (Serva).

Calorimetric standards used were: indium, potassium chromate (Perkin Elmer, Norwalk, Conn., U.S.A.), naphthalene (T. Schuchardt, GmbH, München, West Germany; 99·99 % purity) and benzoic acid (E. Merck, Darmstadt, West Germany; 99·98 % purity).

Solutions

In preliminary experiments milk ultrafiltrate prepared with a Sartorius type SM 115.39.100 membrane (Sartorius, Göttingen, West Germany) was used. Later it was found that indistinguishable calorimetric results were obtained using SMUF, i.e. a salt solution according to Jenness & Koops (1962) which has the following composition (mmol. l^{-1}): Na, 18·3; K, 39·4; Ca, 9·0; Mg, 3·2; Cl, 32·4; phosphate, 11·6; citrate, 9·6; sulphate, 1·0; carbonate, 2·2; lactose, 146·1. The measurements were therefore made using SMUF. The pH of this salt solution was 6·6, its osmolarity 250 ± 2 mosM and electric conductivity at 20 °C $0{\cdot}494 \pm 0{\cdot}003$ S m^{-1}. The pH of the solution was adjusted to $6{\cdot}7 \pm 0{\cdot}1$ after dissolution of the proteins, 0·1 M-KOH or HCl being used for this purpose. The final solutions were passed through filters of 0·45 μm pore width (Millipore Corp., Bedford, Mass., U.S.A.) to remove undissolved and denatured material. A small decrease of pH was observed when SMUF was heated alone. At 80 °C, for example, a pH of 6·42 was measured compared to 6·66 at 20 °C.

Protein concentrations ranged from 3–9 % and were determined by measuring the absorbance of suitably diluted samples in 0·1 M-phosphate buffer, pH 6·9, with a Zeiss PMQ-II spectrophotometer (Carl Zeiss, Oberkochen, West Germany). The following optical factors ($E^{1\%}_{1\,cm}$) and mol. wts were used: β-lg, 9·3 at 278 nm, 18422 (McKenzie, 1971); α-la, 20·6 at 280 nm (Quarfoth & Jenness, 1975), 14176 (Brew, Vanaman & Hill, 1967, corrected by Gordon, 1971); bovine serum albumin, 6·67 at 279 nm (Aoki *et al.* 1973), 66000 (Spahr & Edsall, 1964); lactoferrin, 12·7 (apo form) and 15·7 (Fe saturated form) at 280 nm (Brown & Parry, 1974), 93000 (Weiner & Szuchet, 1975); ribonuclease-A, 7·38 at 278 nm (Scott & Scheraga, 1963), 13683 (Hirs, Moore & Stein, 1956); lysozyme, 26·3 at 281 nm, 14400 (Sophianopoulos *et al.*

1962); α chymotrypsin, 20·3 at 280 nm (Aune & Timasheff, 1971), 25200 (Privalov & Khechinashvili, 1974); γ-globulin, 13·5 at 275 nm, 156000 (Sober, 1968). γ-Globulin contains various types of immunoglobulins (Ig), e.g. IgG, IgM and IgA (Alais & Blanc, 1975). Because IgM and IgA resemble polymers of the basic IgG molecule, the approximate mol. wt of IgG (156000) was assumed for γ-globulin. Extinction coefficients for β-lg and γ-globulin were determined in our laboratory. They are based on dry weight determinations. A mol. wt of 275000 (Hart *et al.* 1970) was assumed for xanthine oxidase and its concentration determined by a modified Folin-Ciocalteu method (Bailey, 1967), using bovine serum albumin as a reference (Behringwerke, Marburg Lahn, West Germany).

Calorimetry

For calorimetric measurements, samples of 15 μl were sealed in aluminium pans (Perkins Elmer volatile sample pans) and weighed. Thermograms (plots of heat flow as a function of temperature) in the temperature range of about 20–110 °C were recorded on a Perkin Elmer model DSC-2 differential scanning calorimeter, equipped with the manufacturer's refrigeration accessories (Perkin Elmer, Intracooler II). The heat capacity of the reference cell was balanced using 15 μl SMUF solution. Leakage of the pans was checked before the DSC scans on an electronic microbalance (Mettler ME-22, Mettler Instruments A.G., Greifensee, Switzerland). It was readily detected by a continuous weight loss.

