Reaction Kinetics of Thermal Denaturation of Whey Proteins in Heated Reconstituted Whole Milk

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Reconstituted whole milk was heated using pilot-scale heating equipment. Kinetic and thermodynamic parameters for the irreversible denaturation of β-lactoglobulins A and B and α-lactalbumin were determined. α-Lactalbumin denaturation was first order, whereas both β-lactoglobulin variants had a reaction order of 1.5. Arrhenius plots for all three proteins showed an abrupt change in temperature dependence. In the low-temperature range, the thermodynamic parameters were ascribed to typical denaturation processes in which the unfolding of the protein tertiary structure is the rate-determining step. At higher temperatures, these parameters were in the range expected for typical condensation reactions, suggesting that aggregation processes may be rate-determining in this temperature range. The rate constants for β-lactoglobulin denaturation were independent of the initial protein concentration at all temperatures. For α-lactalbumin at temperatures below 85 °C the rate constants may have been dependent on the initial α-lactalbumin concentration as higher rate constants were observed with decreasing protein concentrations.

Keywords: *Whey protein; denaturation; whole milk; reconstituted milk*

INTRODUCTION

Milk, in comparison with many other food systems, is remarkably heat stable, which allows for the manufacture of a range of heat-sterilized products. During the heating process, numerous reactions that influence the nutritional and functional properties of the milk and the subsequent products can occur (Walstra and Jenness, 1984). The occurrence and extent of these reactions are dependent on heating conditions (temperature and duration of heat treatment) as well as on factors such as milk composition, concentration, and pH. It is essential to have an understanding of these reactions so that heating conditions can be applied to achieve the desired functional properties in milk products and to keep undesirable reactions to a minimum.

A particularly important group of reactions is the thermal denaturation of the major whey proteins [α-lactalbumin (α-lac), β-lactoglobulin (β-Lg) A, and β-Lg B], as these can have a marked influence on the functional properties of milk products (Walstra and Jenness, 1984). Globular proteins such as the whey proteins retain their native conformation only within relatively limited pH and temperature ranges. Exposing these proteins to extremes of temperature or pH results in denaturation of the proteins, defined as a considerable change in the native conformation in which the three-dimensional (tertiary) structure of the polypeptide chain is converted to a lower state of order. The unfolded conformation exposes amino acid residue side-chain groups that are normally buried within the native structure, and this causes an increase in the reactivity of such groups. Especially important is the increased reactivity of the thiol group of cysteine, which can be involved in disulfide interchange reactions; however, an unfolded protein can be more susceptible to protein−protein interactions via calcium bridging and hydrophobic bonding. In contrast to the globular whey proteins, the open-structured casein proteins are very heat stable; however, the cysteine residues of κ-casein, and presumably α_{s2}-casein, can be involved in sulfhydryl−disulfide interchange reactions with the denatured whey proteins (Fox, 1982; Brown, 1988).

A study of the kinetics of thermal denaturation of the individual whey proteins should lead to a better understanding of the relationship between heat treatment and its effect on the functional properties of milk products. The objective of experimental kinetic studies is the development of mathematical models to describe the rate of particular reactions as a function of various experimental variables and thus to gain an understanding of the thermodynamic parameters important in the reaction process. There are numerous reports of kinetics studies on the denaturation of the major whey proteins in buffer solutions (Gough and Jenness, 1962; El-Shazly et al., 1978; Harwalkar, 1980; Park and Lund, 1984), whey (Hillier and Lyster, 1979; Hillier et al., 1979; Harwalkar, 1986; Kessler and Beyer, 1991), or skim milk (Gough and Jenness, 1962; Lyster, 1970; Hillier, 1976; Hillier and Lyster, 1979; Manji and Kakuda, 1986; Dannenberg and Kessler, 1988a−c; Kessler and Beyer, 1991). All studies to date have reported a first-order reaction for the denaturation of α-lac. In contrast, the findings for the reaction order for the denaturation of β-Lg are not always in agreement. Several researchers (Lyster, 1970; El-Shazly et al., 1978; Hillier and Lyster, 1979; Hillier et al., 1979; Park and Lund, 1984; Harwalkar, 1986; Manji and Kakuda, 1986; Kessler and Beyer, 1991) have reported second-order kinetics for the thermal denaturation of both variants of β-Lg. In contrast, Gough and Jenness (1962) and Luf (1988) found that the denaturation of β-Lg followed first-order reaction kinetics, whereas McKenzie et al. (1971), Sawyer et al. (1971), and Harwalkar (1980) suggested that the denaturation reaction followed a series of consecutive first-order reactions. In detailed studies by Dannenberg and Kessler (1988a−c), a reaction order of 1.5 was reported

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for the denaturation of both variants of β-Lg. The discrepancies among these studies may be explained by several factors: the concentration or composition of the protein solution, the medium of the protein solution (skim milk, whey, or isolated protein in buffer solution), the method and conditions of heat treatment, the method of assay to determine the residual native protein concentration, and insufficient data to accurately determine the reaction order.

