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Kinetics and Mechanism of Lactosylation of α-Lactalbumin

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The lactosylation of α-lactalbumin in aqueous solution was followed at $pH_c = 6.0$, 6.3, 7.0, 7.3, and 7.9 and constant ionic strength ($I = 0.080$) at 50−60 °C by reversed-phase high-performance liquid chromatography (RP-HPLC) and electrospray mass spectrometry (MS). The rate of the lactosylation reaction increased with increasing pH and with temperature most significantly at lower pH. The rate of lactosylation could be described by an acid dissociation curve corresponding to pK_a of the ϵ-amino group of lysine in α-lactalbumin. From initial rates for conditions of excess of lactose, pseudo-first-order rate constants were calculated and further transferred into second-order rate constants by dividing with the lactose concentration. Second-order rate constants for protonated and unprotonated lysine in α-lactalbumin both showed Arrhenius behavior, and using transition-state theory, $\Delta H^{\#} = 31 \pm 2$ kJ/mol and $\Delta S^{\#} = -266 \pm 48$ J/(mol · K) were determined for the unprotonated form and $\Delta H^{\#} = 158 \pm 49$ kJ/mol and $\Delta S^{\#} = 80 \pm 150$ J/(mol · K) for the protonated form, respectively. On the basis of the marked differences in activation parameters, initial formation of a lactosylamine is suggested as rate-determining for reaction between lactose and a protonated lysine in α-lactalbumin, while subsequent water elimination to form a Schiff base becomes rate-determining for the unprotonated form.

KEYWORDS: α-Lactalbumin; lactosylation; pH-effect; activation parameters

INTRODUCTION

Whey protein powders are widely used in food products because of high nutritional value, desirable sensory characteristics, and excellent functional properties (*1*). However, nonenzymatic browning reactions, also known as Maillard reactions, occur easily during drying of whey concentrates and in foods with whey proteins used as ingredients (*2*). The initial step in the Maillard reaction is an addition of a reducing sugar to an amine, a peptide, or a protein. In milk products, the primary reactants are lactose and milk proteins such as caseins, β-lactoglobulin, and especially α-lactalbumin.

Maillard reactions of milk proteins leads to loss of nutritive value because of blockage of lysine residues, which no longer are available for digestion (*3*, *4*). More advanced Maillard reactions also result in formation of antioxidative compounds, compounds with mutagenic properties, and cross-linkage of proteins, which play a role in diabetes (*5*−*7*).

The structure of α-lactalbumin, which is stabilized by calcium ions, is well-characterized (*8*, *9*). The reactive sites of lactosylation in whey proteins are primarily the ϵ-amino group of lysine. Of the 12 lysine residues in α-lactalbumin, the main lactosylation site is the ϵ-amino group of Lys98, but also ϵ-amino groups of Lys5, Lys13, Lys94, Lys108, Lys114, and Lys122 and the NH_2-terminal group are known to react with lactose (*10*, *11*). The reactive form of lactose has been identified as the open-chain form with the carbonyl carbon reacting through nucleophilic addition of the ϵ-amino group of lysine followed by water loss (**Figure 1**) (*12*, *13*). The ring opening of lactose often followed as mutarotation needs also to be considered since

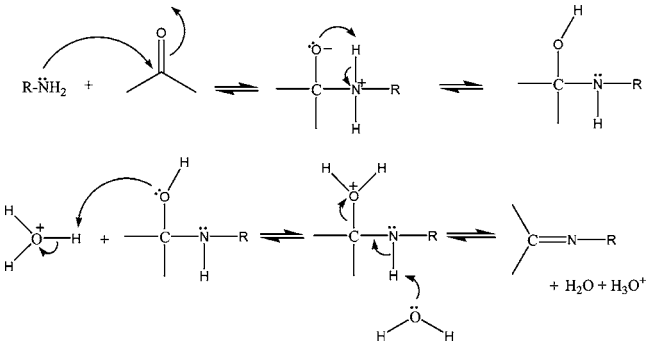

Figure 1. Nucleophilic addition of an amine to a carbonyl carbon to give a tetrahedral carbonyl addition compound (1), followed by dehydration of the tetrahedral carbonyl addition compound and formation of an imine or Schiff base (2).

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it under certain conditions may be the rate-determining step of the lactosylation.

α-Lactalbumin will denaturate in reactions competing with lactosylation at increasing temperature, especially at extremes of pH and for conditions of decreasing calcium and lactose concentrations compared to milk. Other metal ions than calcium such as sodium and magnesium, however, stabilize the structure of α-lactalbumin, and the presence of these metal ions will accordingly protect α-lactalbumin against thermal denaturation. In the pH range 5.2−6.1, the denaturation of α-lactalbumin is at its minimum (*14*−*19*).

Lactosylated α-lactalbumin has previously been detected in skim milk powder, whey protein concentrates, and infant formula and typically in greater amounts than lactosylated *β*-lactoglobulin (*10*, *11*, *20*, *21*). Furthermore, the lactosylation of α-lactalbumin leads to higher solubility and better emulsifying properties compared to the nonlactosylated α-lactalbumin, which makes investigation of lactosylated α-lactalbumin relevant in relation to modification of functional properties (*20*). A kinetic investigation of the reaction between α-lactalbumin and lactose was accordingly initiated in which both temperature and pH were varied to identify the actual reactants, determine the activation parameters, and suggest a mechanism for the lactosylation reaction. A better understanding of the initial step in the Maillard reaction will provide the tools for a better control of Maillard reactions during food processing.