To determine enthalpy changes accompanying the thermal transitions samples were heated at a rate of 10 °C/min and the areas under the endotherms measured with a planimeter. The baselines were drawn as straight lines from the predenaturational to the postdenaturational DSC curve. This method of baseline construction was considered a sufficient approximation because the baseline shift due to the difference between heat capacity of denatured and native proteins was small compared to the heat effect accompanying the thermal transitions (Brennan, Miller & Whitwell, 1969).

The predenaturational changes which have been observed by Privalov, Khechinashvili & Atanasov (1971) to occur before the effective thermal transitions have not been considered in this study. These predenaturational processes are not accompanied by sharp changes of physical parameters. Only gradual increase of heat capacity takes place with the rise in temperature.

Different programming rates were used to determine the temperature of maximum heat absorption in thermograms, commonly referred to as temperature of denaturation (T_D) or temperature of transition (Privalov, 1974; Sturtevant, 1974). This denaturation temperature is a function of heating rate either if the rate of the transition involved is comparable to the scan rate or if the process under study is irreversible (Donovan & Ross, 1973). Therefore, DSC measurements for T_D determinations were made using scan rates of 10, 5, 2·5 and 1·25 °C/min and the temperatures of maximum heat absorption plotted as a function of heating rate. T_D was then estimated by extrapolation to a rate of 0 °C/min. The symbol T_D° is used in this paper for the extrapolated values of temperature of denaturation.

To estimate renaturation of the proteins after thermal treatments, previously denatured samples were cooled in the calorimeter cell at a rate of 5 °C/min to 20 °C

