ABSTRACT

Within each milk protein there are many individual protein variants and marked alterations to milk functionality can occur depending on the genetic variants of each protein present. Bovine A1 and A2 β-casein (β-CN) are 2 variants that contribute to differences in the gelation performance of milk. The A1 and A2 β-CN variants differ by a single AA, the substitution of histidine for proline at position 67. β-Casein not only participates in formation of the casein micelle but also forms an oligomeric micelle itself and functions as a molecular chaperone to prevent the aggregation of a wide range of proteins, including the other caseins. Micelle assembly of A1 and A2 β-CN was investigated using dynamic light scattering and small-angle X-ray scattering, whereas protein functionality was assessed using fluorescence techniques and molecular chaperone assays. The A2 β-CN variant formed smaller micelles than A1 β-CN, with the monomer–micelle equilibrium of A2 β-CN being shifted toward the monomer. This shift most likely arose from structural differences between the 2 β-CN variants associated with the adoption of greater polyproline-II helix in A2 β-CN and most likely led to enhanced chaperone activity of A2 β-CN compared with A1 β-CN. The difference in micelle assembly, and hence chaperone activity, may provide explain differences in the functionality of homozygous A1 and A2 milk. The results of this study highlight that substitution of even a single AA can significantly alter the properties of an intrinsically unstructured protein such as β-CN and, in this case, may have an effect on the functionality of milk.

Key words: β-casein, variant, monomer–micelle equilibrium, aggregation, small-angle X-ray scattering

INTRODUCTION

The genetic variability of the bovine milk proteins is increasingly recognized as playing an important role in the functionality and yield of milk and milk proteins. The study of the genetic variability of milk proteins began over 50 yr ago with the detection of β-LG variants (Aschaffenburg and Drewry, 1957). The nomenclature of the milk proteins in the Bos genus indicates the presence of 9 αS1-CN, 4 αS2-CN, 12 β-CN, 11 κ-CN, 11 β-LG, and 3 α-LA variants (Farrell et al., 2004; Lühken et al., 2009). Although many milk protein variants have been identified, most milk contains only a handful of common variants (12 of 53; Caroli et al., 2009).

The β-CN variants A1 and A2 are 2 milk protein variants that have received much attention from both the scientific community and the wider public for 2 primary reasons: (1) the influence of the protein variants on milk gelation performance, and (2) the proposed health benefits of A2 over A1 β-CN. β-Casein has a charged polar N-terminal region due to the presence of up to 5 phosphoseryl groups, and a less polar C-terminal region, characterized by a high proportion of proline and glutamine (Swaisgood, 1993). The amphiphilic nature of β-CN allows it to self-assemble into micelles with a hydrodynamic radius of ~12 nm above its critical micelle concentration (CMC) of 0.5 mg/mL (O’Connell et al., 2003). Whereas the phosphoseryl clusters allow for binding of calcium within the casein micelle structure (Holt et al., 1996), its amphiphilic structure is thought to play a role in the ability of β-CN to act as a molecular chaperone to prevent the aggregation of a wide range of partially unfolded proteins (Morgan et al., 2005; Zhang et al., 2005; Holt et al., 2013). Molecular chaperones represent a diverse class of proteins that interact with protein intermediates to inhibit protein aggregation. Proteins such as the heat-shock proteins Hsp70 and GroEL can refold proteins to their native state, whereas other chaperones such as α-crystallin and small heat-shock proteins prevent aggregation but cannot restore proteins to their native state (Bhattacharyya and Das, 1999). β-Casein is classified into this second group because it cannot facilitate the refolding of proteins into their native state.

The difference between A1 and A2 β-CN is a single AA change from histidin to proline at position 67, which
may result in altered secondary structure (Kamiński et al., 2007). Proline has the highest propensity to form polyproline II (PPII) secondary structures (Brown and Zondlo, 2012), and β-CN has significant PPII structural features (Syme et al., 2002). Therefore, the presence of an additional proline residue in A2 β-CN, which already contains many such residues, could enhance PPII helix formation and alter the protein’s structural dynamics and self-assembly behavior.

