Heat Treatment of Bovine α-Lactalbumin Results in Partially Folded, Disulfide Bond Shuffled States with Enhanced Surface Activity†

Ramani Wijesinha-Bettoni,‡,§ Chunli Gao,§,|| John A. Jenkins,|| Alan R. Mackie,|| Peter J. Wilde,|| E. N. Clare Mills,|| and Lorna J. Smith*,‡

Department of Chemistry, University of Oxford, Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, U.K., and Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, U.K.

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ABSTRACT: Prolonged heating of holo bovine α-lactalbumin (BLA) at 80 °C in pH 7 phosphate buffer in the absence of a thiol initiator improves the surface activity of the protein at the air:water interface, as determined by surface tension measurements. Samples after 30, 60, and 120 min of heating were analyzed on cooling to room temperature. Size-exclusion chromatography shows sample heterogeneity that increases with the length of heating. After 120 min of heating monomeric, dimeric, and oligomeric forms of BLA are present, with aggregates formed from disulfide bond linked hydrolyzed protein fragments. NMR characterization at pH 7 in the presence of Ca^{2+} of the monomer species isolated from the sample heated for 120 min showed that it consisted of a mixture of refolded native protein and partially folded protein and that the partially folded protein species had spectral characteristics similar to those of the pH 2 molten globule state of the protein. Circular dichroism spectroscopy showed that the non-native species had approximately 40% of the α-helical content of the native state, but lacked persistent tertiary interactions. Proteomic analysis using thermolysin digestion of three predominant non-native monomeric forms isolated by high-pressure liquid chromatography indicated the presence of disulfide shuffled isomers, containing the non-native 61−73 disulfide bond. These partially folded, disulfide shuffled species are largely responsible for the pronounced improvement in surface activity of the protein on heating.

α-Lactalbumin (αLA[1]) is an important calcium binding protein comprising around 20% of the whey fraction of milk (*1*). The native state structure of bovine α-lactalbumin (BLA) possesses two subdomains (Figure 1). The α-domain consists of four α-helices and two short 3_{10} helices, and the *β*-domain contains a triple stranded antiparallel *β*-sheet, a 3_{10} helix, and the calcium binding site. BLA contains four disulfide bonds, two in the α-domain (C6−C120, C28−C111), one in the *β*-domain (C61−C77), and one connecting the α-domain to the *β*-domain (C73−C91).

αLA is one of the most extensively characterized model systems used in protein folding studies. Under various conditions it forms a partially folded "molten globule state" (*2*−*5*). This state is characterized by the presence of nativelike secondary structure and an overall nativelike fold, but it lacks the nativelike packing of amino acid side chains present in the native protein. The molten globule state is somewhat expanded compared to the native form and is highly heterogeneous. Its formation and structure has been studied by numerous techniques, including mutagenesis (*6*−*9*), urea denaturation (*10*−*12*), hydrogen-exchange studies (*13*, *14*), real-time NMR (*15*−*17*), disulfide scrambling experiments (*18*−*20*), and molecular dynamics simulations (*21*−*24*). The molten globule state formed under equilibrium conditions has been shown to have a close similarity to a kinetic intermediate in the folding of the protein (*14*−*17*, *24*).

The formation of a molten globule state may play an important role in determining the physiological functions of

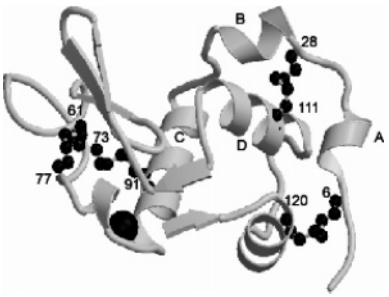

FIGURE 1: Molscript (*67*) representation of the structure of BLA. The α-domain contains four α-helices A, B, C, and D, and the *β*-domain has a three-stranded antiparallel *β*-sheet. The 2.3 Å X-ray structure (1HFZ) was used to generate this diagram. The four disulfide bonds are shown in a ball-and-stick representation, and the Ca^{2+} ion as a black ball.

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* Corresponding author: Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford, OX1 3QR, U.K. Phone: +44-1865-272694. Fax: +44-1865-285002. E-mail: lorna.smith@chem.ox.ac.uk.

‡ University of Oxford.

§ R.W.-B. and C.G. contributed equally to this work.

|| Institute of Food Research.

[1] Abbreviations: αLA, α-lactalbumin; BLA, bovine α-lactalbumin; CD, circular dichroism; HPLC, high-pressure liquid chromatography; LC−MS, liquid chromatography/mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; PFG, pulsed field gradient; R_s, hydrodynamic radius; SEC, size-exclusion chromatography; TOCSY, total correlation spectroscopy.