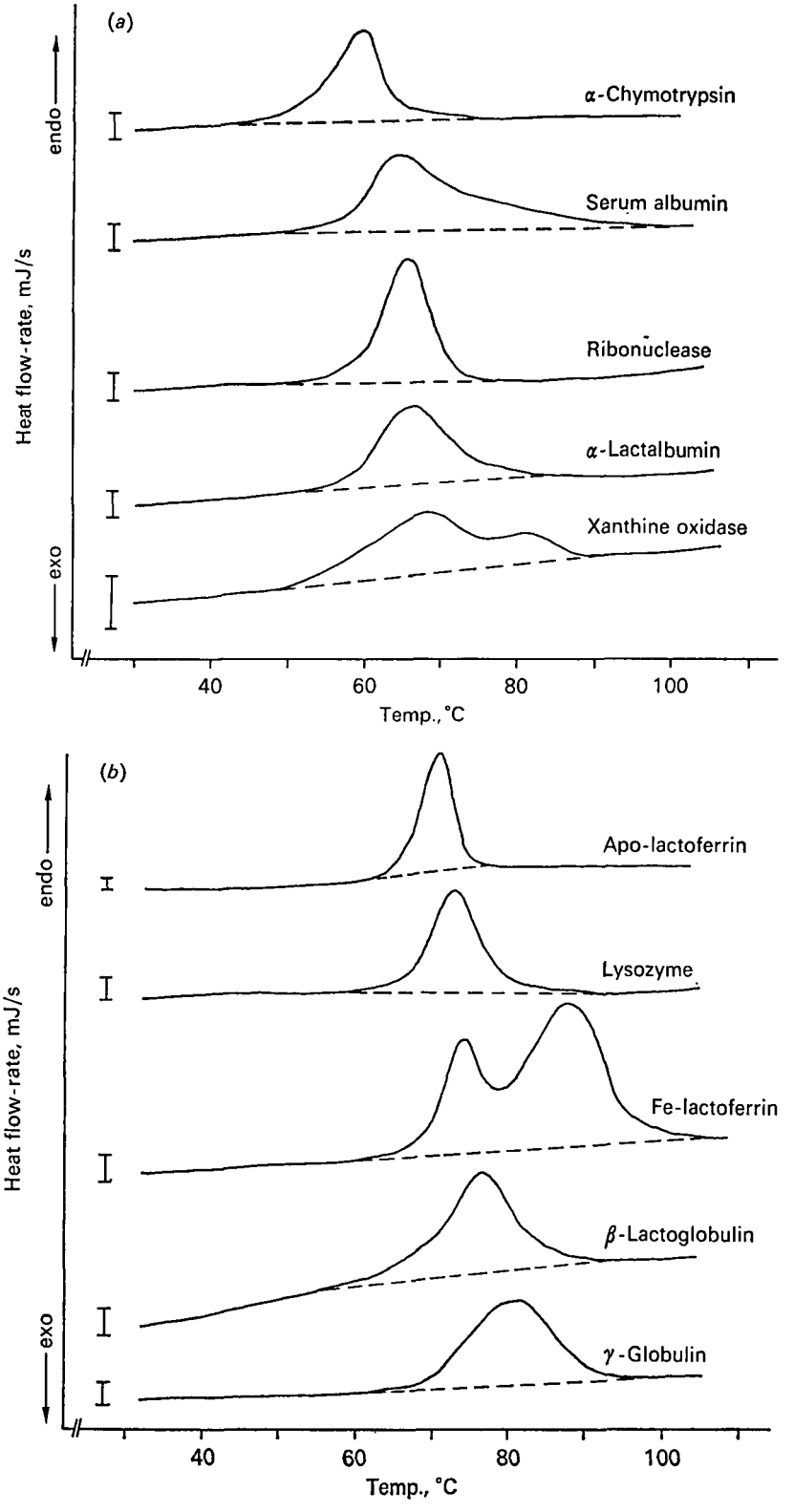

Fig. 1(*a*) and (*b*). For legend see opposite.

Table 1. *Thermodynamic parameters of heat denaturation of whey proteins in simulated milk ultrafiltrate*

Protein	Denaturation temp.,* T_D^0(°C) ± S.D.	Half width denaturation peak, $\Delta T_D^{\frac{1}{2}}$(°C) ± S.D.	Denaturation enthalpy, ΔH_D(kJ/mol) ± S.D.	Renaturation,† % of ΔH_D
α-Chymotrypsin	55·7 ± 0·3	6·5 ± 0·8	458 ± 32	0
Xanthine oxidase‡	61·4 ± 0·8 77·6 ± 0·5	—	3230 ± 300	5–10
Serum albumin‡	62·2 ± 0·5	11·7 ± 0·7	939 ± 88	0
Ribonuclease	62·7 ± 0·2	6·4 ± 0·4	418 ± 18	60–70
Apo-lactoferrin	64·7 ± 0·3	4·9 ± 0·1	2100 ± 95	40–50
α-Lactalbumin	65·2 ± 0·2	10·2 ± 0·7	318 ± 17	80–90
Fe-lactoferrin‡	69·0 ± 0·5 83·5 ± 0·5	—	2920 ± 90	0
Lysozyme	70·5 ± 0·2	7·4 ± 0·2	494 ± 19	10–20
β-Lactoglobulin	72·8 ± 0·4	12·0 ± 1·1	227 ± 21	0
γ-Globulin	72·9 ± 0·4	12·9 ± 0·5	4120 ± 260	0

* Extrapolated values for heating rate 0 °C/min (see Fig. 2).

† Estimated from differential scanning calorimetry thermograms of samples which have been denatured, cooled at a rate of 5 °C/min and rescanned.

‡ Overlapping peaks (see Fig. 1); ΔH_D calculated from total area of unresolved peaks.

S.D., standard deviation.

and rescanned. The ratio of the peak areas was then taken as a measure of the extent of renaturation.

RESULTS AND DISCUSSION

Fig. 1(*a*) and (*b*) show denaturation thermograms of the various whey proteins studied. They are ranked in the order of increasing T_D at a heating rate of 10 °C/min. The position of the denaturation peaks and, to a certain extent, the shape of these endotherms are affected by the heating rate. Fig. 2 shows the dependence of the denaturation temperatures on heating rate. A strong dependence of T_D on heating rate indicates an irreversible process or a rate of reaction which is comparable to the scan rate. In Fig. 2, the solid lines correspond to the best fit of second-order polynomials. The temperatures of denaturation obtained by extrapolation to a scan rate of 0 °C/min are included in Table 1. This table summarizes thermodynamic parameters of the thermal transitions observed for the various whey proteins and enzymes by calorimetry. As in Fig. 1, the proteins are ranked in the order of increasing temperatures of denaturation.