MATERIALS AND METHODS

Reaction Solutions. Reaction solutions were prepared with the following concentrations: 0.080 mM α-lactalbumin, 0.111 M α-D-lactose, 2.04 mM $CaCl_2$, and adjusted to $I = 0.080$ with NaCl. Throughout the study, pH was based on concentration standards, and the definition $pH_c = \log [H^+]$ was used. The buffer concentration was 0.050 M, and for $pH_c = 6.0$, 2-*N*(morpholino)ethanesulfonic acid (MES) buffer was used, for $pH_c = 6.3$ and 7.0, imidazole buffer was used, and for $pH_c = 7.3$ and 7.9, tris(hydroxymethyl)-aminomethane (tris) buffer was used. α-Lactalbumin produced from bovine milk at the Arla Foods Innovation Centre under industrial conditions was purified according to the method of Kristiansen et al. (*22*), and the exact concentration of stock solutions was determined by absorbance measurement at 280 nm using the absorption coefficient of 29 642 $M^{-1}cm^{-1}$ (*23*). Analytical grade α-D-lactose·H_2O, NaCl, $CaCl_2$·H_2O, and tris were obtained from Merck (Damstadt, Germany). MES and imidazole were obtained from Sigma Chemical (St. Louis, MO). The reaction solutions were thermostated in water baths at 50 °C, 55 °C, and 60 °C, and aliquots were withdrawn at various reaction times, cooled on ice to stop the reaction, and subsequently stored in a freezer at −18 °C until analysis. Buffers were prepared to have the desired pH_c values at 50 °C, but since the pK_a value of the buffers decrease with temperature (*24*), the buffers were adjusted with 1 M NaOH for experiments at 55 °C and 60 °C. To determine pH_c in the samples, EMF was measured at the temperature of the experiment (50 °C, 55 °C, or 60 °C), using a combination glass electrode (6.0224.100, Metrohm, Herisau, Switzerland) and concentration standard of hydrochloric acid ($1.0 \cdot 10^{-2}$ and $1.0 \cdot 10^{-3}$ M with 0.080 ionic strength adjusted with NaCl). pH_c was calculated according to

$$pH_c = -\log [H^+]_{sample} = 2.000 + \frac{EMF_{pH\,2} - EMF_{sample}}{EMF_{pH\,2} - EMF_{pH\,3}} \quad (1)$$

The ionic strength was not altered significantly by the addition of NaOH. pH of the samples increased during cooling, and it was necessary to neutralize pH in the samples prior to freezing to prevent high-pH protein denaturation.

Separation by RP-HPLC and Identification by MS. The thawed samples were filtered through a 0.22-μm filter and analyzed with an Agilent (Palo Alto, CA) 1100 Series HPLC system with a C-18 column (Symmetry 300-5, 150 × 2.1 mm i.d.) from Waters (Milford, MA) and a flow of 0.3 mL/min. The HPLC system was coupled to a LC/MSD electrospray mass spectrometer. The quadropole was scanned from *m*/*z* 1750 to *m*/*z* 2850 with 0.89 scan/s. The sample temperature was 4 °C in the autosampler and the column temperature was 60 ± 0.8 °C. The injection volume was 20 μL. Gradient elution was used with eluent A: 0.1% TFA in H_2O and eluent B: 0.08% TFA in acetonitrile and H_2O (90:10). The H_2O was filtered by suction through a Buchner funnel and filter paper with 0.45-μm pores was used, and after mixing, the eluents were treated in an ultrasound bath for 15 min. A gradient program of 55 min was used: 0−2 min with 15% B, 2−20 min from 15% B to 40% B, 20−44 min from 40% B to 49% B, 44−47 min from 49% B to 85% B, 47−48 min from 85% B to 15% B, and 48−55 min with 15% B. All changes in amount of eluent B were linear over time. TFA was obtained from Sigma Chemical (St. Louis, MO), and acetonitrile was obtained from Rathburn (Walkerburn, Scotland).

The absorbance of the samples was measured at 214 nm. The area of a nonheated sample corresponding to zero reaction time was used as concentration standard ($area_0$), and the area of a heated sample to time *t* ($area_t$) was used to calculate the concentration of α-lactalbumin and lactosylated α-lactalbumin (c_t) over time according to eq 2:

$$c_t = (c_0/area_0) \cdot area_t \quad (2)$$

The α-lactalbumin concentration of the nonheated sample corresponding to zero reaction time was determined from the concentration of the stock solution. The initial rate for appearance of lactosylated α-lactalbumin was calculated by linear regression analysis of the concentration versus time using the SAS 8.2 package, SAS Institute, Inc., USA. GraFit 4.0 (Erithacus Software Ltd., Middlesex, U.K.) was used for nonlinear regression analysis of the pH profile and Origin 7.0 (OriginLab Corporation, Northampton, USA) for calculations related to the Arrhenius plot.

