Interactions between κ -Casein and β -Lactoglobulin: Effects of Anions on Covalent Stabilization of the Complex

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Structure breaking anions, trichloroacetate ($-TCA^-$) and thiocyanate ($-SCN^-$), significantly increased the interaction between κ -casein (K-C) and β -lactoglobulin (B-Lg) to give the covalently stabilized K-C/B-Lg tetrameric (A4) complex, whereas "structure making" anions, chloride ($-Cl^-$) and especially sulfate ($-SO_4^-$), reduced it. The reactivity of K-C with B-Lg in the presence of these anions followed the order, $-TCA^- > -SCN^- > -Cl^- > -SO_4^-$ (100 mM). The percentile distribution of the covalently bonded K-C/B-Lg A4 complex after 720 s of heating at 70°C in $-TCA^-$ (100 mM) was one order greater than it was in $-SO_4^-$. During storage of the heated K-C/B-Lg mixture, the B-Lg A3/K-C interaction in $-TCA^-$ was rapid and unaffected by the holding temperature, whereas in $-SO_4^-$, the reaction rate was inversely related to the temperature, being apparently controlled by the relative concentration of monomeric K-C.

Heating of milk reduces its subsequent rennetability,¹⁾ because following the interaction between β -lactoglobulin (B-Lg) and κ -casein (K-C) on the micelle,^{2,3)} the phenylalanine (105)-methionine (106) bond of the K-C becomes inaccessible to chymosin action.

Hydrophobic interactions are the predominant forces initially involved in the interaction between isolated K-C and B-Lg,^{4,5)} and the interaction is enhanced at a higher temperature (70°C), apparently due to an increased potential for hydrophobic interactions.⁶⁾ The Hofmeister, lyotropic or chaotropic series of anions have been used to observe the contribution of hydrophobic forces in protein/protein interaction,^{7~9)} and the possible effects of high concentrations (>0.1 M) of these anions on disruption of the water structure around apolar amino acid residues, following the order of $-SCN^- > -Cl^- > -SO_4^-$, has been reported.^{9,10)} However, at low concentrations (<100 mM), these anions function mainly by binding preferentially to proteins, and the binding affinity follows the Hofmeister order.¹¹⁾ The larger anions bind more strongly to the cationic sites in proteins

and thereby indirectly enhance the potential for hydrophobic interactions.¹²⁾ Therefore, the charge masking effect of these anions at a low concentration should indirectly facilitate hydrophobic interactions. Thus, the effects of low concentrations (100 mM) of these anions on the formation of the complex between K-C and B-Lg were studied.

MATERIALS

A type HA filter, of 0.45 μ m pore size, was purchased from Millipore Corp., Bedford, Massachusetts. The standard protein molecular weight calibration kit (ZEA) was obtained from Pharmacia, Uppsala, Sweden. The ultrafiltration membrane (PM-10) was from Amicon, Lexington, Massachusetts. Analytical grade thiocyanate ($-SCN^-$), sodium sulfate ($-SO_4^-$), sodium trichloroacetate ($-TCA^-$) and sodium chloride ($-Cl^-$) were all obtained from Pfaltz and Bauer, Inc., Stamford, Connecticut. All other chemicals used were of analytical grade.

METHODS

Protein purification. The proteins, B-Lg and K-C, were purified from delipidated milk powder, flash frozen in liquid nitrogen and then stored at -20°C for short periods.⁵⁾

Thermal treatment. In order to study the effects of the anions on the heat induced interaction between K-C and B-Lg, aliquots of the protein solutions (1 mM) in the reaction buffer were mixed together and immediately heated at 70°C for specific times. The reaction was stopped by four-fold dilution with chilled (4°C) reaction buffer, and then the products were analyzed by HPLC.⁶⁾