The A1 and A2 β-CN variants have gained significant attention because of the proteolytic cleavage product β-casomorphin 7 (βCM7) that is formed from A1 β-CN but not A2 β-CN. β-Casomorphin 7 is an opioid peptide (Brantl et al., 1979) that is the subject of debate around possible links between βCM7 ingestion and increases in rates of coronary heart disease (McLachlan, 2001) and type 1 diabetes (Elliott et al., 1999). A review from the available scientific literature surrounding the health effects of βCM7 by the European Food Safety Authority concluded that there is no cause–effect relationship between the oral intake of βCM7 and etiology of disease (Noni et al., 2009). Clearly, more research is required to establish whether such a relationship exists between A1 β-CN consumption and potential health effects.

β-Casein variants have also received attention because casein variants play a major role in curd formation of milk (Bittante et al., 2012). Enzymatic curd formation of milk is achieved by the addition of the protease rennet that hydrolyses κ-CN between Phe<sup>105</sup> and Met<sup>106</sup> (Fox and McSweeney, 1998). κ-Casein is located on the surface of casein micelles, and hydrolysis of κ-CN reduces the stability of micelles to such an extent that aggregation occurs, producing the curd. Milk coagulation is a complicated process that is governed not only by the milk protein variants but also by protein and mineral contents (Fox and McSweeney, 1998). Recent research by multiple research groups has confirmed that milk homozygous for A2 β-CN (usually in combination with the A variant of κ-CN) is the predominant β-CN variant found in poorly or noncoagulating milk in multiple cow breeds (Jensen et al., 2012a,b; Vallas et al., 2012; Poulsen et al., 2013; Gustavsson et al., 2014).

From the literature, a clear link exists between milk protein variants and differences in the gelation performance of milk. However, the reason for the observed differences in milk functionality is not well understood. A possible explanation is that the single AA variation between A1 and A2 β-CN imparts structural and functional differences that lead to alterations in milk functionality. Understanding how individual AA substitutions in an IUP can alter the properties of the protein is important not only for β-CN, but also for other IUP. Therefore the aim of this work was to examine the differences between A1 and A2 β-CN through a systematic investigation into their structural and functional properties. To achieve this, A1 and A2 β-CN were purified from milk homozygous for A1 or A2 β-CN. Fluorescence of 8-anilino-1-napthalenesulfonic acid (ANS) binding was used to assess the exposed hydrophobicity of the proteins. Their hydrodynamic radii and their self-association to micellar aggregates were investigated by solution small-angle X-ray radii and dynamic light scattering (DLS).

**MATERIALS AND METHODS**

Milk was collected from 74 cows from the experimental dairy herd run by the Department of Environment and Primary Industries Victoria (Ellinbank, Victoria, Australia). Milk homozygous for A1 or A2 β-CN genotypes was selected by analysis of individual cow milk samples using capillary electrophoresis. β-Casein (C6905), catalase (Cat; C9322), lysozyme (Lyso; L6876), κ-CN (C0406), α-LA (L5385), and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Protein concentrations were determined spectrophotometrically at 280 nm using a ND-1000 UV-Vis spectrophotometer from NanoDrop Technologies (Wilmington, DE), and absorption coefficients were calculated from the AA sequence using the ProtParam tool (Gasteiger et al., 2005) for β-CN, Cat, Lyso, κ-CN, and α-LA of A<sub>280</sub> (1 mg/mL) = 0.485, 1.08, 2.65, 0.9, and 1.76, respectively.

**Purification of A<sub>1</sub> and A<sub>2</sub> β-CN**

Purification of A1 and A2 β-CN was based on the methods of Ward and Bastian (1996, 1998) and Post et al. (2009) with modification. Fresh full-fat milk with the A<sub>1</sub> and A<sub>2</sub> genotypes was centrifuged (4,000 × g) for 30 min at 4°C and left to cool for 1 h before removal of the cream layer. The skim milk fraction was then collected and filtered through Whatman No. 4 filter paper. Isoelectric precipitation of the casein was achieved by decreasing the pH of the milk to 4.6 dropwise with 1 M HCl while stirring on ice. The precipitated solution was then stirred for 1 h at room temperature to induce full precipitation of the casein. The precipitated casein was collected by centrifugation (3,000 × g) for 10 min. The supernatant was discarded and the pellet resuspended in distilled water. This washing process was repeated 3 times to ensure removal of residual lactose. The final pellet was resuspended in distilled water and the pH was increased to 6.8 with 1 M NaOH to resolubilize the casein. Then, CaCl<sub>2</sub> was then added to a final concentration of 50 mM to precipitate αs<sub>1</sub>- and β-CN. The solution was then centrifuged (3,000 × g) for 10 min. The supernatant containing κ-CN was discarded, and the pellet was washed 3 times again in distilled water.
The final washed pellet was resuspended in distilled water. The solution was then cooled to 4°C and left for 24 h to allow the β-CN to dissociate from the αs-CN and solubilize into the serum phase. The supernatant containing the β-CN was collected by siphoning before heating it to 40°C to precipitate the protein, which was collected and freeze-dried.