RESULTS AND DISCUSSION

Using RP-HPLC with the developed gradient program, it became possible to separate α-lactalbumin and lactosylated α-lactalbumin as will be evident from the two chromatograms shown in **Figure 2**. The most intense peak in the chromatograms ($R_t = 30$ min) displayed a molecular mass of 14176 g/mol, which corresponds to α-lactalbumin. The peaks with lower retention times ($R_t = 28$−29 min) all displayed a molecular mass of 14500 g/mol, which corresponds to monolactosylated α-lactalbumin. Mass spectra of α-lactalbumin and lactosylated α-lactalbumin from the RP-HPLC separation are shown in **Figure 3**. In **Figure 3A**, the *m*/*z* ratios 1773.1, 2026.3, 2363.8, and 2836.3 all correspond to α-lactalbumin ($z = 8$, 7, 6, and 5, respectively). The *m*/*z* ratio 2417.7 corresponds to lactosylated α-lactalbumin ($z = 6$). In **Figure 3B**, the *m*/*z* ratios 2072.9, 2417.8, and 2418.4 correspond to lactosylated α-lactalbumin ($z = 7$, 6, and 6, respectively). The *m*/*z* ratio 2366.7 ($z = 6$) gives the molecular mass 14194 g/mol, which tentatively was assigned to α-lactalbumin with an oxidized methionine moiety. The monoisotopic molecular masses of α-lactalbumin and lactosylated α-lactalbumin are 14177 and 14501 g/mol, respectively.

In the HPLC chromatograms, two separated monolactosylated forms of α-lactalbumin appear. The separation of the two forms indicates different hydrophobicity of the two forms. The most obvious explanation is that the two forms are α-lactalbumin lactosylated at different lysine residues or different groups of lysine residues. As mentioned in the Introduction, the lactosylation sites of α-lactalbumin are the different ϵ-amino groups of lysine and to some extent the NH_2-terminal group (*10*, *11*). Dependent on which amino groups lactose reacts with, the