To achieve short heating and cooling times, all previous studies examined the level of denaturation in very small volumes of milk heated in an oil bath or in specially developed heating apparatus. These conditions are very different from those typically encountered during the processing of milk in which flow characteristics are far removed from idealized plug flows and residence time distributions of the milk samples through the heat exchangers need to be considered. In addition, previous studies have focused on the kinetic and thermodynamic parameters for the individual whey protein denaturation in whey or fresh skim milk systems but not for these proteins in a reconstituted whole or skim milk even though heat treatments are invariably applied to these systems.

The purpose of this study was to determine the levels of denaturation of the major whey proteins (α-lac, β-Lg A, and β-Lg B) in reconstituted whole milk that was heated using pilot-scale processing equipment. Ranges of heating temperatures and times were employed so that the kinetic and thermodynamic parameters for the thermal denaturation of α-lac, β-Lg A, and β-Lg B could be determined under typical processing conditions.

EXPERIMENTAL PROCEDURES

Milk Powder Manufacture. Whole milk powder was manufactured on six occasions through the 1991/1992 manufacturing season at the Waitoa Powder Development Centre, Waitoa, New Zealand. Skim milk and cream were blended to give standardized whole milk of 30% (w/w) fat (dry basis) and 12% (w/w) total solids. The whole milk was preheated to 68 °C by indirect heating and then either given no further heat treatment or heated to a final preheat temperature using direct steam injection and passage through the required number of holding tubes to achieve the specified holding time. The milk was concentrated in a falling film evaporator (modified three effect, Wiegand GmbH, Karlsruhe, Germany), homogenized (7.6 MPa stage one, 3.6 MPa stage two), and then spray-dried to 3% moisture in a modified Niro spray-drier incorporating an integral fluidized bed (MKT, Helsinki, Finland).

Reconstitution and Heat Treatment. Reconstitution and heat treatment were performed at the New Zealand Dairy Research Institute, Palmerston North, using a pilot-scale processing plant. Whole milk powder was reconstituted to 15% (w/w) total solids in water at 45 °C and held for 30 min for hydration prior to pasteurization at 72 °C for 15 s and chilling to below 10 °C. The reconstituted milk was subjected to the appropriate heat treatment using indirect heating to 65 °C, direct steam injection to the required temperature, holding for the specified time, and flash cooling to below 65 °C. Ranges of heating temperatures (70−115 °C) and holding times (0−300 s) were used. The average holding times (residence times) were calculated from the configuration of the heating apparatus and the flow rates of liquid through the system. These calculated residence times were confirmed by experimentally timing the flow of milk and water through the system.

Analyses. The total solids content of the reconstituted whole milk was measured according to the method of Mojonnier and Troy (1925).

The content of individual native whey proteins (α-lac, β-Lg A, and β-Lg B) in unheated (control) and heated reconstituted whole milk samples was determined by using native polyacrylamide gel electrophoresis (native PAGE) in the absence of dissociating or reducing agents. Electrophoresis was performed on a mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA), as described by Andrews (1983). The resolving gel contained 12.5% acrylamide dissolved in 1.5 M Tris-HCl buffer, pH 8.8, and the stacking gel was composed of 4.0% acrylamide made up in 0.5 M Tris-HCl buffer, pH 6.8. Samples were dispersed in 0.5 M Tris-HCl buffer, pH 6.8, with 0.01% bromophenol blue as tracking dye.

The gels were run at 210 V and 70 mA for approximately 2 h (until the tracking dye seeped out of the bottom of the gel) and then stained with Coomassie Brilliant Blue (R-250) in 25% (v/v) 2-propanol and 10% (v/v) acetic acid for 1 h. This was followed by two destaining steps with 10% (v/v) acetic acid and 10% (v/v) 2-propanol for a total of 20 h. Immediately after destaining, the gels were scanned on a computing laser densitometer (Molecular Dynamics Model P.D., Sunnyvale, CA) and the integrated intensities of the α-lac, β-Lg A, and β-Lg B bands were determined using the Molecular Dynamics ImageQuant software. Within each experimental trial, the results were corrected for the small differences in total solids between samples as a consequence of the direct heating.