Freeze-dried A¹ and A² β-CN was dissolved in 50 mM sodium phosphate buffer, 3.3 M urea, pH 7.0 (buffer A), at 1.5 mg/mL and loaded onto a GE Healthcare Mono Q HR 16/10 column (GE Healthcare Bio-Sciences, Pittsburgh, PA) pre-equilibrated with buffer A at a flow rate of 1.5 mL/min. The bound protein was washed with 2 column volumes of buffer A before a linear gradient of buffer B (50 mM sodium phosphate, 3.3 M urea, 1 M NaCl, pH 7.0) was applied. The peak corresponding to β-CN (at ~25% NaCl elution gradient) was collected and extensively dialyzed against distilled H₂O before freeze-drying.

**Capillary Electrophoresis**

Electromigration was carried out with a Beckman P/ACE MDQ system (Beckman, Brea, CA) using a UV detector set at 214 nm. Identification of A¹ and A² β-CN was based on the methods of de Jong et al. (1993). An uncoated capillary (50 μm i.d.) with 10 cm to the detection window and an effective length of 30 cm was used for the analysis. Migrations were run at 35°C, with normal polarity and a separation voltage of 20 kV. Samples (1 mg/mL) were made up in 5 mM trisodium citrate dihydrate, 10 mM dithiothreitol (DTT), and 6 M urea, pH 8. Running buffer contained 20 mM trisodium citrate dihydrate, 0.05% methyl hydroxyethylcellulose (200–300 cP), and 6 M urea, pH 8. Before each injection, the capillary was washed with 0.1 M NaOH and 0.5% SDS, and then equilibrated with running buffer for 1 min per solution.

**Mass Spectrometry**

Electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) was carried out on an Agilent 6510 QTOF ESI-MS coupled to an Agilent 1100 HPLC (Santa Clara, CA). Purified A¹ and A² β-CN (1 mg/mL) in H₂O was diluted with 0.1% trifluoroacetic acid (TFA) and loaded onto a C-18 column pre-equilibrated with 0.1% TFA. The protein was eluted from the column using 50% acetonitrile and loaded directly into the mass spectrometer.

**ANS Fluorescence Binding**

Based on the modified method of Morgan et al. (2005), the increase in fluorescence of the hydrophobic probe ANS upon binding to A¹ and A² β-CN was measured using a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA). A measurement time of 100 ms and an excitation bandwidth of 5 nm were used with excitation and emission wavelengths of 387 and 479 nm, respectively. A stock solution of 66.7 mM ANS in dimethyl sulfoxide was mixed with sample buffer to give a working solution concentration of 10 mM ANS. The working solution (5 μL) was added to 150 μL of sample and the fluorescence measured. To ensure enough dye was present to achieve maximum fluorescence, a further 5 μL of ANS was added to each solution and the samples measured again for stabilized fluorescence.

**DLS**

The DLS was carried out using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at wavelength of 633 nm. Samples were filtered through a 0.2 μm Supor (Pall Corp., Hauppauge, NY) filter and analyzed in a 1-cm-pathlength quartz cuvette using a fixed detector angle of 173°. Samples were allowed to equilibrate to the desired temperature for 10 min before analysis. Solvent viscosity and refractive index were set as H₂O (dependent on temperature). Measurement duration was set to automatic and each sample was measured 3 times, with 3 replicates of each sample averaged. The protein hydrodynamic diameter was calculated using the autocorrelation curves.