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αLA. Thus, it has been proposed that the transition to the molten globule state can be induced in physiological systems by compounds such as bile salts and hormones which have surfactant properties (*25*). It is also adopted by αLA when membrane-bound (*25*−*27*), and consequently the protein can be considered "amphitropic". Both human and bovine (*28*, *29*) αLA have also been shown to selectively cause apoptosis in tumor cells when partially unfolded and bound to oleic acid. Furthermore, a molten globule state of the human protein has been shown to have antimicrobial activity (*30*). As a consequence of these physiological activities bovine milk protein fractions enriched with αLA are now added to infant formula (*31*) as well as specialized enteral and clinical protein supplements, sports nutrition products, and products specific to weight management and mood control (*32*).

Many have suggested that the thermal processes routinely used to improve the interfacial properties of protein ingredients could result in formation of molten globule like structures (*33*, *34*). However, these have not been defined and yet could impact on proteins, such as αLA, where partially folded forms are important for its physiological activity. In this paper, we report studies of non-native states of BLA formed by heat treatment which have increased surface activity. In order to understand the structural changes responsible for this, we characterize the states formed using various biophysical techniques, including high-resolution NMR spectroscopy and mass spectrometry. Our results demonstrate that heat treatment of BLA gives a mixture of disulfide shuffled species with some molten globule like characteristics.

MATERIALS AND METHODS

Protein Samples. Highly purified BLA (type I) was purchased from Sigma Chemical Company, St. Louis (product number L-5385).

Thermal Treatment. BLA (0.05 mM in 0.1 M phosphate buffer pH 7 containing 0.1 M $CaCl_2$) was flushed with argon prior to heating to 80 °C for 30, 60, or 120 min and then concentrated by ultrafiltration (1000 molecular weight cutoff ultrafiltration membrane (Millipore UK Ltd, Watford)). Samples heated for 30 and 60 min were desalted using PD-10 columns (GE Healthcare UK Ltd), and the protein was freeze-dried.

Chromatography. Size-exclusion chromatography (SEC) was performed on a Hiload 16/60 Superdex 75 column (GE Healthcare UK Ltd) equilibrated in 50 mM phosphate buffer pH 7 containing 150 mM NaCl, attached to an ÄKTA (Amersham Biosciences Ltd) using a flow rate of 1 mL/min. Protein was monitored in the eluate by UV absorption at 220 nm. Heated samples or selected SEC fractions were further fractionated using reverse phase HPLC on a Phenomenex Jupiter C4, 300 Å, 5 μm, 250 × 4.6 mm column attached to an ÄKTA chromatography system using 0.1% (v/v) trifluoroacetic acid as solvent A and 90% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid as solvent B. The column was equilibrated in 1% (v/v) solvent B and protein eluted with a linear gradient increasing buffer B from 37% to 48% B over 60 min using a flow rate of 1 mL/min. Eluate was monitored for protein using UV absorption at 220 nm.

Electrophoresis. Alkaline (native)-PAGE was performed with a Novex 12% Tris-Glycine precast mini gel from Invitrogen Ltd. (Paisley, U.K.), run with a Novex Tris-Glycine native running buffer. The samples were diluted 1:2 (v:v) with a Novex Tris-Glycine native sample buffer, and the gel was stained with SimplyBlue SafeStain (Invitrogen). SDS−PAGE was performed with a Nupage 12% Bis-Tris precast mini gel (Invitrogen), run with a Nupage MES running buffer. Samples were reduced by addition of DTT (50 mM) and diluted 1:4 (v:v) with Nupage sample buffer, and the gel was stained with SimplyBlue SafeStain. Marker proteins (Chemical Company, St. Louis) aprotinin (M_r 6500), α-lactalbumin (M_r 14 200), trypsin inhibitor (M_r 20 000), carbonic anhydrase (M_r 29 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 000), β-galactosidase (M_r 116 000), and myosin (M_r 205 000).

Circular Dichorism (CD) Spectroscopy. Far-UV (190-260 nm) and near-UV (250−350 nm) CD spectra were recorded at 20 °C using a J-710 CD spectra polarimeter (Jasco Ltd., Japan). 0.5 and 10 mm cells were used for far-UV and near-UV CD, respectively. Far-UV CD spectra were recorded using 25 μM protein (0.05 M phosphate buffer pH 7 containing 0.05 mM $CaCl_2$) in a 0.5 mm path length quartz demountable cell (Hellma) while near-UV CD spectra were recorded using 50 μM protein (0.1 M phosphate buffer pH 7 containing 0.1 mM $CaCl_2$) in a 10 mm path length cell (Hellma). The instrument was calibrated with *d*-10-camphorsulfonate, and spectra were collected as the average of 4 accumulations at 100 nm/min, with a 2 s time constant, 0.5 nm resolution, and a sensitivity of ±100 mdeg. All CD data were converted to molar ellipticity $[\theta]_M$ as described by Mills et al. 2001 (*35*).

