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Effect of Protein, Nonprotein-Soluble Components, and Lactose Concentrations on the Irreversible Thermal Denaturation of β-Lactoglobulin and α-Lactalbumin in Skim Milk

SKELTE G. ANEMA,*,†,‡ SIEW KIM LEE,†,‡ AND HENNING KLOSTERMEYER†

Lehrstuhl für Chemie der Biopolymere, Technische Universität München, 85350 Freising, Germany, and Fonterra Research Centre, Private Bag 11029, Palmerston North, New Zealand

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The effect of protein, nonprotein-soluble components, and lactose concentrations on the irreversible denaturation of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) in reconstituted skim milk samples was studied over a wide temperature range (75−100 °C). The irreversible thermal denaturation of β-LG had a reaction order of 1.5 and that of α-LA had a reaction order of 1.0 in all systems and under all conditions. The rates of irreversible denaturation of β-LG and α-LA were markedly dependent upon the composition of the milk. At all temperatures, the irreversible denaturations of β-LG and α-LA were enhanced at a higher protein concentration and were retarded when the nonprotein-soluble components and lactose concentrations were increased. The effects of increasing the concentrations of lactose and nonprotein-soluble components were interpreted using the preferential hydration theory and allowed for the interpretation of the changes in the denaturations of β-LG and α-LA when the milk total solids concentration was increased.

KEYWORDS: Milk; β-lactoglobulin; α-lactalbumin; thermal denaturation; lactose; protein; soluble components

INTRODUCTION

The heat treatment of milk at temperatures above about 70 °C results in the irreversible denaturation of the whey proteins. Numerous studies have investigated these denaturation reactions, and many studies have completed full kinetic and thermodynamic evaluations that allow for predictive models to be developed (*1*−*11*).

Most of the studies have investigated the effect of heat on the irreversible denaturation of whey proteins in milk at its natural concentration. However, these denaturation reactions are markedly dependent upon the concentration and composition of the milk. Recent studies have shown that β-lactoglobulin (β-LG) denaturation in skim milk is markedly retarded by increasing the total solids concentration of the milk (*10*), whereas α-lactalbumin (α-LA) denaturation is hardly affected by the milk solids concentration (*11*).

It is uncertain what components of milk are influencing this unusual denaturation behavior when milk is concentrated to 4 times its natural levels, especially the marked differences between β-LG and α-LA. The concentration of milk not only increases the protein concentration but also the nonprotein colloidal components (especially colloidal calcium phosphate) and the nonprotein-soluble components (such as soluble calcium, phosphate, lactose, nonprotein nitrogen components, and the other soluble mineral components). Law and Leaver (*12*) have shown that both β-LG and α-LA denaturation are increased when the milk protein concentration is doubled and the milk is heated at 80 °C only. In a subsequent study, Law and Leaver (*13*) have also shown that the denaturations of both β-LG and α-LA are enhanced when the pH of the milk is increased from the natural pH and are retarded when the pH is decreased.

No full study of the effect of milk protein, nonprotein-soluble components, and lactose concentrations on the irreversible denaturation of β-LG and α-LA over wide concentration ranges and at a range of temperatures and holding times has been reported previously. This paper describes the results of studies on the irreversible thermal denaturation of β-LG and α-LA, in reconstituted skim milk in which the various components (protein, nonprotein-soluble components, and lactose) were concentrated. The temperature and time combinations were chosen to allow for a full kinetic and thermodynamic study on the denaturation reactions to be completed. The results of this study are compared with the previous studies in which all milk components were concentrated (*10*, *11*) and are used to deduce the relative importance of various components on whey protein denaturation and their roles in the denaturation mechanism.

MATERIALS AND METHODS

Skim Milk Samples with Increased Protein Concentrations. The method of Anema et al. (*14*) was used to produce milk samples with increased protein concentrations. Reconstituted skim milk samples of

* To whom correspondence should be addressed: Fonterra Research Centre, Private Bag 11029, Palmerston North, New Zealand. Telephone: +64-(6)-350-4649. Fax: +64-(6)-356-1476. E-mail: skelte.anema@fonterra.com.
† Technische Universität München.
‡ Fonterra Research Centre.

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9.6 total solids (TS) (w/w) were prepared by adding the appropriate quantity of low heat skim milk powder (New Zealand Dairy Board, Wellington, New Zealand) to water [purified through a Milli-Q apparatus (Millipore Corp., Bedford, MA)]. A small quantity (about 0.04%) of sodium azide was added to each of the milk samples as a preservative. The milk samples were allowed to stir for at least 12 h before further use to ensure equilibration (*15*). After equilibration, each milk sample was ultrafiltered using a 10 000 Da (nominal) hollow-fiber membrane cartridge and the associated pumping equipment (Amicon, Inc., Beverly, MA). Samples of the retentate were taken at various concentrations, and the protein content was estimated from the concentration factor and confirmed by electrophoresis.

Skim Milk Samples with Increased Nonprotein-Soluble Components Concentrations. The method of Anema et al. (*14*) was used to produce milk samples with increased nonprotein-soluble components concentrations. Reconstituted skim milk samples of 9.6, 19.2, and 28.8% TS (w/w) were prepared by adding the appropriate quantity of the low heat skim milk powder to purified water, with a small quantity (about 0.04%) of sodium azide added as a preservative. The milk samples were allowed to stir for at least 12 h before further use to ensure equilibration (*15*), and then each sample was ultrafiltered using a 10 000 Da (nominal) hollow-fiber membrane cartridge and the associated pumping equipment (Amicon, Inc., Beverly, MA). Samples of permeate (less than 10% of the total volume) were taken from each of the milk samples. Milk samples with 2, 3, and 4 times the nonprotein-soluble components levels were prepared by reconstituting skim milk powder in the permeate from the 9.6, 19.2, and 28.8% TS milk, respectively, to give an equivalent protein concentration to that of the 9.6% TS (w/w) skim milk. The milk samples were allowed to stir for several hours at room temperature and were then stored at 5 °C for 12 h before use. The protein content of the milk samples was checked by electrophoresis.

Skim Milk Samples with Increased Lactose Concentrations. Lactose solutions containing 5, 10, and 15% (w/w) lactose were prepared by dissolving lactose (BDH Laboratories, Poole, U.K.) in purified water and allowing these solutions to stir until the lactose was completely dissolved. Milk samples with increased lactose levels were prepared by reconstituting skim milk powder [equivalent protein levels to that of the 9.6% TS (w/w) skim milk] in the 5, 10, and 15% lactose solutions. The milk samples with altered lactose levels were allowed to stir for several hours at room temperature and were then stored at 5 °C for 12 h before use. A small quantity (about 0.04%) of sodium azide was added to each of the milk samples as a preservative. The protein content of the milk samples was checked by electrophoresis.

Heat Treatment. The method of heat treatment was similar to that reported in the studies on increased milk solids concentrations (*10*, *11*), because this would allow for a direct comparison between the results. Weighed aliquots (about 100 mg) of the various milk samples were transferred to small sealable plastic tubes. The milk samples were heated at temperatures in the range of 75−100 °C (±0.1 °C) for times from 0 to about 60 min in a thermostatically controlled oil bath. The heat-up time was estimated by inserting a thermocouple in selected sample tubes and monitoring the temperature change during heating. The estimated heat-up time was subtracted from the total heating times. After heat treatment, the milk samples were rapidly cooled in an ice bath for 5 min.