**SAXS Measurements**

The SAXS measurements were performed at the Australian Synchrotron (Clayton, Australia) SAXS/WAXS beamline equipped with a Pilatus 1M detector (170 mm × 170 mm; effective pixel size, 172 μm × 172 μm; Dectris, Baden, Switzerland). The wavelength of the X-rays was 1.0332 Å. A single detector distance of 1,576 mm was used, providing a Q range of 0.01 to 0.4 Å, where Q is the magnitude of the scattering vector, which is related to the scattering angle (2θ) and the wavelength (λ) as follows: $Q = \frac{4\pi}{\lambda} \sin(\theta)$. Data were collected at 2-s intervals using a 1.5-mm quartz capillary at 9 and 25°C under a continuous flow of 50 mM sodium phosphate, pH 7.0 buffer. Two-dimensional intensity plots were radially averaged and normalized to sample transmission, and the background was subtracted.

**SAXS Data Analysis**

Kratky plots, where $Q^2 \times I(Q)$ is plotted against $Q$ and $I(Q)$ is the scattering intensity, were first used as
a model-independent analysis to investigate the micelle formation of A1 and A2 β-CN (Mertens and Svergun, 2010). Data fitting was done using SASview (www.sasview.org). Samples measured at 9°C were modeled using a poly gausscoil (eq. [1]), where the increase in scattering was directly related to an increase in protein concentration (Higgins and Benoit, 1996):

\[
I(Q) = \text{scale} \frac{2\left(1 + Ux\right)^{1/2} + x - 1}{(1 + U)x^2} + \text{bkg},
\]

where \( \text{bkg} \) is the background, \( \text{scale} \) is the scale factor, and the dimensionless chain dimension, \( x \), is

\[
x = \frac{R_s^2 Q^2}{1 + 2U},
\]

and the polydispersity, \( U \), is

\[
U = \frac{M_w}{M_n} - 1,
\]

where \( M_w \) is the weight average molecular weight, which takes into account the molecular weight of a chain in determining contributions to the molecular weight average; and \( M_n \) is the number average molecular weight, which is the statistical average molecular weight of all polymer chains in a sample.

For samples measured at 25°C, the poly gausscoil was used to fit the data for samples below the CMC, whereas fitting the data above the CMC was achieved by combining 2 models: the ellipsoid model and the Debye model. The dual ellipsoid–Debye model is described by sum of the form factors for the ellipsoid and Debye model (Ossowski et al., 2012) (eq. [4]):

\[
I(Q) = \left[ NV^2 \cdot (\Delta p)^2 \cdot P(Q) \right]_{\text{ellipsoid}} + \left[ NV^2 \cdot (\Delta p)^2 \cdot P(Q) \right]_{\text{Debye}} + B,
\]

where \( N \) is the number particle concentration, \( V \) the particle volume, \( \Delta p \) is the difference between the scattering length density of the solvent and the scattering particles, \( P(Q) \) is the form factor of the particles, and \( B \) is the incoherent background.

The ellipsoid model provides radius measurements along the rotation axis of the ellipsoid (\( R_a \)) and the radius perpendicular to the rotation axis (\( R_b \)). The radius of gyration (\( R_g \)) can then be calculated using eq. [5]:

\[
R_g = \sqrt{\frac{R_a^2 + R_b^2}{4}}.
\]

### Molecular Chaperone Assays

The ability of A1 and A2 β-CN to inhibit amorphous and amyloid fibrillar aggregation was determined using the methods of Koudelka et al. (2009) by determining the rate of aggregation for the target protein with and without β-CN present. Chaperone activity was compared by calculating the percentage protection, as described by Koudelka et al. (2009). From this, significance testing via a paired sample \( t \)-test was used to investigate the variation in chaperone activity of both proteins. Target protein concentrations were chosen by performing concentration aggregation series and using the concentration that gave a reproducible aggregation rate over an appropriate period. β-Casein chaperone concentrations were chosen using a concentration series until a decrease in the total aggregation rate of 50% of the target protein was achieved. The chaperone assay conditions for each target protein were as follows:

For lysozyme, 1 mg/mL Lyso in 50 mM sodium phosphate and 0.2 M NaCl was induced to aggregate by the addition of 20 mM DTT at 25°C. A final concentration of 2 mg/mL A1 or A2 β-CN was used to compare molecular chaperone activity. The progression of aggregation was followed by measuring the absorbance at 360 nm due to light scattered by turbidity using a SpectraMax384 plate reader (Molecular Devices, Sunnyvale, CA).

For catalase, 1 mg/mL Cat in 50 mM sodium phosphate and 0.2 M NaCl was induced to aggregate by heating at 60°C. A final concentration of 0.25 mg/mL of A1 or A2 β-CN was used to compare molecular chaperone activity. The progression of aggregation was followed by measuring the absorbance at 360 nm due to light scattered by turbidity using a UV-1700 spectrophotometer (Shimadzu, Tokyo, Japan).