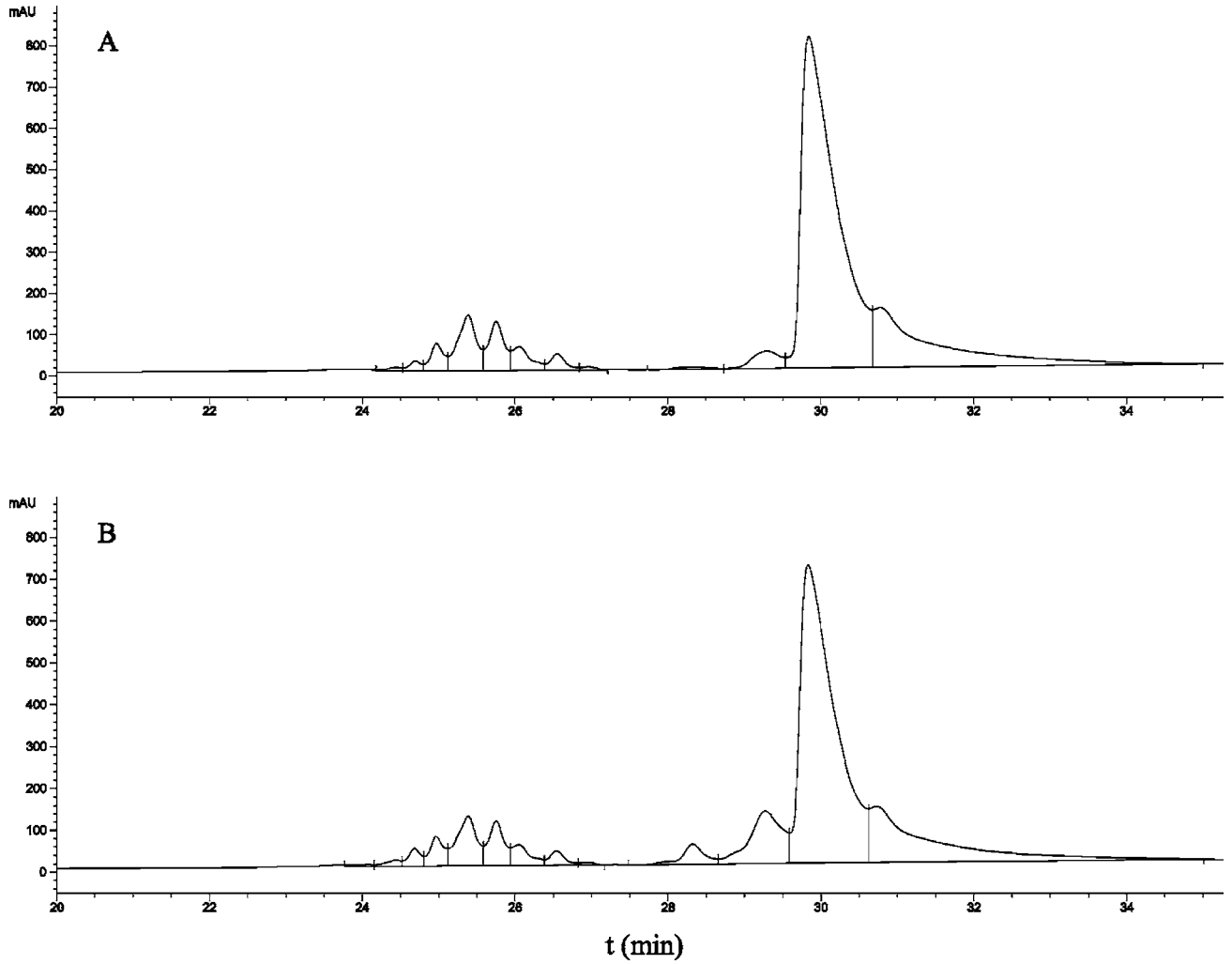

Figure 2. Chromatogram of a sample containing 0.080 mM α-lactalbumin, 111 mM α-D-lactose, 2.04 mM $CaCl_2$, and 0.050 M pH buffer (pH_c = 7.9) with I = 0.080 heated for (A) 0 and (B) 3 h, respectively, at 55 °C. The peak with retention time at 30 min is α-lactalbumin, and the peaks with retention time of 28–29 min are monolactosylated α-lactalbumin.

hydrophobicity of the lactosylation product might be different and thereby cause the observed chromatographic separation of the two forms. It is the experience at Arla Foods Innovation Centre that the whey protein powder produced only contains one monolactosylated form. The formation of the two monolactosylated forms of α-lactalbumin in the present study might be due to other reaction conditions in dilute aqueous solution compared to the reaction conditions during evaporation of water from the whey concentrates. The mobility of lactose is higher in dilute aqueous solution than in products under drying, and therefore lactose might have access to other lysine groups buried in the native structure of α-lactalbumin.

Lactosylation of α-lactalbumin occurs as a bimolecular reaction:

$$\text{α-lactalbumin} + \text{lactose} \xrightarrow{k_2} \text{lactosylated α-lactalbumin (a Schiff base)} \quad (3)$$

The rate of disappearance of native α-lactalbumin depends on lactosylation and on denaturation and may be expressed by eq 4.

$$\frac{-d[\text{α-lactalbumin}]}{dt} = k_2[\text{α-lactalbumin}][\text{lactose}] + k_{\text{den}}[\text{α-lactalbumin}] \quad (4)$$

In the present study, the molar ratio between lactose and α-lactalbumin is 1000:1, and lactose concentration may accordingly be considered to be constant. The reaction can be described as a pseudo-first-order reaction with an observed rate constant ($k_{\text{obs}} = k_2[\text{lactose}] + k_{\text{den}}$) as evident from eq 5:

$$\frac{-d[\text{α- lactalbumin}]}{dt} = k_{\text{obs}}[\text{α-lactalbumin}] \quad (5)$$

When the reaction was allowed to take place at elevated temperatures, the peak of α-lactalbumin and also of lactosylated α-lactalbumin started to decrease with time, especially for high pH conditions. The apparent decrease in concentration can be ascribed to the formation of a molten globule state of α-lactalbumin, which is a predenaturated form, as has previously been described (*19*). The rate of denaturation of α-lactalbumin dominates at temperatures above 60 °C and it is not possible to determine the rate constants for the lactosylation reaction for higher temperatures with any accuracy. Hence, the reaction temperatures were restricted to be lower than temperatures used in industrial processes, for example, below the commonly used pasteurization temperature for milk of 72 °C. It should be clear from **Figure 4** that at 60 °C for the present pH_c of 7.3, the loss of native α-lactalbumin still is similar to the formation of lactosylated α-lactalbumin, but also that 60 °C probably presents the temperature limit for this assumption.