Dilution of Milk Samples. The milk samples (where required) were accurately diluted, by weighing, with water to a concentration comparable with that of the 9.6% TS (w/w) milk samples. Several small glass beads were added to each sample to aid dispersion. The samples were shaken vigorously to ensure homogeneous dispersion of the milk and were allowed to stand for 24 h before analysis. Milk samples at high protein concentrations aggregated during prolonged heat treatment at high temperatures; however, the dispersion method allowed for a representative sample of the milk slurry to be collected.

Polyacrylamide Gel Electrophoresis (PAGE). The level of native β-LG in the control and heat-treated milk samples was determined using native PAGE, as has been described previously (*8*). The casein and denatured whey proteins were removed from the milk by adjusting the pH to 4.6 and centrifuging out the precipitate using a bench centrifuge. The resultant supernatant was used for analysis of residual native whey proteins using the native-PAGE technique. The supernatant and milk samples were accurately diluted, by weight, with the native-PAGE sample buffer.

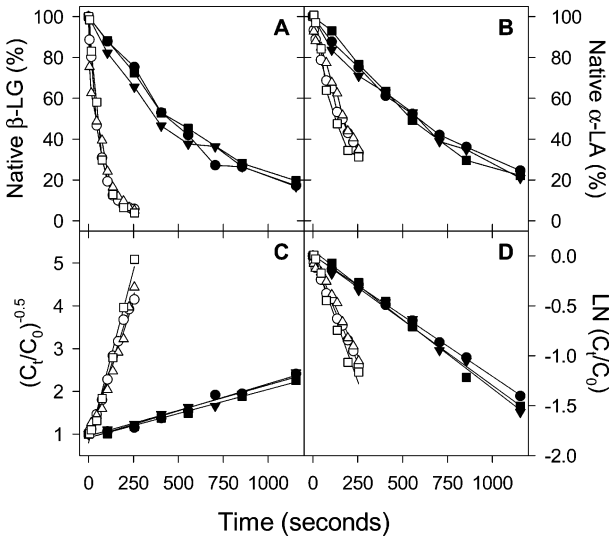

Figure 1. Comparison of the irreversible denaturations of β-LG and α-LA for three separate milk samples at 80 °C (●, ■, and ▼) and 95 °C (○, □, and ▽). (A) Denaturation of β-LG, (B) denaturation of α-LA, (C) kinetic evaluation of the denaturation of β-LG as a 1.5-order reaction, and (D) kinetic evaluation of the denaturation of α-LA as a first-order reaction. (● and ○) Milk 1, (■ and □) milk 2, and (▼ and ▽) milk 3.

After electrophoresis, the gels were stained using 0.1% (w/v) amido black 10B in 10% acetic acid and 25% isopropanol. After the gels were stained for 3 h, they were destained using a 10% acetic acid solution until a clear background was achieved. The gels were scanned using a Molecular Dynamics model P.D. computing densitometer (Molecular Dynamics, Inc., Sunnyvale, CA), and the integrated intensities of the β-LG and α-LA bands were determined using the Molecular Dynamics Imagequant integration software. No attempt was made to separate the two variants of β-LG, because these behaved similarly under the reaction conditions. The changes in the levels of native β-LG and α-LA as a consequence of the heat treatment were determined by comparing the band intensities of the residual proteins in the heated milk samples with the average band intensities of the proteins in two unheated samples, with corrections for differences induced by the various dilution steps in the sample preparations.

The concentrations of β-LG and α-LA in the milk samples were determined by comparing the band intensities of the proteins in the milk samples with standard curves prepared from purified β-LG and α-LA solutions of known concentrations.

RESULTS AND DISCUSSION

The proposed mechanism for the irreversible thermal denaturation of β-LG and α-LA in heated milk is considered to be a multistep process in which the reversible denaturation (unfolding) reaction and the irreversible aggregation reactions play important roles in determining the overall kinetic and thermodynamic processes in the irreversible reaction pathway (*6*, *8*−*11*). Unless otherwise stated, the denaturation reaction discussed throughout this paper refers to the combined process of reversible denaturation (unfolding) and irreversible aggregation and is therefore the irreversible thermal denaturation process.

Reproducibility of Determinations of Residual α-LA and β-LG. Representative gel electrophoresis patterns have been presented in a previous paper (*10*). Because of the scale of the experiments, only selected points were repeated to ensure that consistent results were obtained. In addition, the control milk

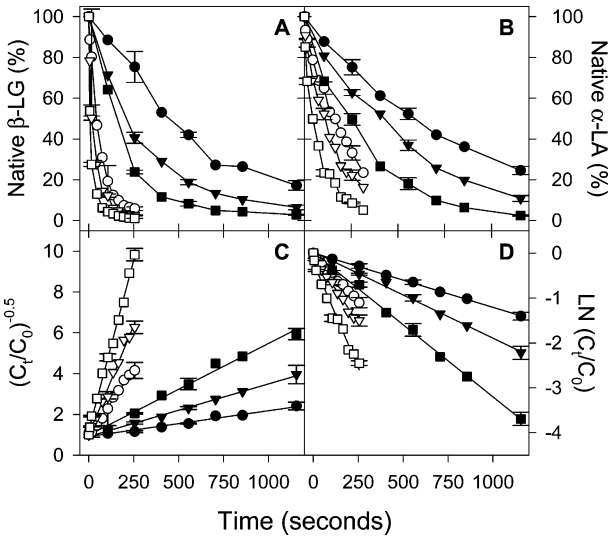

Figure 2. Effect of the protein concentration on the irreversible thermal denaturations of β-LG and α-LA at 80 °C (●, ▼, and ■) and 95 °C (○, ▽, and □). (A) Denaturation of β-LG, (B) denaturation of α-LA, (C) kinetic evaluation of the denaturation of β-LG as a 1.5-order reaction, and (D) kinetic evaluation of the denaturation of α-LA as a first-order reaction. (● and ○) 3.33 mg of β-LG/g and 1.30 mg of α-LA/g (skim milk), (▼ and ▽) 7.93 mg of β-LG/g and 3.10 mg of α-LA/g, and (■ and □) 18.16 mg of β-LG/g and 7.10 mg of α-LA/g.

sample was repeated for each set of experiments (i.e., for the milks with increased protein, increased nonprotein-soluble components, and increased lactose contents), which allowed for full errors to be calculated for this milk sample. Standard deviations were obtained for the repeated samples and are presented as error bars on relevant points in the figures and as standard deviations in the tables.

Figure 1 shows typical results for the level of irreversible denaturation of β-LG (**Figure 1A**) and α-LA (**Figure 1B**) in the control milk samples heated at 80 and 95 °C for various times. The kinetic evaluations of these data for the denaturation of β-LG as a 1.5-order reaction and for the denaturation of α-LA as a first-order reaction are shown in parts **C** and **D** of **Figure 1**, respectively. The degree of denaturation at each heating temperature/time combination was reproducible, with consistently low standard deviations between repeated measurements. For example, for the results in parts **A** and **B** of **Figure 1**, the standard deviation ranged from 0.9 to 6.1 for β-LG and from 1.8 to 6.8 for α-LA, which were typical for the experiments in this study. These results demonstrate the reproducibility of the heating methods and also the electrophoresis method for determining denaturation levels when appropriate running, staining, and destaining methods were employed and appropriate standards were used on the gels.