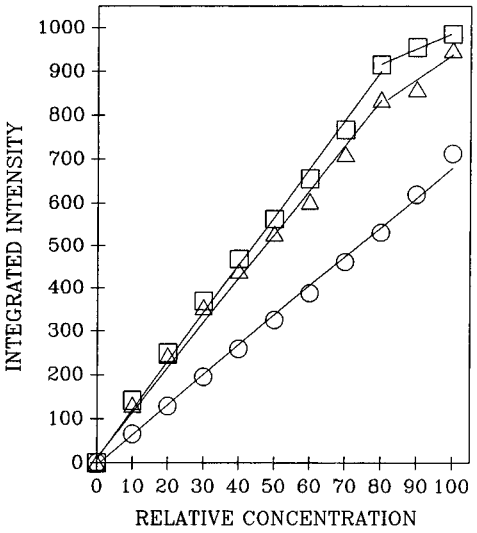

Figure 1. Relationship between relative protein concentration and dye uptake for native whey proteins in milk: ○, α-lac; □, β-Lg A; △, β-Lg B.

RESULTS

Evaluation of the Gel Electrophoresis Procedure. The concentrations of native α-lac, β-Lg A, and β-Lg B were varied by diluting milk into sample buffer over a wide concentration range (1:10 to 1:100 dilution factors). A linear relationship was obtained between integrated intensity and protein concentration for all three whey proteins (Figure 1). At high β-Lg concentrations, the curve plateaued.

Order of Denaturation Reactions. The order of the thermal denaturation reaction for the three whey proteins was determined by integration of the general rate equation

$$-(dC_t/dt) = k_n C_t^n \quad (1)$$

(n = reaction order, k_n = rate constant, and C_t = concentration of native protein at time t) which yields

$$(C_t/C_0)^{1-n} = 1 - (n-1)k_n C_0^{n-1} t = 1 - (n-1)kt \quad (2)$$

$$(\text{where } k = k_n C_0^{n-1})$$

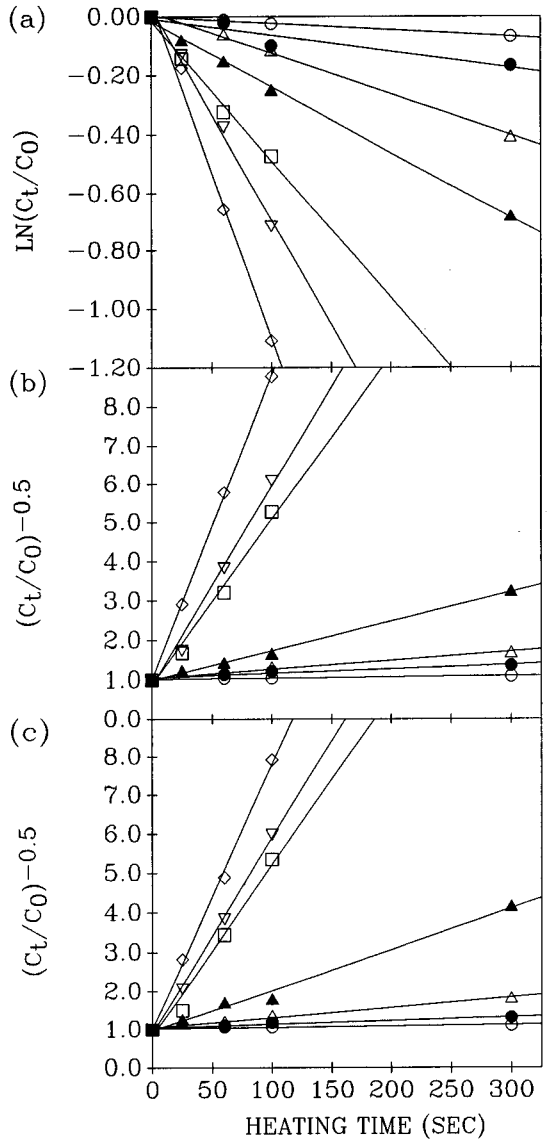

Figure 2. Denaturation of the whey proteins [(a) α-lac; (b) β-Lg A; (c) β-Lg B] in heated milk: ○, 70 °C; ●, 75 °C; △, 80 °C; ▲, 85 °C; □, 100 °C; ▽, 105 °C; ◇, 115 °C.

for $n \neq 1$, and

$$\ln(C_t/C_0) = -k_n C_0^{n-1} t = -k_1 t \qquad (3)$$

when $n = 1$. Note that the product $k_n C_0^{n-1}$ is combined into the rate constant k. When $n \neq 1$, k is dependent on the initial protein concentration.

Equations 2 and 3 were used to analyze the results at each temperature to obtain the overall order, n, for the thermal denaturation reactions of α-lac and the two variants of β-Lg. For α-lac, linear relationships were obtained when $\ln(C_t/C_0)$ was plotted against t, indicating that the denaturation of this protein followed first-order reaction kinetics. A reaction order of 1.5 was observed for both variants of β-Lg, as plots of $(C_t/C_0)^{-0.5}$ against t yielded straight lines. Typical results are shown in Figure 2. The rate constants, k, for the denaturation of α-lac and β-Lg at each temperature were determined from the slopes of the straight lines.