Standard reaction and HPLC buffers. The standard reaction buffer was 20 mM imidazole, pH 6.8, containing 20 mM EGTA. EGTA was used to quench the effect of Ca^{2+} .^{6,13)} The anions, TCA^- , SCN^- , Cl^- and SO_4^- , were added (100 mM) to the above buffer. The reaction buffer plus 2.3 M recrystallized urea was used as the HPLC buffer. B-Lg is not denatured with this concentration of urea.¹⁴⁾ Recrystallized urea (low cyanate) was added to the HPLC buffer to decrease hydrophobic interactions between the proteins and the column materials, and also to detect the formation of the covalently (S-S) bonded K-C/B-Lg A4 complex.⁶⁾ In the presence of 2.3 M urea, only the covalent bounded K-C/B-Lg A4 complex was detected on HPLC, the noncovalently bonded K-C/B-Lg A4 complex being dissociated to the B-Lg A3 complex and K-C.⁶⁾ All HPLC buffers were filtered (Millipore, 0.45 μm) and degassed by sonication.

High performance liquid chromatography (HPLC). The association/dissociation states of K-C and B-Lg and the covalent stabilization of the K-C/B-Lg A4 complex were studied by HPLC. The apparent molecular weights of the proteins corresponding to the HPLC peaks were determined using standard proteins of known molecular weight as described.⁶⁾ A Waters M-45 solvent delivery system was connected to a TSK gel G3000 SW (Toyo Soda, Tokyo, Japan) (60 cm $\times$ 7.5 mm i.d.) hydrophilic glass bead column connected to a Waters model 450 variable wavelength detector and a Hewlett Packard 3390A integrator. The flow rate was 1 ml/minute at 25°C and detection was performed at 280 nm. Following heat treatment, 0.5 ml of the K-C/B-Lg mixture was diluted four-fold to 0.25 mM in the standard HPLC buffer (4°C), followed by mixing with a vortex mixer, and then 2.5 μl of the diluted solution containing 11.5 μg was injected into the HPLC using a Hamilton precision syringe (5 ml). The mean of three experiments was taken. Because the $E_{280}^{1\%,1\text{cm}}$ of K-C and B-Lg are close to each other,^{15,16)} the total area under all the separated protein peaks reflected the detector response for 6.2 mM [(0.25 $\mu\text{M}/1000) \times 2.5$] of the protein (11.5 μg), i.e., following normalization each unit percent of absorbance (1%) represented 0.062 nM of protein.

RESULTS AND DISCUSSION

Upon heating, B-Lg self associates to form the B-Lg A3 complex. The rate of formation of

FIG. 1. Effects of Anions on the Formation of the β -Lactoglobulin A3 and K-C/B-Lg A4 Complexes.

The κ -casein/ β -lactoglobulin mixture was injected directly into the HPLC, elution was performed with the standard HPLC buffer and detection was performed at 280 nm. The results presented are the normalized percentile distributions of the different fractions. The small dotted line (.....) denotes the K-C/B-Lg A4 complex, the larger dotted line (-----) the B-Lg A3 complex and the solid line (—) the dimeric K-C and B-Lg mixture.

the A4 complex increased gradually with the heating time in SO_4^{2-} and reached a plateau at around 360 s (Fig. 1). In TCA^- , there was a sharper increase in the B-Lg A3 complex followed by a decrease with a concomitant increase in covalently bonded (non-dissociated by 2-ME) K-C/B-Lg A4 complex formation. The specific effects of the anions on the covalent bonded K-C/B-Lg A4 complex formation are significant. After 720 s (12 min) of heating at 70°, the percentile distribution of the covalent bonded K-C/B-Lg A4 complex was 45, 27, 10 and 4 in TCA^- , SCN^- , Cl^- and SO_4^{2-} , respectively. In the control, the percentile distribution of the K-C/B-Lg A4 complex was 8.⁶⁾ There was a more than ten-fold increase in covalent bonded K-C/B-Lg A4 complex formation in TCA^- compared to the in SO_4^{2-} .

In all cases, the dimeric A2 complex decreased as the A3 complex increased. This transition through A3 (B-Lg3) in the formation of the covalent bonded K-C/B-Lg A4 was most evident in the presence of SCN^- and TCA^- , especially the latter, with which covalent bonded K-C/B-Lg A4 formation was maximal. In TCA^- , the heat induced re-

action sequence of A2 (K-C and B-Lg)- > A3 (B-Lg)- > covalent bonded A4 (K-C1/B-Lg3) was clear; there was a sharp decrease in A2 and an equally sharp increase in A3 which then decreased as the covalent linkage between A3 and the K-C monomer increased.