For the proteins showing renaturation the van't Hoff enthalpy of denaturation (ΔH_{vH}) was estimated using the formula $\Delta H_{vH} = 4RT_D^2/\Delta T_D^{\frac{1}{2}}$, where R is the gas constant, T_D the midpoint of the thermal transition and $\Delta T_D^{\frac{1}{2}}$ the half width of the

Fig. 1. (*a*) and (*b*) Thermal denaturation of whey proteins in simulated milk ultrafiltrate. Differential scanning calorimetry thermograms of 15 μl samples recorded at a heating rate of 10 °C/min. Protein concentrations (%) are: α-chymotrypsin, 5·36; bovine serum albumin, 7·72; ribonuclease, 4·14; α-lactalbumin, 6·18; xanthine oxidase, 5·64; apo-lactoferrin, 9·60; lysozyme, 7·14; Fe-lactoferrin, 9·83; β-lactoglobulin, 6·24; γ-globulin, 6·33. *Endo* and *exo* indicate the directions of endothermic and exothermic transitions. The bars represent a heat flow of 0·1 mJ/s.

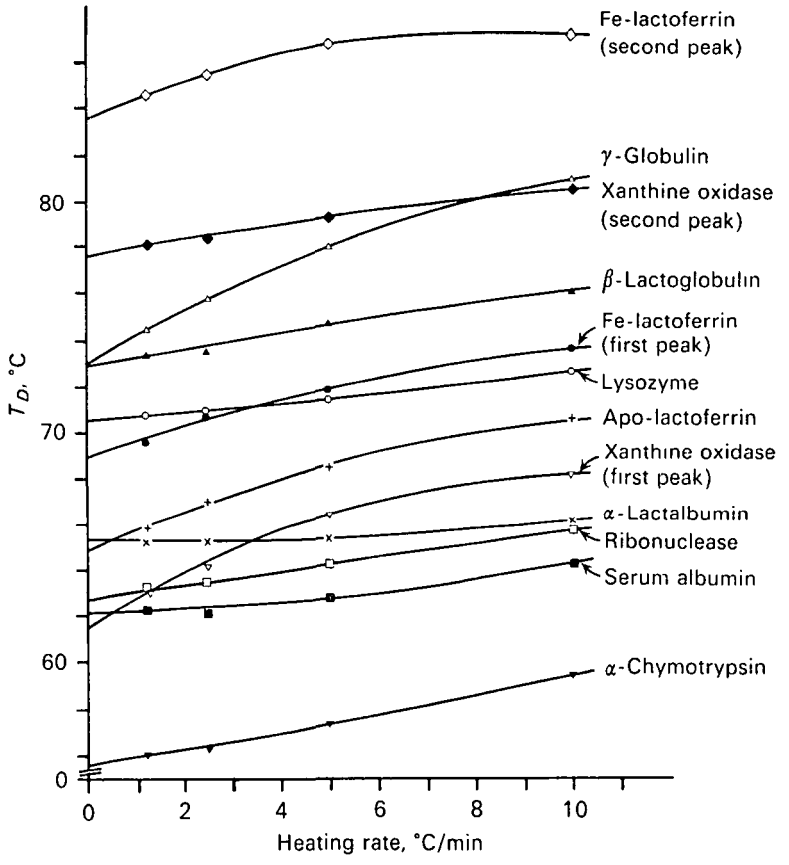

Fig. 2. Dependence of denaturation temperature (T_D) of various whey proteins in simulated milk ultrafiltrate upon heating rate. T_D values are temperatures of maximum deflection in differential scanning calorimetry thermograms.

heat absorption peak (Privalov *et al.* 1971). This approximate formula is derived from the van't Hoff equation and is valid for reversible 2-state processes only. Therefore, comparison of the enthalpy change estimated from the sharpness of the transition (ΔH_{vH}) and the heat effect determined from the peak area (ΔH_D) may be used as a criterion for a 2-state case, i.e. a transition involving only one initial and one final state (Privalov, 1974; Sturtevant, 1974). A lack of correspondence between ΔH_{vH} and ΔH_D indicates decreased cooperativity due to the presence of intermediates or to intermolecular association (Brandts, 1969).