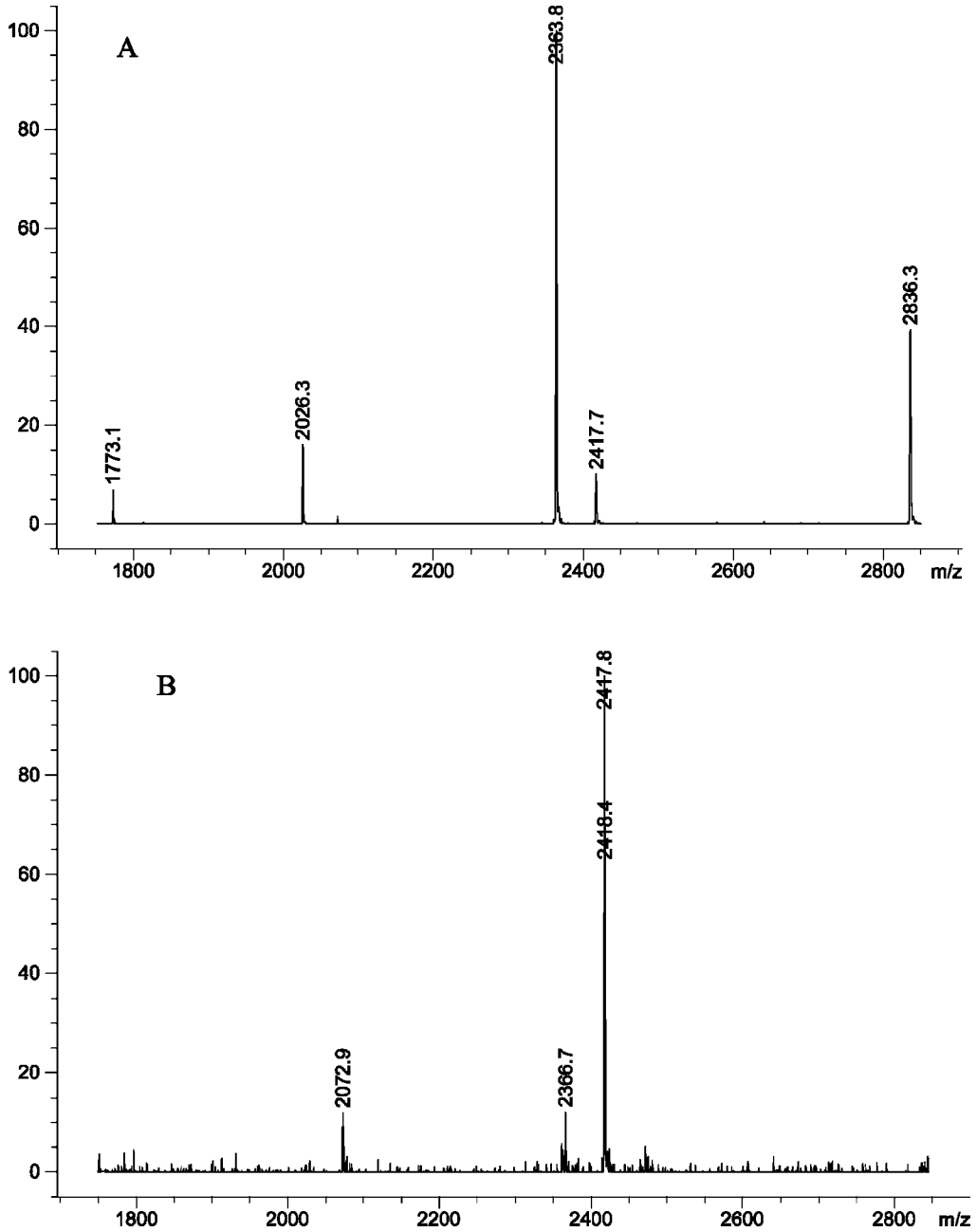

Figure 3. Mass spectra of a sample containing 0.080 mM α-lactalbumin, 111 mM α-D-lactose, 2.04 mM $CaCl_2$, and 0.050 M pH buffer ($pH_c = 7.0$) with $I = 0.080$ heated for 6 h at 60 °C. A is in the interval of the peak of α-lactalbumin ($R_t = 30$ min), and B is in the interval of the peak of lactosylated α-lactalbumin ($R_t = 28–29$ min).

For the more detailed kinetic analysis using moderate temperature and pH conditions, only the rate of formation of lactosylated α-lactalbumin was accordingly considered neglecting denaturation under these conditions:

$$\frac{d[\text{lactosylated α-lactalbumin}]}{dt} = k_{obs}[\text{α-lactalbumin}] \quad (6)$$

Heating of lactose in solution under alkaline conditions leads to isomerization to lactulose via the Lobry de Bryun−van Ekenstein rearrangement by 1,2-enolization and thereby arises the possibility of reaction between α-lactalbumin and both lactose and lactulose. Lactulose will further degrade through 2,3-enolization to galactose, various C5/C6 compounds, and carboxylic acids (mainly formic acid) (*25*, *26*). Such degradation reactions occur mainly at temperatures above 100 °C and have activation energies above 100 kJ/mol. Since the reaction conditions in the present study are no higher than 60 °C, the isomerization and the subsequent degradation will be minimal, and pH is not expected to decrease because of formation of formic acid.

The equilibrium constant for mutarotation of lactose at 50 °C has the value 1.63 and depends on the foreword and backward reaction rates as seen in eq 7 (*27*).

$$K_{50\,°C} = \frac{[\beta\text{-lactose}]}{[\text{α-lactose}]} = \frac{k_\alpha}{k_\beta} = 1.63 \quad (7)$$

The rate constant of mutarotation at 50 °C and pH 6.57 has the value $k_\alpha + k_\beta = 4.63 \cdot 10^{-3}\ s^{-1}$ as extrapolated from values in the temperature range of 0.5−45 °C (*28*). The rate constant for

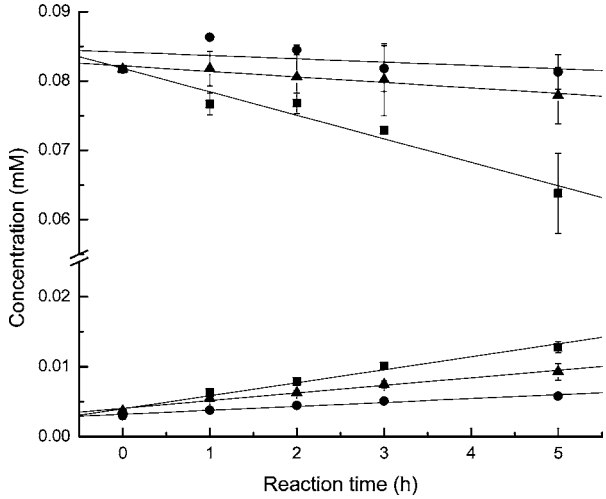

Figure 4. Disappearance of α-lactalbumin (upper curves) and formation of lactosylated α-lactalbumin (lower curves) at $pH_c = 7.3$ at 50 °C (●), 55 °C (▲), and 60 °C (■). The concentrations in the reaction mixture at zero reaction time were 0.080 mM α-lactalbumin, 111 mM α-D-lactose, 2.04 mM $CaCl_2$, and 0.050 M pH buffer ($pH_c = 7.3$) and $I = 0.080$.