Effect of the Protein Concentration on the Irreversible Thermal Denaturation of β-LG and α-LA. Milk samples with increased protein concentrations, prepared by the ultrafiltration of skim milk to various concentrations, were heated at temperatures from 75 to 100 °C for times up to about 60 min before analysis by native PAGE. For selected temperatures and protein concentrations, the levels of native β-LG and α-LA remaining after the various heat treatments are shown in parts **A** and **B** of **Figure 2**, respectively. Equations 1 and 2 were used to analyze the results at each temperature and thereby to obtain the overall order, *n*, for the irreversible thermal denaturation reactions for β-LG and α-LA at each milk protein concentration.

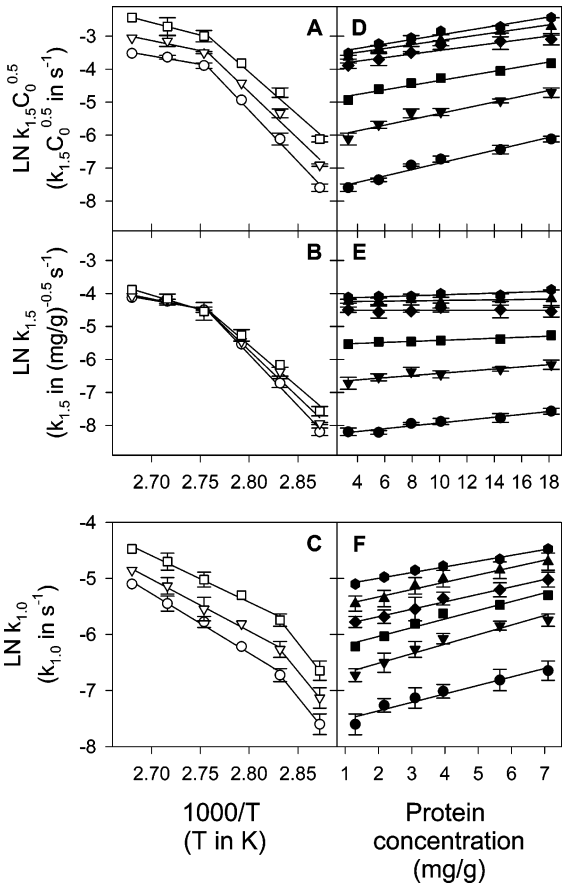

Figure 3. Effect of the protein concentration on the Arrhenius plots and rate constants for the denaturations of β-LG and α-LA. (A) Arrhenius plot for β-LG denaturation, raw data; (B) Arrhenius plot for β-LG denaturation, data corrected for the initial β-LG concentration; (C) Arrhenius plot for α-LA denaturation; (D) relationship between the initial β-LG concentration and the rate constant for β-LG denaturation, raw data; (E) relationship between the initial β-LG concentration and the rate constant for β-LG denaturation, data corrected for the initial β-LG concentration; and (F) relationship between the initial α-LA concentration and the rate constant for α-LA denaturation. (○) 3.33 mg of β-LG/g and 1.30 mg of α-LA/g (skim milk), (▽) 7.93 mg of β-LG/g and 3.10 mg of α-LA/g, (□) 18.16 mg of β-LG/g and 7.10 mg of α-LA/g, (●) 75 °C, (▼) 80 °C, (■) 85 °C, (◆) 90 °C, (▲) 95 °C, and (⬢) 100 °C.

$$\ln(C_t/C_0) = k_f t \quad (1)$$

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

where n = reaction order, k_f = rate constant, C_0 = initial native protein concentration, and C_t = concentration of native protein at time t.

The irreversible denaturation of β-LG was best described as a 1.5-order reaction, and the irreversible denaturation of α-LA was best described as a first-order reaction at all milk protein concentrations and at all temperatures (parts **C** and **D** of **Figure 2**, respectively). The correlation coefficients ranged from 0.97 to 0.99 for both β-LG and α-LA, and the *y* intercepts ranged from 0.89 to 1.2 for β-LG and from −0.21 to 0.04 for α-LA. The determined orders of 1.5 and 1.0 for the thermal denaturations of β-LG and α-LA, respectively, in heated milk systems are in agreement with those reported for milk at its natural concentration (*6*−*8*) and for concentrated milk (*10*, *11*). Oldfield et al. (*9*), using nonlinear regression analysis, reported reaction

Table 1. Effect of the Protein Concentration on the Rate Constants for the Irreversible Thermal Denaturation of β-LG and α-LA[a]

		Rate Constants for β-LG Denaturation [$k_f(C_0)^{0.5} \times 10^3$]					
protein concentration (mg/g)	protein concentration factor	75 °C	80 °C	85 °C	90 °C	95 °C	100 °C
3.33	1.00	0.5 (1)	2.2 (4)	7.2	20 (2)	26 (2)	29
5.55	1.67	0.6 (1)	3.4 (4)	9.9	24 (4)	34 (3)	39
7.93	2.38	1.0 (1)	4.8 (6)	12.1	30 (1)	43 (7)	47
10.09	3.03	1.2 (1)	5.0 (5)	14.0	38 (5)	44 (7)	58
14.45	4.34	1.6 (2)	6.9 (4)	17.4	43 (10)	58 (10)	66
18.16	5.45	2.2 (2)	9.0 (9)	22.1	45 (8)	67 (18)	87
		Rate Constants for α-LA Denaturation ($k_f \times 10^3$)					
protein concentration (mg/g)	protein concentration factor	75 °C	80 °C	85 °C	90 °C	95 °C	100 °C
1.30	1.00	0.5 (1)	1.2 (1)	2.1	3.1 (3)	4.3 (6)	6.1
2.17	1.67	0.7 (1)	1.5 (3)	2.4	3.4 (4)	4.7 (7)	6.9
3.10	2.38	0.8 (1)	1.9 (3)	3.0	3.9 (8)	5.9 (9)	7.8
3.95	3.03	0.9 (1)	2.3 (2)	3.6	4.7 (5)	6.7 (9)	8.4
5.65	4.34	1.1 (1)	2.9 (2)	4.2	5.5 (7)	7.8 (9)	9.5
7.10	5.45	1.3 (1)	3.2 (4)	4.9	6.6 (9)	9.1 (9)	11.4

[a] Standard deviations for repeated measurements are given in parentheses.

orders from 1.0 to 1.6 for β-LG denaturation and from 0.9 to 1.1 for α-LA denaturation in heated skim milk. According to eqs 1 and 2, the reaction rate constants, k_f, for each temperature can be obtained from the slopes of the straight lines, such as those shown in the selected examples in parts **C** and **D** of **Figure 2**. The rate constants for β-LG and α-LA are given in **Table 1**.

For both β-LG and α-LA, the relationship between the observed rate constants and the temperature of the reaction was analyzed using the Arrhenius equation (*6*, *8*). The logarithms of the rate constants [$\ln(k_f)$] were plotted against the reciprocal of the absolute temperature (parts **A** and **C** of **Figure 3**). It should be noted that, for β-LG, the observed rate constant [$k_f(C_0)^{0.5}$] was obtained directly from the experimental results, whereas the true rate constant (k_f) was corrected for the initial β-LG concentration, which was determined experimentally for each milk sample. Because α-LA appeared to follow first-order reaction kinetics, no correction for the protein concentration was made.