β-Lactoglobulin

Thermograms of β-lg in SMUF reveal a single and almost symmetrical denaturation peak, similar to that observed when pure water was used as solvent (Rüegg, Moor & Blanc, 1975). When compared to the other proteins studied, the dependence of T_D and $\Delta T_D^{\frac{1}{2}}$ on pH was most pronounced for β-lg. However, there was no significant variation of the heat of denaturation in the pH range tested (pH 6·4–7·3). Fig. 3 shows denaturation thermograms of β-lg in SMUF at 3 different pH values. Corresponding mean T_D and $\Delta T_D^{\frac{1}{2}}$ values are reported in Table 2. β-Lactoglobulin

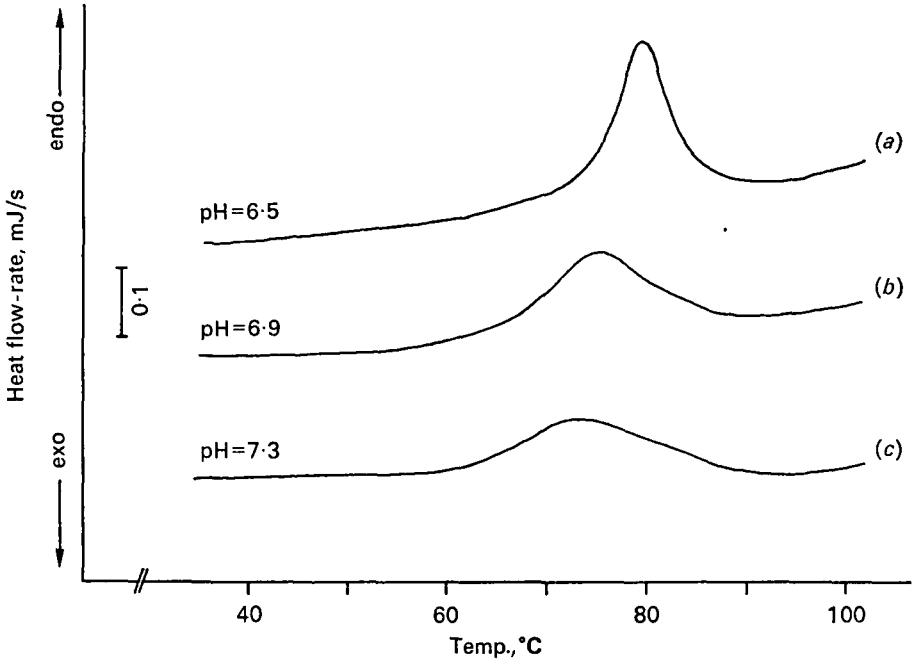

Fig. 3. pH dependence of thermodenaturation of β-lactoglobulin in simulated milk ultrafiltrate. Differential scanning calorimetry thermograms of 15 μl samples recorded at a heating rate of 10 °C/min. Protein concentrations (%) are: (*a*), 4·41; (*b*), 5·36; (*c*), 4·55.

Table 2. *Effect of pH on heat denaturation of β-lactoglobulin in simulated milk ultrafiltrate. Differential scanning calorimetry data obtained at a heating rate of 10 °C/min.*

pH	Denaturation temp., T_D (°C) ± S.D.	Half width of denaturation peak, $\Delta T_{\frac{1}{2}}$ (°C) ± S.D.
6·46	79·9 ± 0·5	6·6 ± 0·4
6·87	76·1 ± 0·6	12·0 ± 1·1
7·25	74·4 ± 0·4	15·6 ± 1·2

S.D., standard deviation.

is known to undergo irreversible thermodenaturation due to aggregation involving sulphur groups, followed by nonspecific aggregation (Sawyer, 1968; McKenzie, 1971). The complex pathway for the heat denaturation of this protein might explain the strong dependence of the denaturation temperature and sharpness of the denaturation peak on heating rate and pH.

α-Lactalbumin

Among the proteins tested, α-la showed the greatest extent of renaturation: 80–90% reversibility was observed at protein concentrations of 3–9% and for the particular experimental procedure used in this study. This high degree of renaturation presumably accounts for the fact that α-la has been considered as the most resistant of all the whey proteins (Shukla, 1973). The midpoint of the thermal transition is

significantly lower than that for various other whey proteins including β-lg, lactoferrin, lysozyme and γ-globulin and remains independent of heating rate up to a rate of about 5 °C/min.

The enthalpy of denaturation of α-la in milk ultrafiltrate, $\Delta H_D = 318$ kJ/mol, is higher than that observed in pH 6·9 phosphate buffer solution, $\Delta H_D = 272$ kJ/mol (Rüegg *et al.* 1977). This suggests a conformation stabilizing effect of lactose, citrate or Ca^{2+}.