The six milk powders used to manufacture the reconstituted whole milk samples contained native whey proteins at a range of initial concentrations due to preheat treatments applied during powder manufacture and to the natural variation in expression levels of these proteins. For reactions other than those of a first order,

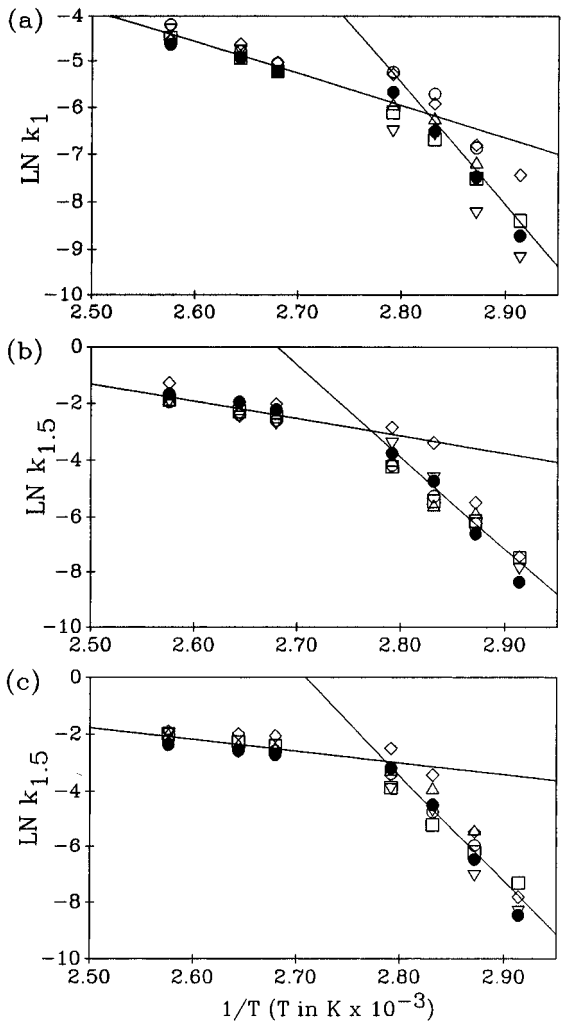

Figure 3. Arrhenius plot for the denaturation of the whey proteins [(a) α-lac; (b) β-Lg A; (c) β-Lg B] in heated milk: ○, trial 1; △, trial 2; □, trial 3; ▽, trial 4; ◇, trial 5; ●, trial 6.

the rate constants, k, calculated from eq 2 are the product of the true rate constant (k_n) and a function of the initial reactant concentration (C_0^{n-1}). Therefore, for the thermal denaturation of β-Lg A and β-Lg B, the rate constants, k, were dependent on the initial native protein concentration. Although absolute initial native whey protein concentrations in the six samples were unknown, the concentration (C_0) relative to one trial could be determined using native PAGE. For β-Lg A and β-Lg B, $k_{1.5}$ was calculated by dividing k by $C_0^{0.5}$ (i.e., corrected for concentration) using protein concentrations relative to trial one. The average rate constants $k_{1.5}$ for β-Lg A and β-Lg B and k_1 for α-lac are given in Table 1, along with a comparison with those reported by Dannenberg and Kessler (1988a,b).

Temperature Dependence. The relationship between the observed rate constants and the temperature of the reaction is given by the Arrhenius equation

$$k = k_0 \exp(-E_a/RT) \qquad (4)$$

(E_a = activation energy, R = universal gas constant, k_0 = frequency factor, and T = absolute temperature). The logarithms of the rate constants ($\ln k_n$) obtained from the slopes of the lines from Figure 2 and corrected for protein concentration differences (Table 1) were plotted against the reciprocal of absolute temperature for all six trials (Figure 3). For each trial, the relationship (ln

Table 1. Kinetic Parameters for α-lac, β-Lg A, and β-Lg B Denaturation, with a Comparison with Those Reported by Dannenberg and Kessler (1988a−c) (D and K)