The relationship between $\ln(k_f)$ and $1/T$ was linear within certain temperature ranges, with a marked change in the temperature dependence at about 90 °C for β-LG and at about 80 °C for α-LA, which is consistent with previous papers (*5*−*11*). From the plots in **Figure 3**, the activation energies (E_a), enthalpies of activation ($\Delta H^{\ddagger}$), entropies of activation ($\Delta S^{\ddagger}$), and free energies of activation ($\Delta G^{\ddagger}$) were calculated using the appropriate equations (*6*, *8*). Selected results are presented in **Table 2**.

The results in **Figures 2** and **3** and **Tables 1** and **2** demonstrate the effect of the milk protein concentration on the irreversible denaturations of β-LG and α-LA and on the kinetic and thermodynamic properties of these denaturation reactions. For both β-LG and α-LA, the level of denaturation at any particular temperature/time combination, as measured by the fraction of native protein remaining after heating, increased as the milk protein concentration in the milk samples was increased (**Figure 2**). When the experimental rate constants for β-LG denaturation (parts **A** and **D** of **Figure 3** and **Table 1**) and α-LA denaturation (parts **C** and **F** of **Figure 3** and **Table 1**) are compared, it is apparent that, at each temperature, the rate constants increased linearly with an increasing protein concentration. Law and Leaver (*12*) examined the effect of the protein concentration on the denaturation of α-LA and β-LG upon heating at 80 °C, over a narrower concentration range than used here, and also observed linear increases in the rate constants as the protein concentration of the milk samples was increased.

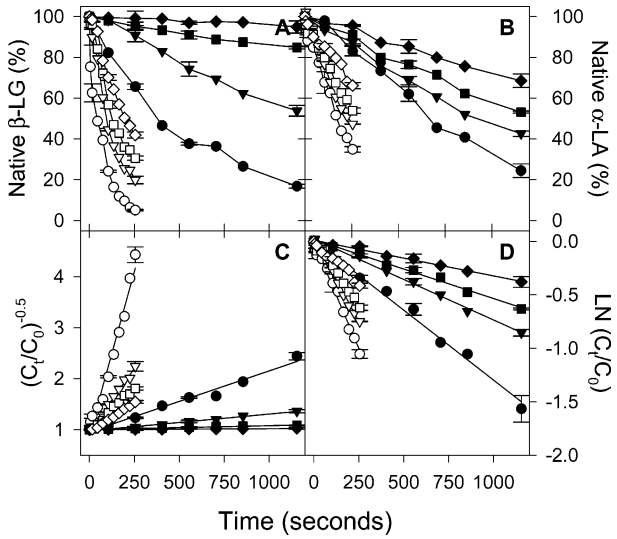

Figure 4. Effect of the nonprotein-soluble components concentration on the irreversible thermal denaturations of β-LG and α-LA at 80 °C (●, ▼, ■, and ◆) and 95 °C (○, ▽, □, and ◇). (A) Denaturation of β-LG, (B) denaturation of α-LA, (C) kinetic evaluation of the denaturation of β-LG as a 1.5-order reaction, and (D) kinetic evaluation of the denaturation of α-LA as a first-order reaction. (● and ○) 1× concentration factor (skim milk), (▼ and ▽) 2× concentration factor, (■ and □) 3× concentration factor, and (◆ and ◇) 4× concentration factor.

The effects of the concentration on the rate constants for the denaturation of β-LG obtained by Law and Leaver (*12*) were very similar to those observed here; however, the rate constants for the denaturation of α-LA were much lower than those found in the current study, even for milk at the natural concentration.

Because the irreversible denaturation of β-LG appeared to follow a reaction order of 1.5, according to eq 2, the experimental rate constant $k_{1.5}(C_0)^{0.5}$ was dependent upon the initial protein concentration. The concentration-independent rate constant, $k_{1.5}$, was calculated from the concentration of β-LG in the milk. The concentration-independent rate constants, $k_{1.5}$, for β-LG denaturation at each temperature were relatively independent of the initial protein concentration (parts **B** and **E** of **Figure 3**), although small increases in the rate of denaturation were observed at 75 and 80 °C. If the denaturation of α-LA followed true first-order reaction kinetics, the rate constants for denaturation should be independent of the protein concentration. However, the results in **Table 1** and **Figure 2** show a clear

Table 2. Effect of the Protein Concentration on the Kinetic and Thermodynamic Parameters for the Irreversible Thermal Denaturation of β-LG and α-LA[a]

protein concentration (mg/g)	protein concentration factor	temperature range (°C)	E_a (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (kJ mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ (kJ/mol)
			β-LG			
3.33	1.00	75–90	262 (22) a	259 (22) a	0.44 (7) a	102 (2) a
		90–100	42 (8) b	39 (7) b	–0.19 (5) b	101 (1) a
5.55	1.67	75–90	253 (24) a	250 (18) a	0.41 (7) a	103 (3) a
		90–100	52 (6) b	49 (5) b	–0.14 (3) b	101 (3) a
7.93	2.38	75–90	234 (18) a	232 (18) a	0.36 (6) a	102 (4) a
		90–100	50 (8) b	47 (7) b	–0.14 (5) b	100 (3) a
10.09	3.03	75–90	240 (19) a	236 (18) a	0.38 (8) a	102 (2) a
		90–100	47 (8) b	44 (8) b	–0.15 (6) b	100 (3) a
14.45	4.34	75–90	226 (24) a	223 (25) a	0.34 (5) a	101 (3) a
		90–100	49 (9) b	46 (9) b	–0.14 (4) b	99 (2) a
18.16	5.45	75–90	210 (27) a	207 (27) a	0.30 (9) a	100 (6) a
		90–100	61 (9) b	58 (10) b	–0.11 (3) b	99 (2) a
			α-LA			
protein concentration (mg/g)	protein concentration factor	temperature range (°C)	E_a (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (kJ mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ (kJ/mol)
1.30	1.00	75–80	185 (12) a	182 (12) a	0.22 (3) a	107 (1) a
		80–100	81 (13) b	78 (12) b	–0.08 (5) b	107 (1) a
2.17	1.67	75–80	155 (20) a	152 (20) a	0.13 (8) a	106 (2) a
		80–100	79 (7) b	76 (7) b	–0.08 (2) b	107 (2) a
3.10	2.38	75–80	176 (15) a	174 (15) a	0.19 (4) a	106 (1) a
		80–100	76 (8) b	74 (7) b	–0.09 (3) b	106 (1) a
3.95	3.03	75–80	191 (14) a	189 (14) a	0.24 (3) a	105 (2) a
		80–100	75 (15) b	72 (14) b	–0.09 (2) b	105 (2) a
5.65	4.34	75–80	198 (19) a	195 (18) a	0.26 (2) a	105 (2) a
		80–100	70 (11) b	67 (11) b	–0.11 (2) b	105 (2) a
7.10	5.45	75–80	184 (16) a	181 (16) a	0.22 (3) a	104 (2) a
		80–100	74 (8) b	71 (8) b	–0.09 (2) b	105 (2) a

[a] Standard deviations for repeated measurements are given in parentheses. For each protein, values with the same letter within a column are not significantly different ($p < 0.05$), as determined by ANOVA.

dependence of α-LA denaturation on the protein concentration, which indicates that this was only an apparent reaction order.