For α-LA, 2 mg/mL α-LA in 50 mM sodium phosphate and 0.2 M NaCl was induced to aggregate by the addition of 20 mM DTT at 25°C (Carver et al., 2002). A final concentration of 1 mg/mL A1 or A2 β-CN was used to compare molecular chaperone activity. The progression of aggregation was followed using the same method as for the Cat assay.

For κ-CN, 3.175 mg/mL κ-CN in 50 mM sodium phosphate and 0.2 M NaCl was induced to form amyloid fibrils by the addition of 20 mM DTT at 37°C (Thorn et al., 2005a). A final concentration of 3.175 mg/mL A1 or A2 β-CN was used to compare molecular chaperone activity. Thioflavin T was added to a final concentration of 2.5 μM and amyloid formation moni-
tored by measuring the increase in fluorescence of thioflavin T with excitation and emission wavelengths of 450 and 485 nm, respectively, with a measurement time of 100 ms and an excitation bandwidth of 5 nm using a Varioskan Flash microplate reader (Thermo Scientific).

RESULTS AND DISCUSSION

Purification and Identification of A1 and A2 β-CN

Investigating the structural and functional differences of A1 and A2 β-CN first required the purification and identification of both proteins. The chaperone activity of β-CN depends on protein concentration (Morgan et al., 2005) and the level of phosphorylation (Koudelka et al., 2009). It was critical, therefore, that A1 and A2 β-CN were of high purity and had the same level of phosphorylation to ensure that any differences in the dynamic structural assembly and chaperone activity of the proteins were due to the single AA change at position 67 and not from differences in the purity or level of phosphorylation.

After isolating β-CN from raw milk, capillary electrophoresis was used to identify the 2 protein variants by running the A1 and A2 samples separately in a 1:1 mixture and by comparison with a Sigma β-CN standard (Supplemental Figure S1; http://dx.doi.org/10.3168/jds.2014-8800). After confirming identification, the proteins were further purified by high-performance anion exchange chromatography (Supplemental Figure S2; http://dx.doi.org/10.3168/jds.2014-8800). Mass spectrometry was used to determine the mass of each protein, with the masses matching well with the theoretical masses (Table 1; Farrell et al., 2004). The results also confirmed that both β-CN variants contained 5 phosphoserine groups, the most common phosphorylation level for β-CN (Farrell et al., 2004).

A2 β-CN Has Less Exposed Hydrophobicity than A1 β-CN

Hydrophobic interactions are proposed to be important in the chaperone action of molecular chaperones such as β-CN that prevent target proteins from aggregating (Bhattacharyya and Das, 1999; Morgan et al., 2005; Zhang et al., 2005). Hydrophobic interactions are also thought to be involved in the association of β-CN in micelle formation (O’Connell et al., 2003). Surface hydrophobicity was therefore probed using ANS, a fluorescent surface hydrophobicity probe (Figure 1). At all protein concentrations, below and above the published CMC of 0.5 mg/mL (O’Connell et al., 2003), ANS fluorescence intensity was greater for A1 than for A2 β-CN, indicating that A1 β-CN has more exposed hydrophobicity than A2 β-CN. The difference in the ANS fluorescence intensities between the A1 and A2 β-CN also became greater at higher protein concentrations (e.g., 1 mg/mL; Figure 1).

DLS

The DLS data showed that the hydrodynamic diameter of the A1 β-CN micelles was 2 to 3 nm greater than that of the A2 β-CN micelles (Figure 2), with the largest difference seen at 4 mg/mL in A1 β-CN due to an outlying measurement. As well as confirming that A1 β-CN forms larger micelles above the CMC, DLS was used to examine changes in micelle size with increasing temperature. The A1 and A2 variants showed very similar trends above the CMC, with both variants increasing in size in a temperature range of 30 to 40°C (Figure 2B), which has been observed by others (O’Connell et al., 2003; Faizullin et al., 2013); A1 β-CN again maintained a larger micelle size throughout the increase in temperature.