Table 1. *P*-Values of Linear Regression Analysis of the Data Obtained from RP-HPLC Experiments for Formation of Lactosylated α-Lactalbumin

heating temperature	50 °C	55 °C	60 °C
$pH_c = 6.0$	<0.0001	0.0018	<0.0001
$pH_c = 6.3$	0.0033	0.0005	<0.0001
$pH_c = 7.0$	0.0014	<0.0001	<0.0001
$pH_c = 7.3$	<0.0001	<0.0001	<0.0001
$pH_c = 7.9$	<0.0001	<0.0001	0.0005

mutarotation of α-lactose to yield β-lactose has accordingly the value $k_\alpha = 2.87 \cdot 10^{-3}\ s^{-1}$ as calculated from eq 7. From a comparison with the rate constant found for the lactosylation of α-lactalbumin at 50 °C and $pH_c = 6.3$ which have the value $k_{obs} = 5.1 \cdot 10^{-8}\ s^{-1}$, it is concluded that the rate of mutarotation of lactose will not be rate-determining for lactosylation since a factor of 10^5 separates the two reaction steps.

In **Figure 4**, the formation of lactosylated α-lactalbumin with time is presented for reaction conditions of $pH_c = 7.3$. The rate of formation of lactosylated α-lactalbumin increases with increasing temperature. The initial rate of reaction was calculated for all reaction conditions by linear regression analysis. *P* values obtained from linear regression analysis are shown in **Table 1**, and all values indicate that the curves of lactosylated α-lactalbumin are linear at all heating conditions. The rate for lactosylation was determined from the slopes of the curves for formation of lactosylated α-lactalbumin.

The reaction was followed for up to approximately 10% of lactosylation. The initial reaction rate (in $mM \cdot s^{-1}$) determined as shown in **Figure 4** was converted to pseudo-first-order rate constants k_{obs} (s^{-1}) by division with the initial α-lactalbumin concentration (0.080 mM in all cases) in agreement with eq 6. The pseudo-first-order rate constant determined for varying pH_c and temperature was further converted to second-order rate constants by division with the lactose concentration of 111 mM used in all experiments to ensure pseudo-first-order conditions. This rate constant denoted as k_{obs}/[lactose] still depends on pH at each temperature as seen in **Figure 5**.

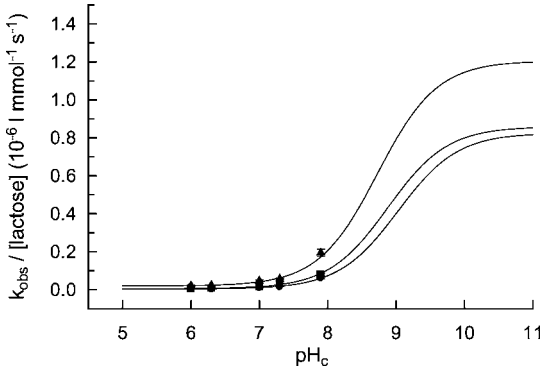

Figure 5. Second-order rate constant (k_{obs}/[lactose]) for lactosylation of α-lactalbumin as a function of pH at 50 °C (●), 55 °C (■), and 60 °C (▲). Full lines calculated by nonlinear regression analysis according to eq 7 using the acid dissociation constant for lysine in α-lactalbumin.

Table 2. Second-Order Rate Constants, Activation Enthalpy, and Activation Entropy for Reaction of Lactose with Protonated α-Lactalbumin and Unprotonated α-Lactalbumin Calculated from Energy of Activation and Pre-Exponential Factors from the Arrhenius Plot in **Figure 6**[a]

parameter		pK_a	k_2^A (protonated form)	k_2^B (unprotonated form)
k_2 /L mol^{-1} s^{-1}	50 °C	9.0	$3.3 \pm 0.3 \cdot 10^{-9}$	$8.3 \pm 0.3 \cdot 10^{-7}$
	55 °C	8.9	$5.1 \pm 0.1 \cdot 10^{-9}$	$8.6 \pm 0.8 \cdot 10^{-7}$
	60 °C	8.7	$20 \pm 2 \cdot 10^{-9}$	$1.2 \pm 0.1 \cdot 10^{-6}$
$\Delta H^{\#}$ /kJ mol^{-1}			158 ± 49	31 ± 2
$\Delta S^{\#}$ /J mol^{-1} K^{-1}			80 ± 150	-266 ± 48

[a] The dissociation constants (pK_a) were extrapolated from (*25*) to 50 °C, 55 °C, and 60 °C using the Van't Hoff equation.

k_{obs}/[lactose] is expected to depend on the dissociation of the reactive ϵ-amino group of lysine in α-lactalbumin according to eq 8:

$$\frac{k_{obs}}{[\text{lactose}]} = \frac{k_2^B K_a + k_2^A [H^+]}{[H^+] + K_a} \qquad (8)$$

In eq 8, k_2^B is the second-order rate constant for the nonprotonated ϵ-amino group, k_2^A is the second-order rate constant for the protonated ϵ-amino group, and K_a is the acid dissociation constant of the ϵ-amino group of lysine (*29*). The dissociation constant (pK_a) has been determined to be 10.43 at 10 °C and 9.86 at 25 °C (*30*), and these values were extrapolated to 50 °C, 55 °C, and 60 °C using the van't Hoff equation. As an ammonium ion, the pK_a value for lysine is insensitive to changes in ionic strength, and no corrections were applied.