For β-LG and α-LA in both temperature ranges, E_a and $\Delta H^{\ddagger}$ were relatively unaffected by the protein concentration in the milk; however, for β-LG in the low-temperature range, E_a and $\Delta H^{\ddagger}$ did appear to decrease as the protein concentration in the milk was increased, although the differences were not statistically significant. Some caution is required in interpreting the results for α-LA in the low-temperature range because these were calculated from two points in the Arrhenius plots (**Figure 3C**), although the trends observed are probably indicative of the changes occurring. The E_a, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ values for the milk at the natural protein concentration were in the range reported previously (*4*−*6*, *8*−*11*).

Effect of the Nonprotein-Soluble Components Concentration on the Irreversible Thermal Denaturation of β-LG and α-LA. Milk samples with increased nonprotein-soluble components concentrations, prepared by reconstituting skim milk powder in milk permeates from milks of different concentrations, were heated at temperatures from 75 to 100 °C for times up to about 60 min before analysis by native PAGE. For selected temperatures and nonprotein-soluble components concentrations, the levels of native β-LG and α-LA remaining after the various heat treatments are shown in parts **A** and **B** of **Figure 4**, respectively. Equations 1 and 2 were used to analyze the results at each temperature and thereby to obtain the overall order, n, for the thermal denaturation reactions for β-LG and α-LA at each nonprotein-soluble components concentration. As with the effect of the protein concentration (**Figure 2**) and milk total solids concentration (*10*, *11*), the denaturation of β-LG was best described as a 1.5-order reaction and the denaturation of α-LA was best described as a first-order reaction at all concentrations of nonprotein-soluble components and at all temperatures (parts **C** and **D** of **Figure 4**, respectively). The correlation coefficients and the y intercepts were in a similar range to that observed when milk protein concentrations were varied. For β-LG and α-LA, the reaction rate constants, k_f, for each temperature are given in **Table 3**.

Figure 5 shows the analysis of the effects of temperature on the rate constants for both β-LG and α-LA using the Arrhenius relationship (*6*, *10*), where $\ln(k_f)$ values, obtained from the straight lines, such as those shown in parts **C** and **D** of **Figure 4**, are plotted against the reciprocal of the absolute temperature. The plots of $\ln(k_f)$ against $1/T$ showed the same linear relationships within certain temperature ranges as those obtained on changing protein concentrations (**Figure 3**) or milk total solids concentrations (*10*, *11*), with the break occurring at about 90 °C for β-LG and about 80 °C for α-LA. Using the appropriate equations (*6*, *8*), E_a, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ were calculated from the straight lines shown in **Figure 5**. Selected results are presented in **Table 4**.

The results in **Figures 4** and **5** and **Tables 3** and **4** demonstrate the effect of the nonprotein-soluble components on the irreversible denaturations of β-LG and α-LA and on the kinetic and thermodynamic properties of these denaturation reactions. For both β-LG and α-LA, the level of denaturation at any particular temperature/time combination, as measured by the fraction of native protein remaining after heating, decreased as the concentration of the nonprotein-soluble components in the milk samples was increased (**Figure 4**). When the experi-

Table 3. Effect of the Concentration of Nonprotein-Soluble Components on the Rate Constants for the Irreversible Thermal Denaturation of β-LG and α-LA[a]

protein concentration factor	Rate Constants for β-LG Denaturation [$k_f(C_0)^{0.5} \times 10^3$]					
	75 °C	80 °C	85 °C	90 °C	95 °C	100 °C
1	0.60 (13)	2.40 (32)	5.6	17 (3)	25 (5)	29
2	0.14 (2)	0.60 (9)	2.0	6.4 (9)	10 (2)	17
3	0.04 (1)	0.16 (2)	0.8	3.4 (5)	6.9 (9)	12
4	0.01 (1)	0.04 (1)	0.2	1.8 (2)	4.4 (6)	11
	Rate Constants for α-LA Denaturation ($k_f \times 10^3$)					
protein concentration factor	75 °C	80 °C	85 °C	90 °C	95 °C	100 °C
1	0.50 (9)	1.2 (2)	2.0	3.1 (8)	4.3 (7)	6.1
2	0.31 (5)	0.80 (9)	1.5	2.3 (4)	3.1 (3)	4.7
3	0.21 (2)	0.61 (9)	1.2	1.7 (3)	2.3 (3)	3.4
4	0.11 (2)	0.35 (7)	0.6	1.0 (1)	1.6 (1)	2.1

[a] Standard deviations for repeated measurements are given in parentheses.

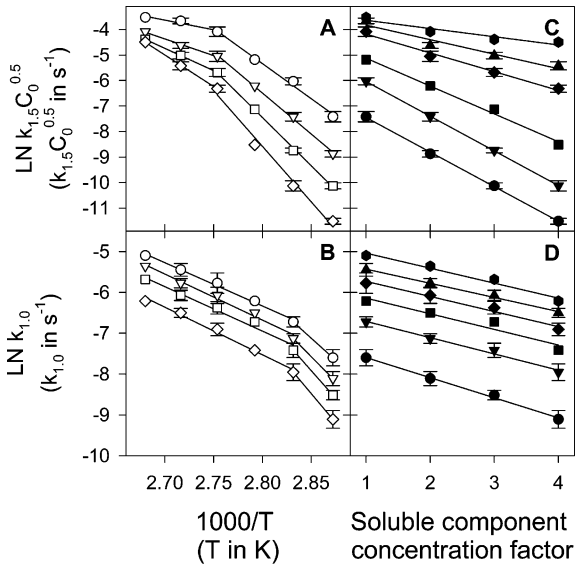

Figure 5. Effect of the nonprotein-soluble components concentration on the Arrhenius plots and rate constants for the denaturations of β-LG and α-LA. (A) Arrhenius plot for β-LG denaturation, (B) Arrhenius plot for α-LA denaturation, (C) relationship between the concentration factor for nonprotein-soluble components and the rate constant for β-LG denaturation, and (D) relationship between the concentration factor for nonprotein-soluble components and the rate constant for α-LA denaturation. (○) 1× concentration of nonprotein-soluble components (skim milk), (▽) 2× concentration of nonprotein-soluble components, (□) 3× concentration of nonprotein-soluble components, (◇) 4× concentration of nonprotein-soluble components, (●) 75 °C, (▼) 80 °C, (■) 85 °C, (◆) 90 °C, (▲) 95 °C, and (⬢) 100 °C.

mental rate constants for β-LG denaturation (parts **A** and **C** of **Figure 5** and **Table 3**) and α-LA denaturation (parts **B** and **D** of **Figure 5** and **Table 3**) are compared, it is apparent that, at each temperature, the rate constants decreased linearly with an increasing nonprotein-soluble components concentration. For α-LA, the effect of increasing the nonprotein-soluble components was the same at all temperatures, so that a series of parallel lines was obtained when the rate constants at each concentration were plotted against $1/T$ (**Figure 5B**) or when the rate constants