Differences in Self-Assembly of A1 and A2 β-CN into Micellar Aggregates

Because β-CN is an aggregated and heterogeneous IUP, conventional techniques for structural studies such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are not suitable. Therefore, we used SAXS to examine the solution structures of A1 and A2 β-CN at concentrations above and below their CMC and to determine whether they differed in their degree of association to micellar aggregates. The SAXS data were first plotted as Kratky plots \( I(Q) \times Q^2 \) vs. \( Q \) to explore differences in the CMC of both proteins. Kratky plots provide information on the folded-unfolded state of proteins (Doniach, 2001). Kratky plots give characteristic curves for different types of overall protein folds. For example, folded globular proteins exhibit a prominent peak at low \( Q \), whereas unfolded or flexible proteins show an increase in \( I(Q) \times Q^2 \) with \( Q \), and a flexible, multi-domain protein can display a mixture of both types of curves (Doniach, 2001).

Kratky plots of A1 and A2 β-CN at 9°C showed no globular structural features at low \( Q \) at any concentra-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Theoretical mass (Da)</th>
<th>Experimental mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-5P</td>
<td>24,023</td>
<td>24,023.14</td>
</tr>
<tr>
<td>A2-5P</td>
<td>23,983</td>
<td>23,982.97</td>
</tr>
</tbody>
</table>
tion, and displayed an increase in \( I(Q) \times Q^2 \) with \( Q \), indicating that only flexible protein monomers existed at this temperature (Figure 3A and B). Conversely, at 25°C, both the A1 and A2 β-CN Kratky plots showed the formation of a peak at <0.05 \( Q \) from 0.75 mg/mL upward (Figure 3C and D), consistent with the formation of a micelle structure. The results agree with the CMC value of 0.5 mg/mL for β-CN reported by O’Connell et al. (2003).

When the SAXS data for the 1 mg/mL A1 and A2 β-CN solutions (i.e., above the CMC) at 9 and 25°C were plotted together, as shown in Figure 4, subtle differences became apparent between A1 and A2 β-CN at 25°C with increasing \( Q \). The reduction in scattering intensity of A1 β-CN at \( Q > 0.1 \) measured at 25°C compared with that at 9°C indicated a significant reduction of monomer protein concentration in solution due to formation of the micelle structure. On the other hand, the upturn in scattering data with increasing \( Q \) for A2 β-CN measured at 25°C and 9°C was almost identical. Thus, because of a change in the monomer–micelle equilibrium, more A2 β-CN molecules were present as flexible monomers in solution above the CMC compared with that for A1 β-CN.

As monomeric β-CN is present in solution, even above the CMC, a dual ellipsoid–Debye model (eq. [4]) was used to further analyze the SAXS data (Ossowski et al., 2012), as the individual ellipsoid and Debye models did not provide a good fit to the data. In fitting the dual ellipsoid–Debye model to the 1 mg/mL A1 and A2 β-CN SAXS data (i.e., above the CMC), the radius of gyration (\( R_g \)) obtained for both proteins was the same, whereas the \( R_g \) Debye was ~1.2 nm larger for A1 β-CN compared with A2 β-CN (Table 2); that is, the A1 β-CN micelle was larger than the A2 β-CN micelle. To gain a good fit of the A2 β-CN SAXS data, a higher polydispersity was needed (Table 2). This, together with the upturn of scattering intensity with increasing \( Q \), provides strong evidence of a difference in the packing of A2 β-CN molecules within the micelle.
and a difference in the equilibrium between micelle-bound protein and free monomer protein, compared with A1 β-CN.

**A2 β-CN Is a More Efficient Chaperone Above the CMC**

The molecular chaperone ability of β-CN is well documented in protecting a range of proteins against aggregation under a variety of stress conditions (Morgan et al., 2005; Zhang et al., 2005). Thus, measuring chaperone activity provides a means for testing for a potential difference in a functional property of A1 and A2 β-CN arising from their subtle structural differences. Here, a range of target proteins was used to test the ability of both β-CN proteins to protect against 2 different types of aggregation; that is, the formation of amorphous aggregates [Cat, Lyso, and α-LA (a whey milk protein)], and amyloid fibrils (κ-CN). For these target proteins, elevated temperature (Cat) and reduc-
tion of disulfide bonds to free thiol groups (κ-CN, Lyso, and α-LA) induced partial unfolding and subsequent aggregation. A representative example of the chaperone assays showing the scattering of light arising from heating 0.25 mg/mL Cat in the presence of increasing amounts of A1 β-CN is shown in Figure 5A to illustrate how the percentage protection was determined.