The van't Hoff enthalpy of denaturation, $\Delta H_{vH} = 376$ kJ/mol, as estimated from the sharpness ($\Delta T_D^{\frac{1}{2}}$) and position (T_D) of the denaturation peak, is higher than the corresponding calorimetric ΔH_D. This indicates that the thermal transition observed is not a simple 2-state process. Similar conclusions were drawn by Baer, Oroz & Blanc (1976*b*) from their immunochemical measurements of the heat denaturation of α-la. Intermolecular interactions may explain in part the deviation from the 2-state case (Shukla, 1973).

Bovine serum albumin

The thermal analysis of serum albumin reveals fairly complex thermograms with several overlapping endothermic peaks over a wide temperature range. Privalov & Monaselidze (1963), when studying human serum albumin in buffer solution of pH 7, obtained 3 denaturation peaks at about 55, 67 and 75 °C. They calculated a total heat of denaturation of 883 kJ/mol. This value may be compared to that obtained for bovine serum albumin in SMUF, $\Delta H_D = 939 \pm 88$ kJ/mol. However, the temperature interval and peak shapes are different for the albumins in buffer solution and milk ultrafiltrate.

The complex behaviour of serum albumins upon heating has been explained in terms of microheterogeneity and formation of heat-stable intermediate forms due to SH-SS exchange reactions (Aoki *et al.* 1973; Privalov & Monaselidze, 1963).

γ-Globulin

Despite the biochemical heterogeneity of γ-globulin, denaturation thermograms reveal a single and symmetrical peak in a relatively small temperature interval ($\Delta T_D^{\frac{1}{2}} = 12{\cdot}9$ °C). The temperature of denaturation is strongly dependent on heating rate. A difference of about 8 °C is observed for T_D at a heating rate of 10 °C/min and T_D° at a rate of 0 °C/min.

The high temperature of denaturation observed in thermograms is consistent with the well known heat resistance of immunoglobulins. For example, when immunsera are heated at temperatures as high as 62 °C for up to 3 min, the antibody activity of the immunoglobulins seems not to be affected (Kwapinski, 1972).

Lactoferrin

Whereas apo-lactoferrin exhibits only one denaturation peak with a midpoint around 65 °C, iron-saturated lactoferrin shows a complex denaturation thermoprofile with maxima at 69 and 83 °C. The binding of iron to lactoferrin not only shifts thermal transitions to higher temperatures but also increases the enthalpy of denaturation from about 2100 kJ/mol to approximately 2900 kJ/mol. A certain contribution to the increase in enthalpy of denaturation might be attributed to the