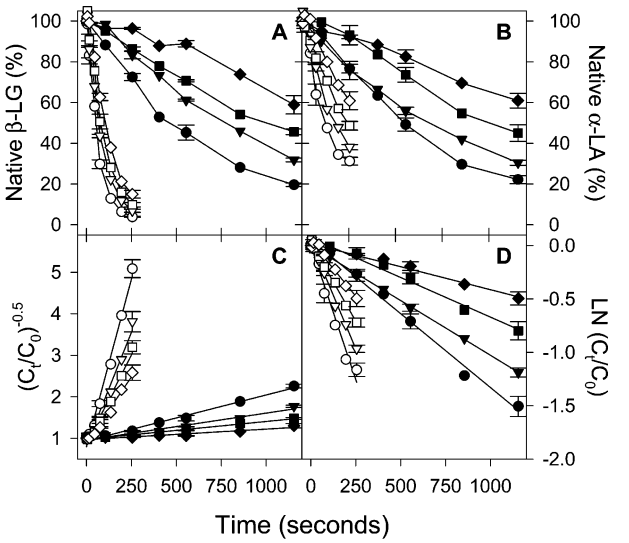

Figure 6. Effect of the lactose concentration on the irreversible thermal denaturations of β-LG and α-LA at 80 °C (●, ▼, ■, and ◆) and 95 °C (○, ▽, □, and ◇). (A) Denaturation of β-LG, (B) denaturation of α-LA, (C) kinetic evaluation of the denaturation of β-LG as a 1.5-order reaction, and (D) kinetic evaluation of the denaturation of α-LA as a first-order reaction. (● and ○) No added lactose (skim milk), (▼ and ▽) 5% (w/w) added lactose, (■ and □) 10% (w/w) added lactose, and (◆ and ◇) 15% (w/w) added lactose.

at each temperature were plotted against the concentration factor (**Figure 5D**). Although the rate of denaturation of β-LG was retarded as the concentration of nonprotein-soluble components increased, the effect became less pronounced as the temperature was increased. As a consequence, the lines observed when the rate constants at each concentration were plotted against $1/T$ tended to converge as the temperature increased (**Figure 5A**) and the slopes of the lines decreased as the temperature increased when the rate constants at each temperature were plotted against the concentration factor (**Figure 5C**).

When the E_a, $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta G^\ddagger$ values are examined, some trends are apparent (**Table 4**). For β-LG, E_a, $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta G^\ddagger$ tended to increase in both temperature ranges as the concentration of nonprotein-soluble components increased. Of particular interest is the observation that, in the high-temperature range, E_a and $\Delta H^\ddagger$ increased markedly and $\Delta S^\ddagger$ changed sign from negative to positive as the nonprotein-soluble components concentration increased. Similar effects were observed when the total solids concentration of the milk was increased (*10*). For α-LA in the low-temperature range, E_a, $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta G^\ddagger$ increased as the concentration of nonprotein-soluble components increased, whereas, in the high-temperature range, E_a, $\Delta H^\ddagger$, and $\Delta S^\ddagger$ remained relatively constant and $\Delta G^\ddagger$ increased slightly (**Table 4**). Again, these results for α-LA in the low-temperature range should be treated with some caution because they were calculated from two points in the Arrhenius plots (**Figure 5B**), although the trends observed are probably indicative of the changes occurring.

The unusual temperature dependence of the rate constants for both β-LG and α-LA (**Figures 3** and **5**) and the calculated thermodynamic parameters (**Tables 2** and **4**) for milk at its natural concentration have been interpreted using a multistep reaction mechanism with different rate-determining steps in the two temperature ranges. This interpretation suggests that the thermodynamic parameters are consistent with a mechanism in which the reversible denaturation reaction (unfolding) is rate-limiting in the low-temperature range and irreversible aggrega-

Table 4. Effect of the Concentration of Nonprotein-Soluble Components on the Kinetic and Thermodynamic Parameters for the Irreversible Thermal Denaturation of β-LG and α-LA[a]

β-LG					
concentration factor for nonprotein-soluble components	temperature range (°C)	E_a (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (kJ mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ (kJ/mol)
1	75–90	262 (18) a	259 (22) a	0.44 (6) a	102 (2) a
	90–100	42 (7) d	39 (7) d	–0.19 (2) d	101 (1) a
2	75–90	266 (11) a	263 (10) a	0.44 (4) a	108 (2) b
	90–100	108 (7) e	106 (6) e	0.002 (8) e	105 (3) b
3	75–90	314 (16) b	311 (16) b	0.56 (4) b	111 (3) b
	90–100	146 (13) f	143 (13) f	0.10 (6) f	106 (3) b
4	75–90	361 (16) c	358 (16) c	0.68 (4) c	115 (4) b
	90–100	205 (16) g	202 (15) g	0.26 (6) g	107 (4) b
α-LA					
concentration factor for nonprotein-soluble components	temperature range (°C)	E_a (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (kJ mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ (kJ/mol)
1	75–80	185 (8) a	182 (8) a	0.22 (3) a	107 (1) a
	80–100	81 (13) d	78 (12) d	–0.08 (5) c	107 (1) a
2	75–80	201 (11) a,b	198 (11) a,b	0.25 (3) a,b	106 (2) a
	80–100	94 (11) d	81 (11) d	–0.05 (4) c	108 (2) a,b
3	75–80	225 (13) b,c	222 (13) b,c	0.32 (4) b	109 (2) a,b
	80–100	80 (8) d	87 (8) d	–0.06 (5) c	109 (1) a
4	75–80	236 (16) c	233 (16) c	0.35 (6) b	111 (2) b
	80–100	97 (9) d	94 (9) d	–0.05 (4) c	111 (2) b

[a] Standard deviations for repeated measurements are given in parentheses. For each protein, values with the same letter within a column are not significantly different ($p < 0.05$), as determined by ANOVA.

tion reactions involving denatured whey proteins are rate-limiting at higher temperatures (*6*, *8*−*11*). Assuming that this interpretation is correct, this suggests that increasing the nonprotein-soluble components concentration (**Table 2**) or the total solids concentration (*10*) of the milk results in a change in the rate-determining step for β-LG in the high-temperature range from one where irreversible aggregation reactions are rate-limiting (negative $\Delta S^{\ddagger}$ and low values for E_a and $\Delta H^{\ddagger}$) to one where reversible denaturation (unfolding) reactions are rate-limiting (positive $\Delta S^{\ddagger}$ and high values for E_a and $\Delta H^{\ddagger}$). Interestingly, this change in the rate-determining step in the high-temperature range is not observed for α-LA when the concentration of nonprotein-soluble components is increased (**Table 2**).

Effect of the Lactose Concentration on the Irreversible Thermal Denaturation of β-LG and α-LA. A major component, although not necessarily the most important component, of the nonprotein-soluble components is lactose. Milk samples with increased lactose concentrations, prepared by reconstituting skim milk powder in lactose solutions of different concentrations, were heated at temperatures of 80 and 95 °C for times up to about 60 min before analysis by native PAGE. The levels of native β-LG and α-LA remaining after the various heat treatments are shown in parts **A** and **B** of **Figure 6**, respectively. As discussed previously, eqs 1 and 2 were used to analyze the results at each temperature and thereby to obtain the overall order, *n*, for the irreversible thermal denaturation reactions for β-LG and α-LA at each lactose concentration. As with the effect of the protein concentration (**Figure 2**), nonprotein-soluble components concentration (**Figure 4**), and milk total solids concentration (*10*, *11*), reaction orders of 1.5 and 1.0 best described the denaturations of β-LG (**Figure 6C**) and α-LA (**Figure 6D**), respectively. Similarly, the correlation coefficients and the *y* intercepts were in a similar range to that observed when milk protein concentrations or soluble components concentrations were varied. For β-LG and α-LA, the reaction rate constants, k_f, for each temperature are given in **Table 5**.