The A2 variant decreased the aggregation of Lyso and α-LA more effectively than did A1 (Figure 5B). In these chaperone assays, the β-CN concentration used to achieve ~50% reduction in total aggregation was above the CMC (2 and 1 mg/mL, respectively). From the SAXS data, at concentrations above the CMC, A2 β-CN had a higher concentration of monomer

present than A1 β-CN, which could explain its greater chaperone activity. Similarly, dissociation of small heat-shock molecular chaperone proteins (sHsp; e.g., αB-crystallin) from their oligomeric state has been proposed as a mechanism that regulates sHsp chaperone action (Kumar and Reddy, 2009). This conclusion is supported by the observation that at concentrations below the CMC where only β-CN monomers exist, as in the Cat chaperone assay, both A1 and A2 β-CN had similar chaperone activity (Figure 5B).

For the κ-CN chaperone assay where the concentration of β-CN used was 3.175 mg/mL (i.e., above the CMC), A1 and A2 β-CN had similar chaperone activity. At this concentration, we anticipated that A2 β-CN would have greater chaperone activity. This difference may be explained by κ-CN aggregating to form amyloid fibrils (Thorn et al., 2005b), in contrast to the other target proteins, which all aggregate amorphously. The type of target protein aggregation may have a significant effect on β-CN chaperone ability as we observed for αB-crystallin interacting with target proteins that are aggregating via these 2 types of pathways (Ecroyd et al., 2007; Ecroyd and Carver, 2008).

The above functional and structural data indicate that A2 β-CN has subtle structural differences compared with A1 β-CN that lead to smaller micelles, an altered monomer–micelle equilibrium leading to more monomer in solution, and enhanced chaperone ability. The DLS data corroborate the data obtained by SAXS. The hydrodynamic diameter of the A1 β-CN micelles was greater than that of the A2 β-CN micelles, as determined by DLS (Figure 3), consistent with the larger Rg Debye calculated from SAXS data (Table 2). The difference in hydrophobicity of A1 and A2 β-CN provides a mechanism to explain the shift toward smaller micelles for A2 β-CN compared with A1 β-CN. Because of the shift in the monomer–micelle equilibrium of A2 β-CN above the CMC, A2 β-CN bound less ANS than

Figure 4. Small-angle X-ray scattering (SAXS) profiles of A1 and A2 β-CN comparing 1 mg/mL A1 and A2 β-CN at 9 and 25°C to show differences in scattering profiles: A2 β-CN contained more monomer protein in the serum phase, as indicated by the upturn at high Q (where Q = magnitude of the scattering vector). (A) Kratky plot; (B) scattering intensity. Color version available online.

Table 2. Values (means ± SD) obtained from small-angle X-ray scattering (SAXS) data fitted to a dual ellipsoid and Debye model for 1 mg/mL β-CN solutions at 25°C

<table>
<thead>
<tr>
<th>Item</th>
<th>A1 β-CN</th>
<th>A2 β-CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rg (Å)</td>
<td>43.2 ± 0.8</td>
<td>42.9 ± 0.8</td>
</tr>
<tr>
<td>Rg (Å)</td>
<td>131.0 ± 4.9</td>
<td>112.8 ± 5.0</td>
</tr>
<tr>
<td>Rg Ellipsoid (Å)</td>
<td>60.5 ± 4.4</td>
<td>60.3 ± 2.5</td>
</tr>
<tr>
<td>Rg Debye (Å)</td>
<td>81.8 ± 0.3</td>
<td>70.0 ± 0.3</td>
</tr>
<tr>
<td>Rg Polydispersity</td>
<td>0.2 ± 0.05</td>
<td>0.3 ± 0.06</td>
</tr>
<tr>
<td>Rg Polydispersity</td>
<td>0.2 ± 0.05</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>X²</td>
<td>0.13</td>
<td>0.11</td>
</tr>
</tbody>
</table>