Comparison of the effects of $-\text{SO}_4^-$ and $-\text{TCA}^-$ showed that the initial B-Lg A3 complex concentration before heating was inversely related to the reported binding affinities of the anions for proteins, being higher for $-\text{SO}_4^-$ than for $-\text{SCN}^-$,¹¹⁾ and this trend was reversed upon heating. These observations suggest that electrostatic association was responsible for the B-Lg A3 formation in the unheated K-C/B-Lg, solution but heating rapidly enhanced the contribution of hydrophobic interactions. The formation of the K-C/B-Lg A4 complex was facilitated by hydrophobic interactions.

At elevated temperatures, the proteins tend to minimize the hydrocarbon/aqueous interface through intermolecular association or intramolecular rearrangement.¹⁷⁾ Generally, specific anions, *i.e.*, chaotropic anions, perturb the entropically driven protein conformation by destructuring water.^{8,18)} At low concentrations, anions may only minimally affect the water structure but mainly affect charge-charge interactions. The preferential binding of the larger anions to cationic sites^{19,20)} indirectly influences the potential for hydrophobic interaction. Hence, at a given temperature, pH and concentration, the potential for intermolecular association should be more for $-\text{TCA}^-$ or $-\text{SCN}^-$ than for $-\text{Cl}^-$ or $-\text{CO}_4^-$, because of their greater charge masking effects. The increase in the A3 complex formation in the initial stages of heating may therefore reflect the contribution of hydrophobic forces to the intermolecular (B-Lg) interaction (Fig. 1).

The anion specific effect on the B-Lg A3 complex and K-C/B-Lg A4 complex formation could also be due to anion specific structural changes in the proteins resulting in complexes of different sizes and/or shapes. Purcell and Susi²¹⁾ have reported three different denatured

states of B-Lg in alkaline solution. The initial interaction between K-C and B-Lg is spontaneous and non-covalent.⁵⁾ To determine the effects of the anions on the sizes and/or structures of the complexes, the HPLC retention times of the B-Lg A3 and K-C/B-Lg A4 complexes were determined. The changes in elution time of a macromolecule through a gel filtration column reflect changes in its size and/or shape, provided no electrostriction or hydrophobic interactions occur with the column material. The HPLC column (G3000SW) used in this study has a low surface charge and is hydrophilic, especially in the presence of the 2.3M urea and 100 mM salts in the HPLC elution buffer.

The retention time of the B(Lg A3 complex varied inversely with the reported preferential binding of the anions to proteins; $-\text{TCA}^- > -\text{SCN}^- > -\text{Cl}^- > -\text{SO}_4^-$ (Fig. 2). If the retention times of the B-Lg A3 complex in the presence of different anions indicate changes in only the size of the complex, *i.e.*, a longer retention meaning a smaller B-Lg A3 complex, then the rate of K-C/B-Lg A4 complex formation was related to the size of the A3 complex, *i.e.*, a larger size of A3 enhanced the rate of covalent formation of the K-C/B-Lg A4 complex (Fig. 1). The retention time of the B-Lg A3 complex with $-\text{Cl}^-$ or $-\text{SO}_4^-$ was greater than in the control, presumably because of their effects on the molecular structure of B-Lg. Hence, electrostriction effects of the column material seem unlikely. Thus hy-

FIG. 2. The HPLC Retention Times of the B-Lg A3 and K-C/B-Lg A4 Complexes in the Presence of Anions.

The solid bars denote the B-Lg A4 complex and the hatched bars the K-C/B-Lg A3 complex.

FIG. 3. Effects of Anions on the Covalently Stabilized K-C/B-Lg A4 Complex during Storage of Heated K-C/B-Lg.

The solid bars denote the K-C/B-Lg A4 complex formation with $-TCA^-$ and the shaded bars the K-C/B-Lg A4 complex formation with $-SO_4^-$.