Table 5. Effect of the Concentration of Lactose on the Rate Constants for the Irreversible Thermal Denaturation of β-LG and α-LA[a]

Rate Constants for β-LG Denaturation [$k_f(C_0)^{0.5} \times 10^3$]		
lactose concentration factor	80 °C	95 °C
1	2.2 (4)	32 (6)
2.1	1.3 (3)	22 (3)
3.2	0.9 (2)	17 (2)
4.3	0.50 (6)	13 (2)
Rate Constants for α-LA Denaturation ($k_f \times 10^3$)		
lactose concentration factor	80 °C	95 °C
1	1.4 (2)	5.0 (5)
2.1	1.0 (2)	4.1 (6)
3.2	0.74 (9)	2.9 (3)
4.3	0.54 (6)	2.1 (2)

[a] Standard deviations for repeated measurements are given in parentheses.

As with increasing the nonprotein-soluble components concentration, increasing the lactose concentration decreased the rate of irreversible denaturation of both β-LG and α-LA at any particular temperature/time combination (**Figure 6**). When the experimental rate constants for both β-LG (**Figure 7A** and **Table 5**) and α-LA (**Figure 7B** and **Table 5**) are compared with the concentration of lactose in the samples relative to that in the original skim milk, it is apparent that the rate of denaturation at any particular temperature decreased linearly with an increasing lactose concentration. For α-LA, the effect of lactose appeared to be similar at both temperatures investigated, whereas for β-LG, the rate of denaturation was retarded to a greater extent at 80 °C than at 95 °C (**Figure 7** and **Table 5**). This is similar to the effect observed when the nonprotein-soluble components were concentrated (**Figure 5** and **Table 3**) or when the total solids concentration of the milk was increased (*10*, *11*). Plock and Kessler (*16*) reported that the denaturation of β-LG was retarded when the concentration of sweet whey was increased, whereas the denaturation of α-LA was unaffected by the whey concentration. In subsequent studies, Plock et al.

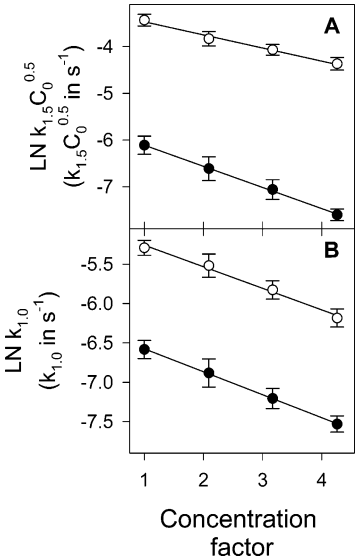

Figure 7. Effect of the lactose concentration on the rate constants for the denaturations of β-LG and α-LA. (A) Relationship between the concentration factor for the lactose concentration and the rate constant for β-LG denaturation and (B) relationship between the concentration factor for the lactose concentration and the rate constant for α-LA denaturation. (○) 80 °C and (●) 95 °C.

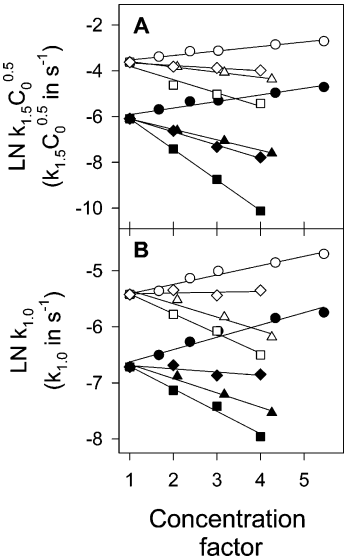

Figure 8. Comparison of the effects of the concentrations of protein (● and ○), nonprotein-soluble components (■ and □), lactose (▲ and △), and total solids (◆ and ◇) on the rate constants for the denaturation of β-LG (A) and α-LA (B) at 80 °C (●, ■, ▲, and ◆) and 95 °C (○, □, △, and ◇). The rate constants for the effect of total solids were derived from Anema (*10*, *11*).

(*17*, *18*) found that increasing lactose concentrations retarded the denaturation of β-LG, whereas lactose had little effect on the denaturation of α-LA.

Although intercomponent interactions will be important, the results of this study, with comparisons with those observed when the total solids concentration of milk is increased (*10*, *11*), can be used to gain some understanding of the effects of increasing the concentration of various milk components on the thermal denaturations of β-LG and α-LA. To aid the discussions, the rate constants obtained at 80 and 95 °C for β-LG and α-LA when the concentrations of milk protein, nonprotein-soluble components, lactose, and total solids are increased relative to those observed in the milk at its natural concentration are compared in **Figure 8**.

When the total milk concentration was increased, the rate of β-LG denaturation was retarded; however, this retardation was less pronounced as the temperature of heating was increased (*10*). In contrast, the denaturation of α-LA appeared to be unaffected by the milk concentration, with similar rates of denaturation at all milk concentrations regardless of the heating temperature (*11*). From these denaturation results, the known self-association behavior of β-LG (*19*), and the observation that the dissociation of dimeric β-LG to monomeric species is the first essential step in the denaturation reaction (*20*−*22*), Anema (*10*) proposed that the differences in the denaturation behavior between β-LG and α-LA may be due to the increased self-association of β-LG as a consequence of the increased lactose concentration when the milk solids concentration is increased. Because α-LA does not self-associate, no retardation in denaturation was observed when the milk concentration was increased, and because the aggregation reactions were rate-limiting at higher temperatures, the self-association of β-LG was less important in stabilizing the denaturation reactions at these higher temperatures.

However, when the effects of the protein concentration (**Figures 2** and **3**) and the effects of the nonprotein-soluble components concentration (**Figures 4** and **5**) are separated, it is evident that this hypothesis by Anema (*10*) needs to be re-evaluated. Clearly, increasing the protein concentration resulted in an increase in the rate of denaturation of both β-LG and α-LA (**Figures 2**, **3**, and **8**), and this increase was similar at all temperatures investigated (**Figures 3** and **8**). Interestingly, when the rate constants for β-LG were corrected for the protein concentration, on the basis of a reaction order of 1.5, there was little effect of the concentration, as expected for the concentration-independent rate constant $k_{1.5}$. The increase in the rate of denaturation of α-LA is not expected for a first-order reaction, indicating that the irreversible denaturation is more complex and has only apparent first-order kinetics. Hillier et al. (*4*) also suggested that the denaturation of α-LA was pseudo-first-order. In a recent study, Wehbi et al. (*23*) showed that the rate of denaturation of α-LA was dependent upon the initial protein concentration, with an increased rate at higher protein concentrations. The level of residual α-LA was measured by an immunoreactivity method, and these authors suggested that a higher initial α-LA concentration may result in protein aggregation, which would lead to a lower level of accessible epitopes in the protein and hence a decreased immunoreactivity. This cannot explain our results, because the electrophoresis method does not rely on immunoreactivity but rather the level of protein remaining in the native configuration.