1Rg = radius along the rotation axis; Rg = radius perpendicular to the rotation axis; Rg Ellipsoid = radius of gyration for the ellipsoid; Rg Debye = radius of gyration for the Debye; Rg Polydispersity = polydispersity index (unitless) for a radius of ellipsoid; Rg Polydispersity = polydispersity index for b radius of ellipsoid.
A1 β-CN (Figure 1) because fewer A2 β-CN micelles were present. These results support the SAXS results (Figure 3) showing the increased number of flexible protein monomers above the CMC for A2 β-CN. As hydrogen bonding and hydrophobic interactions are proposed to participate in micelle formation (Holt et al., 2013; Thorn et al., 2014), the lower hydrophobicity of A2 β-CN compared with A1 β-CN would contribute to this altered equilibrium. Hydrophobic interactions are also thought to be important for the chaperone action of β-CN; however, from the ANS binding results, we would expect A1 β-CN to have enhanced chaperone activity due to its increased hydrophobicity. However, the ANS results did not correlate with the better chaperone ability of A2 β-CN (Figure 5), implying that the degree of exposed hydrophobicity is not directly related to chaperone effectiveness. Similar conclusions have been drawn about the relationship between exposed hydrophobicity of the sHsp α-crystallin and its chaperone ability (Reddy et al., 2006).

A major factor that may regulate β-CN interactions is the greater PPII helix conformation in A2 β-CN due to the proline at residue 67 compared with the histidine at that position in A1 β-CN. The extended PPII helix is favored and prevalent in unstructured proteins such as caseins (Holt et al., 2013) because it imparts flexibility to the polypeptide chain (Adzhubei et al., 2013). The PPII helix is also a common participant in protein–protein interactions (Adzhubei et al., 2013), and we have proposed that PPII-mediated interactions are crucial determinants in casein micelle formation, including of β-CN, via their large tracts of P,Q-rich regions undergoing main-chain hydrogen-bonding interactions with each other (Holt et al., 2013; Thorn et al., 2014). Accordingly, the additional proline in A2 β-CN compared with A1 β-CN (within its P,Q-rich region) would promote PPII helix formation. As a result, the interactions of β-CN with other proteins, particularly other caseins, would be enhanced, leading to tighter packing within the casein micelle and hence a smaller micelle when interacting with itself and stronger interactions when acting as a molecular chaperone to prevent target protein aggregation.

β-Synuclein is another example of an IUP in which a single AA substitution alters functionality. In β-synuclein, a mutation at position 123 from a proline to a histidine (i.e., the same AA substitution as between A2 and A1 β-CN) results in Lewy body pathology (Ohtake et al., 2004), a pathological hallmark of Parkinson’s disease (Lang and Lozano, 1998). Ohtake et al. (2004) hypothesized that alteration in the secondary structure of β-synuclein due to the change from the sterically constrained proline to a histidine residue at residue 123 might explain the progression of Parkinson’s disease observed in patients with this mutation. Specifically, the loss of the proline may reduce PPII helix formation in β-synuclein, with a concomitant reduction in its interactions with other proteins (i.e., α-synuclein). This conclusion is consistent with the
observation that β-synuclein regulates the aggregation of α-synuclein (Bertoncini et al., 2007), the major component of Lewy bodies. In a broader context, IUP have a diversity of important functional roles (Uversky and Dunker, 2010), despite their lack of structure. Intriguingly, the substitution of a single AA in an IUP can have a dramatic effect on the protein’s function, as shown with β-CN and β-synuclein.

Milk functionality is known to be influenced by casein genetic variants, and the data presented here demonstrate the effect that even a single AA substitution can impart on the structure and function of the intrinsically unstructured protein β-CN. The mechanism of how casein genetic variants affect milk functionality remains unknown, but the influence of the His to Pro substitution in A1 and A2, respectively, on β-CN micelle formation and molecular chaperone activity illustrates potential mechanisms that could lead to observed changes in milk functionality.

CONCLUSIONS

In this study, we investigated structural and functional differences between bovine A1 and A2 β-CN using a variety of biophysical techniques and molecular chaperone assays. The results showed that A2 β-CN forms smaller micelles than A1 β-CN, with the monomer–micelle equilibrium of A2 β-CN being shifted toward the monomer. This shift most likely arises from structural differences and leads to A2 β-casein having enhanced chaperone activity above its critical micelle concentration. Alterations in structure (most likely associated with the adoption of more PPII helices in A2 β-CN) led to a decrease in the size of the micelle and in their number, which was also associated with decreased exposed hydrophobicity of A2 β-CN. The enhanced chaperone ability and structural differences of A2 versus A1 β-CN may provide a rationale for differences in milk functionality between milk homozygous for A1 or A2 β-CN.

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