	E_a (kJ mol^{-1})			k (s^{-1} × 10^3)		$\Delta H^‡$ (kJ mol^{-1})		$\Delta G^‡$ (kJ mol^{-1})		$\Delta S^‡$ (kJ mol^{-1} K^{-1})	
protein	this work	D and K	T (°C)	this work	D and K	this work	D and K	this work	D and K	this work	D and K
β-Lg A	263.49	265.21	70	0.44		260.64	262.36	105.81	109.53	0.45	0.445
			75	2.31		260.60	262.32	102.86	107.88	0.45	0.444
			80	10.84		260.55	262.28	100.98	104.31	0.45	0.447
			85	26.81		260.51	262.24	99.42	102.60	0.45	0.446
	51.14	54.07	100	92.57		48.04	50.97	99.56	101.23	−0.14	−0.135
			105	111.22		48.00		100.34		−0.14	
			115	175.08		47.91		101.64		−0.14	
β-Lg B	296.46	279.96	70	0.36	0.16	293.61	277.10	107.21	109.29	0.54	0.484
			75	2.62	0.57	293.57	277.06	103.36	107.35	0.55	0.487
			80	14.61	3.43	293.52	277.02	99.93	103.65	0.55	0.491
			85	39.63	12.82	293.48	276.98	98.23	101.23	0.55	0.491
	33.87	47.75	100	85.02	69.63	30.77	44.65	99.81	100.35	−0.19	−0.149
			105	93.14		30.73		100.91		−0.19	
			115	126.92		30.64		102.61		−0.19	
α-lac	195.11	268.56	70	0.27	0.12	192.26	265.71	108.50	110.49	0.24	0.452
			75	0.72	0.55	192.22	265.66	106.97	108.72	0.25	0.451
			80	2.02	1.56	192.17	265.62	105.39	105.97	0.25	0.452
	57.51	69.01	85	3.37	1.81	54.53	66.04	105.50	107.05	−0.14	−0.115
			100	5.70	5.66	54.41	65.91	108.13	108.14	−0.14	−0.113
			105	8.14		54.37		108.51		−0.14	
			115	11.94		54.24		110.24		−0.14	

k_n against $1/T$) was linear within certain temperature ranges; however, a marked change in temperature dependence occurred at about 80 °C for α-lac and at about 85 °C for both variants of β-Lg. For each protein, the linearity within the two temperature ranges allowed the activation energies (E_a) for the six trials to be calculated. The average values for E_a for the six trials, and those reported by Dannenberg and Kessler (1988a−c), are given in Table 1.

Thermodynamic Considerations. The enthalpy of activation ($\Delta H^‡$), the free energy of activation ($\Delta G^‡$), and the entropy of activation ($\Delta S^‡$) for the thermal denaturation of α-lac, β-Lg A, and β-Lg B were calculated using the Eyring equation

$$k = (k_b T/h) \exp(-\Delta G^‡/RT) \quad (5)$$

and the relationships

$$\Delta H^‡ = E_a - RT \quad (6)$$

$$\Delta G^‡ = \Delta H^‡ - T\Delta S^‡ \quad (7)$$

(k_b = Boltzmann's constant and h = Plank's constant). For each protein, the average for $\Delta H^‡$, $\Delta G^‡$, and $\Delta S^‡$ for the six trials, and those reported by Dannenberg and Kessler (1988a−c), are given in Table 1.

Concentration Dependence. As the six reconstituted whole milk samples had a range of native whey protein levels, it was possible to investigate the effect of initial native protein concentration on the thermal denaturation of β-Lg and α-lac. Using protein concentrations (C_0) relative to trial one, plots of ln $k_{1.5}$ against native β-Lg concentration for each reaction temperature yielded essentially horizontal lines (Figure 4). This indicated that the rate constants, $k_{1.5}$, at each temperature were independent of the initial β-Lg concentration; however, a slightly higher rate constant was observed at very low β-Lg concentrations. In contrast, a plot of ln k_1 against the native α-lac concentration (C_0) indicated that the rate constants for α-lac denaturation in the temperature range 70−85 °C may have been dependent on the initial native protein concentration, as a higher denaturation rate was obtained at lower initial native protein concentrations. For the 85−115 °C temperature range, the rate constants were essentially independent of the protein concentration (Figure 4).

DISCUSSION

For each protein and within certain concentration ranges, a linear relationship was obtained between protein concentration and the integrated intensity obtained from native PAGE and laser densitometry. At high protein concentrations, this relationship failed and the curves plateaued. By maintaining protein concentrations within the linear range, the gel electrophoretic method proved to be suitable for determining the degree of denaturation of the individual whey proteins.

The observation of first-order kinetics for the thermal denaturation of α-lac (Figure 2) is in agreement with earlier reports for this protein in skim milk (Lyster, 1970; Hillier, 1976; Hillier and Lyster, 1979; Manji and Kakuda, 1986; Dannenberg and Kessler, 1988a,b; Luf, 1988; Kessler and Beyer, 1991). The determined order of 1.5 for the thermal denaturation of both variants of β-Lg (Figure 2) is in agreement with that reported by Dannenberg and Kessler (1988a,b) and Kessler and Beyer (1991) for the denaturation of these proteins in skim milk; however, orders of 1 (Gough and Jenness, 1962; Luf, 1988) or 2 (Lyster, 1970; Hillier, 1976; Hillier and Lyster, 1979; Manji and Kakuda, 1986) have also been reported.