When the nonprotein-soluble components were concentrated, the denaturations of both β-LG and α-LA were retarded; however, the effects on these two proteins were somewhat different (**Figures 4**, **5**, and **8**). For β-LG, increasing the nonprotein-soluble components caused a substantial retardation of denaturation in the lower temperature range, and this effect became less pronounced at higher temperatures (parts **A** and **C** of **Figures 4** and **5**). In contrast, for α-LA, the retardation upon increasing the nonprotein-soluble components was less pronounced than for β-LG and was similar at all temperatures investigated (parts **B** and **D** of **Figures 4** and **5**).

From these results, it appears that, upon increasing the total solids concentration of milk (both protein and nonprotein-soluble

components), the increase in the denaturation rate for α-LA upon increasing the protein concentration was almost exactly offset by the retardation of the reaction rate by increasing the nonprotein-soluble components concentration (**Figure 8B**). This effect was similar at all temperatures, and as a consequence, increasing the total solids appeared to have little effect on the rate of denaturation of α-LA (*11*). For β-LG in the low-temperature range, the increase in the rate of denaturation upon increasing the protein concentration was not sufficient to offset the retardation in the rate of denaturation upon increasing the nonprotein-soluble components concentration (parts **A** and **C** of **Figure 5** and **Figure 8A**); therefore, the denaturation of β-LG was retarded by increasing the total solids concentration of the milk. However, as the temperature was increased, the nonprotein-soluble components were less effective in retarding the denaturation of β-LG (**Figures 4** and **5**). As a consequence, the increase in the total solids concentration appeared to have a smaller effect on the denaturation of β-LG at the higher temperatures and particularly above about 90 °C (*10*).

Lactose is the major component of the nonprotein-soluble components. Increasing the lactose concentration had a similar but not identical effect to increasing the nonprotein-soluble components concentration. The denaturation of α-LA was retarded upon increasing the lactose concentration, and a similar effect was observed at both temperatures investigated (**Figures 6B** and **7B**). The denaturation of β-LG was also retarded upon increasing the lactose concentration; however, the effect was less pronounced at 95 °C than at 80 °C (**Figures 6A** and **Figure 7A**). When the rate constants were compared, it was evident that, for both β-LG and α-LA, increasing the lactose concentration had less of an effect than increasing the nonprotein-soluble components concentration (**Figure 8**). Clearly, other nonprotein-soluble components such as calcium, citrate, and phosphate play some role in the irreversible denaturation of the whey proteins, possibly through their effects on the pH, ionic strength, or buffering properties of the milk.

The effect of the nonprotein-soluble components concentration or lactose concentration on the denaturation of β-LG and α-LA can be explained using the preferential hydration theory of Arakawa and Timasheff (*24*, *25*). For globular proteins such as β-LG and α-LA, increased levels of solutes such as sugars increase the ordering of the water structure around the protein molecules. This effectively excludes the sugar from the protein environment and results in unfavorable increases in the free energy of the system. Because these effects will increase with increases in the surface area of the proteins, unfolded proteins will have more unfavorable protein−water interactions than native proteins. Therefore, the native structure of the protein is stabilized. The results in **Figures 4**−**7** clearly demonstrate that increasing the level of nonprotein-soluble components or lactose retards the denaturation of both β-LG and α-LA.

The effects of these components on protein self-association could explain the contrasting effect of temperature on β-LG and α-LA denaturation when the nonprotein-soluble components concentration or lactose concentration is increased. The preferential hydration theory predicts that increased levels of certain solutes (such as sugars) will favor the associated state for proteins that can undergo self-association (*24*). The formation of contacts between the protein molecules decreases the total surface area and therefore the free energy of the system and, thus, is a favored conformation. Because β-LG is known to undergo self-association under certain conditions (*19*) and because the dissociation of dimeric β-LG to monomeric species is the first step in irreversible denaturation (*20*−*22*), the stabilizing effect of the increased milk solids/nonprotein-soluble components/lactose concentration could be due to a shift in the equilibrium between the monomer and dimer to a state that favors the dimer.

In the low-temperature range for β-LG (75−90 °C), the denaturation reaction (unfolding) is considered to be the rate-determining step in the reaction mechanism (*6*−*8*, *10*). The first step in the denaturation of β-LG involves the separation of dimeric β-LG to the monomeric form (*19*, *22*). Therefore, the increased self-association of β-LG upon increasing the lactose, nonprotein-soluble components, or total solids concentration of the milk would result in a reduced rate of denaturation in the low-temperature range because this self-association effectively reduces the concentration of the reactive species (β-LG monomer). In the higher temperature range for β-LG (above 90 °C), aggregation reactions involving the unfolded β-LG are considered to be rate-determining (*6*−*8*, *10*); therefore, the self-association of β-LG may be less effective in stabilizing the denaturation reaction at these higher temperatures. This explanation allows for the same overall reaction mechanism to hold for all milk compositions and therefore is consistent with the unchanged reaction order at all milk compositions investigated.

Under this hypothesis, increasing the nonprotein-soluble components or lactose concentration stabilizes α-LA and β-LG to thermal denaturation through an increase in the ordering of the water structure around the protein molecules, which favors the native state of the protein. This effect would be similar at all temperatures. However, increasing the nonprotein-soluble components or lactose concentration also increases the self-association of β-LG, which may stabilize β-LG to thermal denaturation at low temperatures but may be less effective at higher temperatures. The overall effect is that increasing the nonprotein-soluble components or lactose concentration will stabilize α-LA to a similar extent at all temperatures, whereas the stabilization of β-LG will be temperature-dependent with a diminished effect at higher temperatures.

This study has demonstrated that the irreversible denaturation of the major whey proteins, β-LG and α-LA, is strongly dependent upon the composition of the milk system. An unusual difference between the denaturations of β-LG and α-LA when the milk total solids concentration was increased was observed, where the denaturation of β-LG was retarded by increasing the milk concentration (*10*) and the denaturation of α-LA was unaffected (*11*). When the composition of specific milk components was changed, it was possible to demonstrate that both β-LG and α-LA denaturation were enhanced by increasing the protein concentration and that the effect was similar for both proteins. Increasing the nonprotein-soluble components concentration or lactose concentration retarded the irreversible denaturations of both β-LG and α-LA; however, for α-LA, the effect was similar at all temperatures, whereas for β-LG, these components retarded the denaturation to a greater effect at lower temperatures (<90 °C) than at higher temperatures. The effect of nonprotein-soluble components and lactose on denaturation could be explained by the preferential hydration theory of Arakawa and Timasheff (*24*, *25*), with the difference between β-LG and α-LA being due to the tendency for β-LG to self-associate, which may be temperature-dependent. It appears that, when milk is concentrated, the effect of increasing the nonprotein-soluble components concentration (decreased rate of denaturation) and the effect of increasing the protein concentration (increased rate of denaturation) almost exactly compensate each other for α-LA, so that the denaturation appears to be unaffected by increasing the milk concentration. In contrast, for β-LG, the

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increase in the nonprotein-soluble components concentration has a larger effect than the increase in the protein concentration, so that the denaturation of β-LG appears to be retarded at increased milk concentrations.

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