FIG. 4. Effect of the Storage Temperature with $-SO_4^-$. The heated K-C/B-Lg mixture was stored with $-SO_4^-$ (100 mM) at various temperatures for up to 72 hr.

drophobic interactions are apparently involved in the stabilization of the K-C/B-Lg A4 complex, and this was affected by structural changes in the proteins, as reflected by changes in the retention time.

To further elucidate the mechanism of thermal association of the two proteins, samples were heated (70°C for 48 min) and then stored in the presence $-TCA^-$ and $-SO_4^-$ at various temperatures for 24 hr (Fig. 3). The formation of the K-C/B-Lg A4 complex with $-TCA^-$ reached a maximum after 24 hr and was not influenced by the storage temperature. The storage temperature had a significant influence on the formation of the covalently stabilized A4 complex, on storage in the presence of $-SO_4^-$. Long term storage with $-SO_4^-$ revealed the strong effect of the storage temperature on covalently stabilized A4 formation (Fig. 4). From the slope of the plot of

A4 complex formation vs. storage time, the apparent rate of formation of covalent bonded K-C/B-Lg was 53, 18, and $3 \times 10^{-2} \text{ hr}^{-1}$ at 5, 15 and 25°C , respectively. These data are consistent with the finding of other workers¹⁾ that the holding temperature of heated milk influences the extent of interactions between the B-Lg A3 complex and K-C during subsequent storage.

In conclusion, the increased reaction between K-C and B-Lg in the presence of low concentrations of different anions apparently reflects their effects on the molecular structure of B-Lg. The possibility that the anions further to the left of $-SO_4^-$ (citrate, phosphate) in the Hofmeister series⁷⁾ might even more efficiently decrease the covalent bonded K-C/B-Lg A4 complex formation is being studied.

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APPENDIX

Calculation of the reaction rates. Since the structural alteration of B-Lg is a prerequisite for the formation of the K-C/B-Lg A4 complex, a simple N to U approach was taken, *i.e.*, that the native (N) proteins could not polymerize and the unfolded proteins (U) polymerized to give the K-C/B-Lg complex. $[U]_i$ was the concentration of U at time t and $[U]_\infty$ corresponded to the final concentration of U. If the reaction is considered to be irreversible and stoichiometric, $([U]_\infty - [U]_t)$ is the concentration of [N] remaining at time t .

Then

$$\frac{d[U]}{dt} = -\frac{d[N]}{dt} \\ = k[N]_t = k([U]_\infty - [U]_t)$$

Therefore

$$\frac{d[U]}{([U]_\infty - [U]_t)} = k dt$$

Therefore, by integration

$$\ln([U]_\infty - [U]_t) = -kt + c$$

When $t=0$, $[U]=0$ since we started with N, then $c = \ln[U]_\infty$. Therefore, after integration

$$\ln\left(\frac{[U]_\infty}{[U]_\infty - [U]_t}\right) = kt \quad (1)$$

Since the A4 detected by HPLC was the denatured [U] form, by replacing U with the A4 concentration and by plotting

$$\ln\left(\frac{[A4]_x}{[A4]_x - [A4]_t}\right) \text{ vs. } t \quad (2)$$

we get a straight line, and the rate constant (k) was derived from the slope assuming that the process of denaturation was first order. $[A4]_x$ was the concentration of the complex after 96 min of heating at 70°C.

Free energy of activation. The free energy of activation ($G^\ddagger$) for the formation of the complexes was calculated using the following equation.

$$\Delta G^\ddagger = -RT \ln K^\ddagger$$

Since,

$$K^\ddagger = \frac{hk}{k_b T}$$

$$\Delta G^\ddagger = -RT \ln \frac{hk}{k_b T} \quad (3)$$

where $K^\ddagger$ is the equilibrium constant of the transition state, k the rate constant, k_b the Boltzmann constant, h the Planck constant, T the absolute temperature and R the gas constant.

The rate constant was 2.51, 3.94, 6.37 and 6.55 10^{-1} sec^{-1} for NaSO_4^- , Cl^- , $-\text{SCN}^-$ and $-\text{TCA}^-$, respectively. $\Delta G^\ddagger$ in $-\text{TCA}^-$ was about 3 kJ mol^{-1} less than it was in $-\text{SO}_4^{2-}$.

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