As may be seen from **Figure 5**, a reasonable fit was obtained for pH_c up to 8 and for all three temperatures. Notably, k_2^A is significant different from zero as is seen in **Table 2**, in which the two second-order rate constants are reported for all three temperatures. The temperature dependence of k_2^B and k_2^A was analyzed according to the Arrhenius equation, and the activation energies were determined from the linear plot obtained for both reactions (*31*).

From the transition-state theory, the activation enthalpy ($\Delta H^{\#}$) and activation entropy ($\Delta S^{\#}$) of the lactosylation of α-lactalbumin were calculated at 25 °C according to the Eyring equation (*31*) and presented in **Table 2**. The higher activation barrier for lactosylation of the protonated lysine residue is in accordance with the amino group being a better nucleophile when it is unprotonated ($R-NH_2$) at alkaline pH values (*32*). At alkaline

Table 3. Activation Energies for Reaction of Lactose with Milk Proteins

system studied	pH	temperature range (°C)	medium	reaction measured	E_a (kJ mol^{-1})	reference
lactose + whey	n.s.[a]	25–45	a_w = 0.33–0.65	loss of lysine residues	24–33	(*35*)
lactose + whey	n.s.[a]	25–45	a_w = 0.33–0.65	color formation	29–34	(*35*)
lactose + casein	6.5	37–60	a_w = 0.52	loss of lysine residues	125	(*36*)
lactose + α-lactalbumin	6.0–7.9	50–60	aqueous solution, ionic strength = 0.080 (NaCl)	formation of Schiff base	acidic/alkaline: 161 ± 49/34 ± 2	present study

[a] Not specified.

pH, the reaction will proceed rapidly even at low temperatures, but at acidic pH heating is required to overcome the higher activation energy barrier for the protonated form.

For the unprotonated amino group, $\Delta S^{\#}$ was −266 ± 48 J/(mol · K), while the protonated form had a value close to zero with $\Delta S^{\#}$ = 80 ± 150 J/(mol · K). The negative value of $\Delta S^{\#}$ for the unprotonated form corresponds to a bimolecular reaction and indicates that the two molecules during the reaction obtain a more ordered transition state compared to the reactants. For the protonated form of lysine, $\Delta S^{\#}$ was positive but close to zero, indicating a transition state with slightly decreased order. The positive activation entropy could indicate an increase in solvation as water bound to reactants prior to reaction may form free water molecules during reaction. Considering a reaction involving a nucleophilic attack on a carbonyl carbon atom, protonation of the carbonyl carbon facilitates the attack, as the carbonyl carbon has an increased electron deficiency and will hereby be more reactive toward nucleophiles such as amines (*6*, *32*). Another way of describing the reaction of the protonated carbonyl group at acidic pH is that a H_3O^+ molecule is bound to the carbonyl group with hydrogen bonds and that H_3O^+ molecules are released when the positive charged carbonyl group reacts with the ammonium ion. Again, considering a reaction involving a nucleophilic attack of an amino group on a carbonyl carbon atom, the nucleophilic attack is the rate-determining step at acidic pH, as protonation of the amino group causes decreased nucleophilicity of the amino group and thereby a lower reaction rate. At alkaline pH, the rate-determining step is the dehydration of water, as this requires an acidic catalyst (*32*). This corresponds to the values found for the activation parameters in the present study and the reaction mechanism described can be related to the reaction between ϵ-amino group of lysine in α-lactalbumin and the acyclic carbonyl form of lactose.

On the basis of these considerations, it is suggested that at acidic pH, the nucleophilic attack of the protonated amine with a hydrated carbonyl group forming a lactosylamine becomes rate-determining. In contrast, the rate-determining step at alkaline pH is elimination of water from the rapidly formed lactosylamine to yield the Schiff base, as this requires an acidic catalyst (*32*).

The rate of formation of the lactosylamine should be highest between the pK_a of the carbonyl group and the pK_a of the amino group, where the product of the concentration of $R-NH_2$ and $R-C{=}OH^+$ is at maximum. The optimum pH depends, however, on the structure of the reactants but will be at a weakly acidic pH in the reaction involving an aldose and an amine (*6*, *32*). From the pH profile, it was assumed that the protonation of the ϵ-amino group of lysine accounted for the observed pH dependency of the lactosylation. For experiments at pH_c below 4, α-lactalbumin will denaturate (*33*). In conclusion, the reaction investigated involved two consecutive steps of which the initial reaction, that is, formation of the lactosylamine, is rate-determining at low pH, while the second reaction, that is, elimination of water, is rate-determining at higher pH. An energy profile illustrating the shift between the two transition states is shown in **Figure 7**.