The fractional order of reaction indicates that the irreversible thermal denaturation process for β-Lg is complex, possibly involving a number of successive reaction steps (McKenzie et al., 1971; Sawyer et al., 1971; Ruegg et al., 1977). It is unlikely that the actual denaturation process, independent of other milk components, has an order of 1.5 as the reaction order is known to be dependent on the composition of the reaction medium. Denaturation of β-Lg in buffer solu-

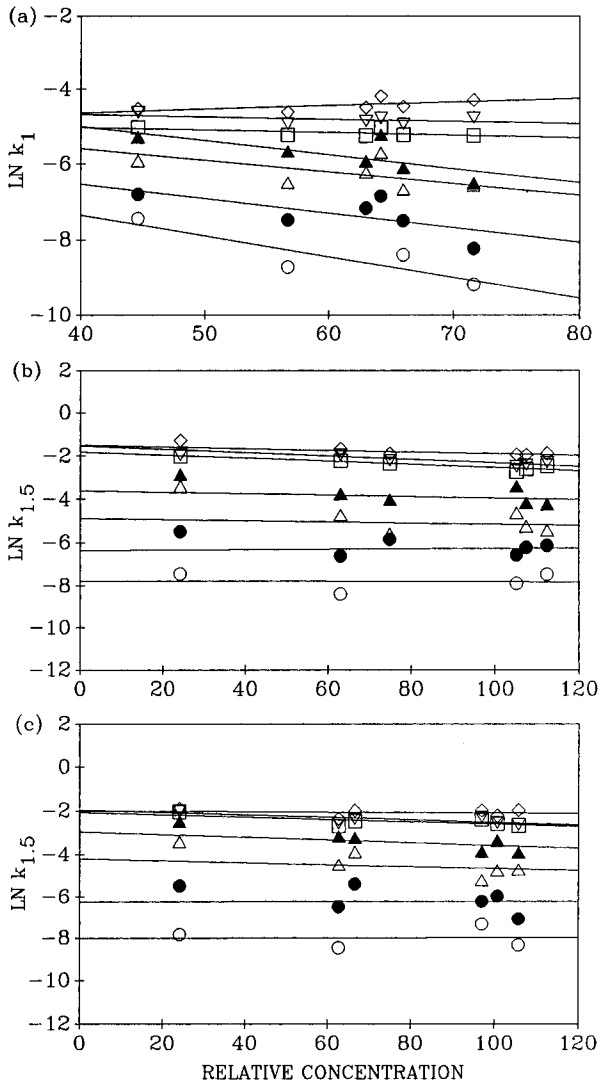

Figure 4. Relationship between initial native protein concentration and the rate constant, k_n, describing the denaturation of (a) α-lac, (b) β-Lg A, or (c) β-Lg B: ○, 70 °C; ●, 75 °C; △, 80 °C; ▼, 85 °C; □, 100 °C; ▽, 105 °C; ◇, 115 °C.

tions has been found to have a reaction order of 1 (Gough and Jenness, 1962) or 2 (Harwalkar, 1980). In sweet whey, the denaturation process for β-Lg has a reaction order of 2, and the order gradually decreases to 1.5 as the casein to whey protein ratio increases (Kessler and Beyer, 1991).

The rate and extent of irreversible denaturation of α-lac (Table 1) were comparable with those reported by Dannenberg and Kessler (1988a,b) but considerably higher than those calculated from the results of Hillier and Lyster (1979) and Luf (1988). In contrast, the rate and extent of irreversible denaturation of β-Lg (Table 1) were significantly higher than that reported by Dannenberg and Kessler (1988a,b). At any particular temperature below 100 °C, the B variant of β-Lg was irreversibly denatured to a greater extent than the A variant, whereas the reverse was observed at higher temperatures (Figures 2 and 3; Table 1). A similar observation was reported by Hillier and Lyster (1979); however, Dannenberg and Kessler (1988a,b) and Manji and Kakuda (1986) noted that the B variant was less heat stable than the A variant over the entire temperature range they studied.

As first reported by Lyster (1970), the thermal denaturation reaction for all three whey proteins showed a marked change in temperature dependence at about 80 °C for α-lac and at about 85 °C for β-Lg (Figure 3). This resulted in marked differences in the E_a values in the two temperature ranges (Table 1). For α-lac, the E_a for the 70−80 °C range was considerably lower than that reported by Dannenberg and Kessler (1988a,b) but comparable with that reported by Manji and Kakuda (1986) and Luf (1988). The E_a for the 80−115 °C temperature range was in good agreement with that reported by Dannenberg and Kessler (1988a,b) and comparable with that determined from the data of Hillier and Lyster (1979) and Manji and Kakuda (1986), although lower than that found by Luf (1988). For β-Lg variants A and B, the calculated E_a values in the 70−85 and 85−115 °C temperature ranges were similar to those reported by Dannenberg and Kessler (1988a,b), Gough and Jenness (1962), Manji and Kakuda (1986), and Luf (1988).