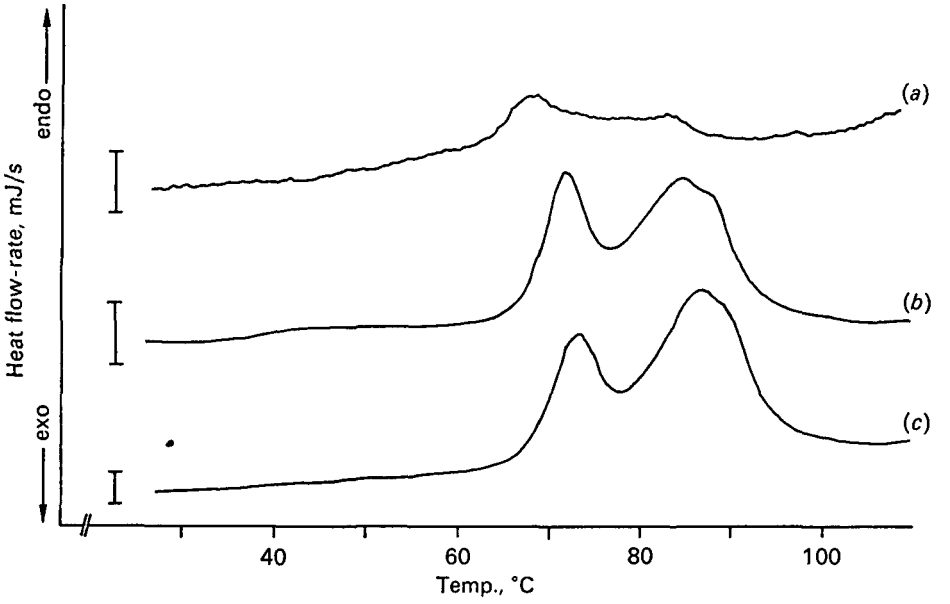

Fig. 4. Effect of heating rate on differential scanning calorimetry thermograms of Fe-lactoferrin. Protein concentration: 9·83%; solvent: simulated milk ultrafiltrate, pH = 6·6. Heating rates are (*a*), 1·25 °C/min; (*b*), 5 °C/min; (*c*), 10 °C/min. The bars represent a heat flow of 0·1 mJ/s.

enthalpy of binding of iron to lactoferrin, but the increase of ΔH_D with the rise in temperature must also be taken into account (Privalov, 1974).

It may be seen from Fig. 4 that the relative size of the endotherms observed for Fe-lactoferrin was affected by heating rate. This indicates that equilibrium conditions do not exist as iron-lactoferrin solutions are heated at high scan rates.

Donovan & Ross (1975) and Donovan *et al.* (1976) when studying ovotransferrins at different Fe to protein ratios by DSC at heating rate 10 °C/min observed 4 endotherms at characteristic temperatures. The 4 endotherms, in the order of increasing temperature, were attributed to the apo-, 2 different monoferric- and Fe_2-lactoferrin species respectively. The position of the second endotherm attributed to monoferric ovotransferrin species, 76·5 °C, and that for the Fe_2-ovotransferrin, 83·5–89·5 °C, may be compared to the positions of the 2 endotherms observed in thermograms of iron-lactoferrin in SMUF, 73·6 and 87·2 °C respectively. Furthermore, the enthalpy of denaturation for Fe_2-ovotransferrin, 2917 kJ/mol, agrees well with ΔH_D calculated from the total area of the endotherms for iron-saturated lactoferrin (2920 kJ/mol). These analogies appear to indicate that both proteins have comparable iron binding properties and that the low temperature peak in denaturation thermograms of iron-saturated lactoferrin is due to the presence of monoferric species. Formation of iron-phosphate complexes upon heating could explain the occurrence of monoferric species in SMUF solutions of iron-saturated lactoferrin. The dependence of the relative size of the 2 peaks on heating rate as shown in Fig. 4 would be consistent with a dissociation of iron from lactoferrin during the DSC scan.

Lysozyme

Thermodenaturation of lysozyme in 0·05–0·5 % solutions at pH 2–4·5 was found to be totally reversible (Privalov & Khechinashvili, 1974). However, in milk ultrafiltrate at pH 6·7 and at protein concentrations of 3–9 %, reversibility was poor. Only 10–20 % renaturation was observed for the particular thermal treatment described in the experimental section. Aggregation of denatured molecules is in many cases one of the major factors which reduces the reversibility of thermal transitions. These aggregations are, as a rule, accompanied by negative enthalpy changes and might be responsible for the small difference between the calculated van't Hoff enthalpy of denaturation, $\Delta H_{vH} = 537$ kJ/mol, and the calorimetric heat of denaturation, $\Delta H_D = 494$ kJ/mol, which represents the sum of the enthalpy processes over the temperature range of transition.

The calculated value for the van't Hoff enthalpy of denaturation, 537 kJ/mol, agrees well with values determined by other authors from optical measurements, 532 kJ/mol (Takesada, Nakanishi & Tsuboi, 1973) and 515 kJ/mol (Delben & Crescenzi, 1969). The latter values were determined using phosphate buffer solutions of pH 5·6 and 5·37, respectively. The agreement between the ΔH_{vH} values suggests that the major components of SMUF, which are not present in phosphate buffer solutions, e.g. lactose, citrate and Ca, do not influence the thermal transition of lysozyme.

α-Lactalbumin is known to have a close structural similarity to lysozyme and some physical and chemical properties are analogous (Shukla, 1973). Thermal stability, however, is strikingly different for these proteins. The enthalpy of denaturation as well as the temperature of denaturation is higher for lysozyme than for α-la. It has been suggested that α-la has a lower number of intramolecular H-bonds (Takesada *et al.* 1973). This would explain in part the lower heat of denaturation found for α-la.