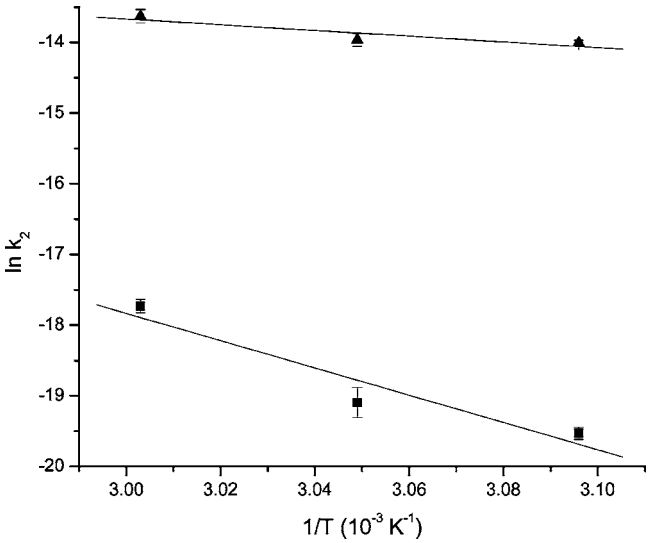

Figure 6. Second-order rate constant for reaction of the protonated α-lactalbumin (k_2^A) (■) and the nonprotonated α-lactalbumin (k_2^B) (▲) plotted accordingly to the Arrhenius equation. The two regression lines were $y = -19307x + 40.09$ and $y = -4052.9x - 1.509$, respectively.

Activation parameters at 25 °C were determined for protonated and unprotonated lysine for the lactosylation of α-lactalbumin corresponding to extreme pH values. In food systems, pH is usually 3−8, and in milk pH is 6.6−6.8 (*34*), for which conditions an intermediate value will be valid for comparison with activation energies reported in the literature and shown in **Table 3**. Notably, the activation energy found for unprotonated lysine agrees with the reported activation energies for the loss of lysine residues. The high activation energy of the lactosylation of caseins indicates that this reaction is more temperature dependent than the lactosylation of α-lactalbumin and explains why α-lactalbumin preferentially is lactosylated during normal processing conditions.

The pK_a values of the ϵ-amino group of lysine in α-lactalbumin were used in the calculation of the second-order rate constants of the lactosylation and thereby the activation parameters and has been extrapolated from values obtained at 10 °C and 25 °C (*30*). As the protein most likely undergoes structural changes from 10 to 25 °C to 50 to 60 °C, the true pK_a values might, however, be different from the estimated values. It was not possible to estimate the pK_a values directly from eq 7 by regression analysis, as the pH interval of the data was too narrow to estimate a function over the full pH interval, as originally intended, since the rate of denaturation of α-lactalbumin at pH > 8 becomes dominating rather than lactosylation. Furthermore, it cannot be excluded that the NH_2-terminal group (glutamic acid) might react with lactose to some lesser extent, and the pK_a value of this (pK_a = 9.47) is different from that of the ϵ-amino group of lysine (*9*).

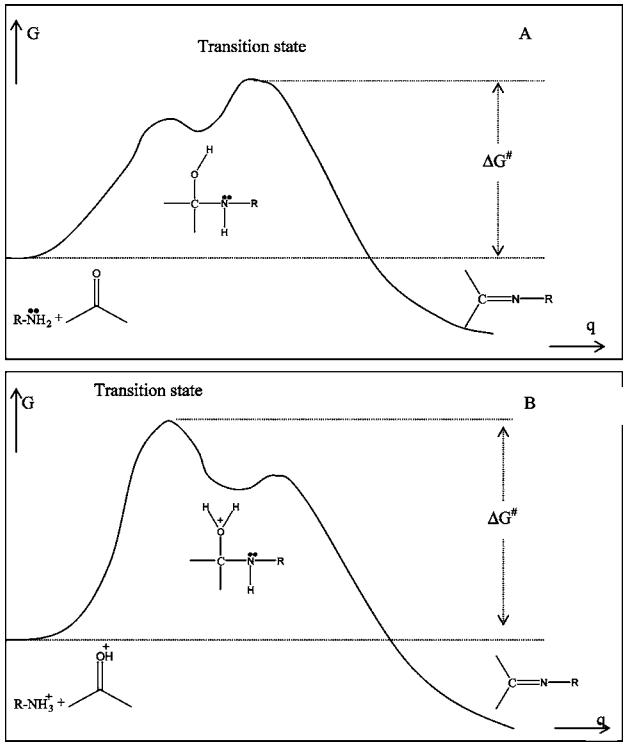

Figure 7. Energy profile of reaction between α-D-lactose and α-lactalbumin during alkaline (A) and acidic (B) conditions in aqueous solutions with different reaction intermediates shown.

When an analytical method for the separation of native, lactosylated, denaturated, and lactosylated denaturated α-lactalbumin becomes available, it will be possible to determine the rate constants of the denaturation of α-lactalbumin and more importantly also the rate constant of the lactosylation of denaturated α-lactalbumin. An unfolding of the protein might alter the rate of the lactosylation by exposure of more lysine groups, and as milk products are heated during processing, a lactosylation of the denaturated α-lactalbumin may become dominating.

ABBREVIATIONS USED

RP-HPLC, reversed-phase high-performance liquid chromatography;

R_t, retention time;

MS, mass spectrometry;

MES, 2-*N*(morpholino)ethanesulfonic acid;

m/*z*, mass-to-charge ratio (for MS);

tris, tris(hydroxymethyl)-aminomethane;

z, charge.

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