The average $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, and $\Delta S^{\ddagger}$ at each temperature (Table 1) were comparable with those reported by Gough and Jenness (1962), Dannenberg and Kessler (1988a,c), and Luf (1988). The E_a and $\Delta H^{\ddagger}$ for the denaturation of β-Lg B were higher than those of the A variant at temperatures in the 70−80 °C range but lower at higher temperatures. These tendencies were also observed by Dannenberg and Kessler (1988a,c) and Luf (1988). For all three proteins and at all temperatures, the $\Delta G^{\ddagger}$ values were very similar at about 100 kJ mol^{-1}. This value of $\Delta G^{\ddagger}$ appears to be characteristic for protein denaturation reactions as relatively constant values, also about 100 kJ mol^{-1}, were reported for the thermal denaturation of 18 different proteins (Labuza, 1980). For all three proteins, a positive $\Delta S^{\ddagger}$ was obtained in the low-temperature range, whereas negative values were obtained at higher temperatures.

Protein denaturation reactions and subsequent aggregation reactions are expected to give significantly different values for kinetic and thermodynamic parameters, which may allow a prediction as to which of these processes is rate-determining in the reaction mechanism. A denaturation process in which the tertiary structure of the protein is disrupted to give randomly coiled molecules involves the rupture of a large number of (weak) intramolecular bonds. This major conformational change in the protein is expressed as high values for both E_a and $\Delta H^{\ddagger}$ and a positive $\Delta S^{\ddagger}$, reflecting the lower state of order in the molecule. In contrast, lower E_a and $\Delta H^{\ddagger}$, along with a negative $\Delta S^{\ddagger}$, are expected for an aggregation process in which a few intermolecular bonds are formed and the state of order of the system is increased.

The thermodynamic parameters for the low-temperature range were typical of a reaction mechanism with the denaturation (thermal unfolding) of the protein as the rate-determining process, i.e. high E_a and $\Delta H^{\ddagger}$ along with a positive $\Delta S^{\ddagger}$ (Table 1). In the high-temperature range, the thermodynamic parameters were in the range expected if a typical chemical reaction process was the rate-determining step in the reaction mechanism and were therefore ascribed to an aggregation process. Both E_a and $\Delta H^{\ddagger}$ were considerably lower than at lower temperatures, and the negative $\Delta S^{\ddagger}$ indicated an increase in order as the reactants proceeded to the aggregated complex (Table 1).

The unusual temperature dependence of the rate constants (Figure 3) could be a consequence of different rate-determining steps in the two temperature ranges for a reaction mechanism involving at least two con-

secutive steps. Steventon et al. (1991) suggested three distinct stages in the denaturation process of globular proteins, with the rate of irreversible aggregation depending on the slowest process

denaturation/unfolding

$$P_n \rightleftharpoons D \tag{8a}$$

initiation of aggregation

$$2\ D \rightleftharpoons D_2 \tag{8b}$$

propagation of aggregation

$$D + D_x \rightarrow D_{x+1} \tag{8c}$$

(P_n = native protein, D = denatured protein, and D_x = aggregate of x denatured protein monomers).

It is possible to explain the temperature dependence of the thermal denaturation of whey proteins using the mechanism shown in eqs 8a−c. In the low-temperature range, the reversible disruption of the native molecular structure of the protein (i.e. conformation change or unfolding) is the rate-determining process and is associated with an increase in $\Delta S^{\ddagger}$ (eq 8a). At higher temperatures, irreversible chemical processes, probably aggregation reactions, become rate-determining (eq 8b or 8c). In a complex medium such as milk, the aggregation reaction is nonspecific and can occur between alike whey proteins, between different whey proteins, or between whey proteins and casein. According to eq 8a−c, native proteins cannot react other than to become denatured (unfolded) and only this unfolded protein can aggregate. Therefore, in the high-temperature range, the aggregation reaction must still proceed through the reversible first step of the reaction mechanism shown in eq 8a.

Experiments in which the denaturation kinetics of the whey proteins in sweet whey and skim milk were compared have shown that the casein proteins were not responsible for the temperature dependence of the rate constants as both systems displayed a marked change in slope of the Arrhenius diagrams for all three whey proteins (Kessler and Beyer, 1991). Interestingly, the thermodynamic parameters determined at low temperatures for the thermal denaturation of all three whey proteins fit in the range reported by Wood (1956) for the thermal denaturation of 18 different proteins, whereas those for the high-temperature range are significantly lower. This supports the hypothesis that in the low-temperature range the rate-determining step is the reversible unfolding of the protein tertiary structure, whereas at higher temperatures irreversible aggregation processes involving the unfolded protein become rate-determining.