Ribonuclease

It has been reported that this enzyme is relatively resistant to heat and is normally not destroyed by pasteurization or even ultra high temperature treatments (Alais & Blanc, 1975; Shahani, 1966; Zittle, 1964). If one takes into account the conformational change observed by calorimetry at about 63 °C the heat resistance of this enzyme must probably be explained by a high degree of renaturation after thermal treatments. This conclusion is supported by the results obtained from thermal measurements of the isolated enzyme in SMUF. Renaturation of 60–70 % was observed after rapid cooling and a short temperature equilibration at 20 °C.

The symmetrical denaturation peak for ribonuclease has a maximum around 63 °C, a value which may be compared to that observed by other authors in water (Delben, Crescenzi & Quadrifoglio, 1969) or NaCl solutions (Tsong *et al.* 1970), i.e. 60–62 °C. Also the ΔH_D value of about 418 kJ/mol found in SMUF solutions is in agreement with data obtained by DSC in the pH range 6–9 using water as solvent, $\Delta H_D = 414$ kJ/mol (Delben *et al.* 1969). The enthalpy of denaturation reported by Tsong *et al.* (1970) for ribonuclease denaturation at pH 7 in 0·2 N-NaCl is significantly higher, i.e. 703 kJ/mol. However, this value refers to the temperature of

transition, whereas the DSC data are average values over the temperature range of transition. It should also be considered that the DSC data were obtained using 3–5 % solutions, whereas Tsong *et al.* (1970) used more dilute solutions. Comparison of the DSC data obtained in water (Delben *et al.* 1969) and in SMUF suggests that heat denaturation of ribonuclease is little affected by constituents of milk ultrafiltrate.

α-Chymotrypsin

Heat denaturation of α-chymotrypsin has been extensively studied by calorimetry in the pH 2–4 range (Brown, 1971; Privalov & Khechinashvili, 1974). In this pH range the thermal transition was reversible and apparently a simple 2-state process (Privalov & Khechinashvili, 1974). In SMUF solutions, however, the heat denaturation was found to be irreversible.

A high temperature shoulder in denaturation thermograms of chymotrypsin has been observed when sucrose was present in the solvent (Cassel, 1973). A similar shoulder is observed in SMUF which contains lactose (Fig. 1). At protein concentrations higher than about 6 %, reproducibility of the denaturation peak shape was poor, possibly due to aggregations which are favoured at high protein concentrations and at pH values in the vicinity of 7.

Xanthine oxidase

Thermograms of xanthine oxidase reveal 2 endothermic transitions with maxima at about 61 and 78 °C. The relative size of the 2 endotherms did not change significantly with different heating rates. Upon reheating previously denatured samples small peaks recurred in the same position as in the first scan. This indicates that there is some renaturation of the metallo-flavoprotein.

It has been reported that xanthine oxidase in milk is moderately resistant to heat and, in contrast to other enzymes, activated after certain heat treatments (Shahani, 1966; Zittle, 1964). When comparing these findings with the conformational changes observed by calorimetry, one must consider that xanthine oxidase in milk is concentrated on the fat globule membrane and that generally, isolated and purified enzymes are more heat labile (Zittle, 1964). The observed heat stability of xanthine oxidase in milk is probably due to both renaturation and a protective effect of other milk constituents. The presence of a heat resistant xanthine oxidase activator has also been discussed (Shahani, 1966).

CONCLUSIONS

Calorimetry allows one not only to follow the heat denaturation of proteins, but also to obtain information about reversibility and, in favourable cases, the mechanism of the reactions involved in thermal transitions.

In general, a fair correlation between the results obtained by DSC and other more classical methods was found, but for some proteins (α-la, lysozyme, ribonuclease and xanthine oxidase), which have been considered so far as relatively thermostable, calorimetry reveals conformational changes starting at temperatures as low as about 45 °C. In these cases, thermostability must be interpreted in terms of renaturation and not of high temperatures of denaturation.

In this study, isolated milk serum proteins have been investigated in simulated milk ultrafiltrate. When applying the results obtained to whole milk, interactions between proteins and the various milk constituents which are not present in SMUF must be considered.

We are grateful to Dr A. Baer for providing samples of lactoferrin and to Dr M. Casey for purifying the α-la and for his linguistic assistance.

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Printed in Great Britain