For both β-Lg A and β-Lg B, the rate constants, $k_{1.5}$, obtained at a range of temperatures were essentially independent of the initial native protein concentration, although a somewhat higher rate constant was observed at very low β-Lg concentrations (Figure 4). Kessler and Beyer (1991) reported that the rate constants for the thermal denaturation of β-Lg were independent of the protein concentration in both sweet whey and skim milk systems; however, at very low initial β-Lg concentrations the denaturation reaction had a higher order than at higher protein concentrations. They attributed this to a change in reaction mechanism at these lower protein concentrations. The deviation of the rate constants at low β-Lg concentrations may have been a reflection of this change in reaction mechanism; however, there were insufficient data in these experiments to detect a small change in reaction order.

In contrast, the rate constants, k_1, for α-lac denaturation may have been dependent on the initial protein concentration in the temperature range 70−85 °C, as generally a higher denaturation rate was obtained at lower protein concentrations. At higher temperatures, the rate constants were independent of the protein concentration (Figure 4). Ruegg et al. (1977), using calorimetry, have shown that isolated α-lac is one of the least heat stable of the milk proteins; however, the denaturation at thermization temperatures is substantially reversible, which accounts for its apparent high thermal stability (Larson and Rolleri, 1955; Shulka, 1973). Kuwajima and Sugai (1978) have shown that major structural changes occur in α-lac at temperatures above about 50 °C. The reversible denaturation of α-lac at lower temperatures is due to calcium ion dissociation from and reassociation with the protein, which is normally a calcium metalloprotein (Hiraoka et al., 1980; Bernal and Jelen, 1984, 1985). At higher temperatures, further unfolding of the tertiary structure allows irreversible aggregation reactions to occur.

At low temperatures, the unfolding of the tertiary structure of the protein is the rate-determining step in the irreversible denaturation of α-lac. As the magnitude of the perturbations involved in the thermal denaturation of proteins can range from a reversible change in the spatial location of a single amino acid to the essentially irreversible total destruction of the secondary and tertiary structure, it is possible that renaturation of α-lac after heat treatment does not completely restore the native conformation. Alternatively, a partial unfolding of α-lac may have occurred during the processing to milk powder. In both cases, this does not influence the analytical determination of the protein but results in a more rapid denaturation on subsequent heating. At higher temperatures, irreversible chemical reactions involving aggregation of the unfolded protein molecules constitute the rate-determining process in the reaction mechanism. As a consequence, differences in the rate of unfolding of α-lac would have little influence on the irreversible aggregation reactions of α-lac. Both of these suppositions are supported by the observation that E_a and $\Delta H^{\ddagger}$ for the denaturation of α-lac at low temperatures were significantly lower than those reported by Dannenberg and Kessler (1988a−c), whereas E_a and $\Delta H^{\ddagger}$ in the higher temperature range were comparable with those in the earlier studies.

Further investigations are required to assess the nature of this concentration dependence of the rate constants for α-lac denaturation. It is interesting to note that this change in dependence does not correspond to the change in the slope of the Arrhenius plot for α-lac (about 80 °C) but coincides with that for β-Lg (about 85 °C), indicating that the irreversible denaturation of α-lac may be dependent on that of β-Lg, possibly through sulfhydryl−disulfide interchange reactions involving the free sulfhydryl groups of denatured β-Lg. It must be remembered that no other compositional differences between the reconstituted whole milk samples have been taken into consideration. Differences in composition could account for the slight scatter in the plots or the apparent concentration dependence of the rate constants for α-lac denaturation (Figure 4). Hillier et al. (1970) and Kessler and Beyer (1991) have shown that

the composition of the solution under study can have a marked effect on the reaction order and rate constants for the thermal denaturation of α-lac and β-Lg.

The results of this study have demonstrated that the processing of whole milk to a dried powder, the subsequent reconstitution, the native protein concentration, and the presence of milk fat do not markedly affect the reaction mechanism for β-Lg denaturation as the determined kinetic and thermodynamic parameters were similar to those reported for fresh milk systems. In contrast, the reaction mechanism for α-lac denaturation may be influenced by these factors as lower E_a and $\Delta H^{\ddagger}$ values than those reported in earlier studies were observed and, at low temperatures (70−85 °C), these parameters generally decreased in value when more severe processing conditions were employed. The results of this study confirm the complexity of whey protein denaturation in milk, which is manifested by a fractional order of β-Lg denaturation and a marked change in temperature dependence of the rate constants, resulting in very different kinetic parameters in the two temperature ranges.

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