Fluorescence Spectroscopy. Fluorescence measurements were performed at 20 °C with a LS 55 luminescence spectrometer (Perkin-Elmer, Wellesley, MA) using a quartz cuvette with a 1.0 cm path length and 0.5 μM BLA samples in 1 mM phosphate pH 7 containing 1.0 μM $CaCl_2$. An excitation wavelength of 280 nm was used, and emission spectra were collected between 300 and 400 nm using excitation and emission slit widths of 5 nm and a scan speed of 100 nm/min.

Surface Activity Measurements. The surface tension (γ) was measured using an FTA200 pulsating drop tensiometer (First Ten Ångstroms, Portsmouth, VA). The technique measures the surface tension using the pendant drop technique. An image of a liquid droplet hanging from the tip of a syringe is captured. The shape of the drop (determined by its density and the surface tension) is analyzed using a derivation of the Young−Laplace equation (equation of capillarity) to give the surface tension. The initial drop volume was 12 μL. The syringe volume was 100 μL, fitted with a Teflon coated, flat ended tip of 0.94 mm in diameter. The applied surface area oscillations had a relative amplitude of 5% to avoid excessive perturbation of the interfacial layer, and the measurement frequency was 0.05 Hz. All measurements were made at room temperature (approximately 20 °C).

ANS Binding. Protein surface hydrophobicity (S_0) was measured using the binding of 1-(aniline)naphthalene-8-sulfonate (ANS) according to the method of Haskard and Li-Chan (*36*). Values of S_0 were determined from the initial slope of the relative fluorescence of an 8 μM solution of ANS versus protein concentration over the range 0 to 20 μM.

NMR Spectroscopy. NMR experiments were performed using BLA before or after heating for 30 or 60 min, or monomer isolated by SEC from the sample heated for 120 min. Samples (2 mM protein) were prepared in 95% H_2O/5% ^2H_2O containing 4 mM $CaCl_2$ in shigemi tubes. NMR experiments were carried out on home-built spectrometers at the Oxford Centre for Molecular Sciences, at 500, 600, or 750 MHz. A sweep width of 8000 Hz was used at 500 MHz and scaled accordingly for higher fields. Two-dimensional TOCSY (*37*) spectra (74 ms mixing time) were collected with 350 complex t_1 increments of 1024 points. The data were zero-filled to 2 K in both dimensions. NOESY (*38*) spectra (200ms mixing time) were collected with 256 complex t_1 increments of 2048 points. The data were zero-filled twice to 1 K in the t_1 dimension and zero-filled once to 2 K in the t_2 dimension. Resolution enhancement was by Gaussian multiplication in the t_2 dimension and by a trapezoidal multiplication (TOCSY) or a shifted sinebell window function (NOESY) in the t_1 dimension.

Hydrogen-Exchange Experiments. Hydrogen exchange was initiated by dissolving the samples (30 min heated sample and isolated 120 min monomer at 2 mM, 60 min heated sample at 3.5 mM) in 50 mM deuterated imidazole buffer, pH 7, containing a 2 molar excess of $CaCl_2$ over protein in ^2H_2O. A series of 10 short mixing time (25 ms) TOCSY spectra were collected with 256 complex t_1 increments of 1024 points. The acquisition time was 6.05 h per spectrum, with the spectral acquisition beginning 1 h after dissolution of protein. Hydrogen-exchange rates were determined from two parameter exponential fits of peak heights in the processed TOCSY spectra as a function of time. Protection factors were determined by dividing the intrinsic exchange rates (k_{int}) by the observed exchange rates (k_{ex}) (*39*).

Bulk hydrogen-exchange measurements were carried out on the misfolded fractions, on an unheated control sample in the absence of urea and in 10 M urea. All samples were first dissolved in H_2O at pH 2 and lyophilized. After dissolution of the protein in ^2H_2O or 10 M urea in ^2H_2O at pH 2 a series of 1D NMR spectra were acquired at 20 °C over a period of time. Each sample was then briefly heated to 50 °C and cooled to 20 °C and a reference spectrum collected. Only the resonances of nonexchangeable aromatic protons remain in the downfield region of this reference spectrum. This spectrum was used to determine the intensity per proton on the basis of the known number of nonexchangeable aromatic protons. This reference spectrum was subtracted from each of the hydrogen-exchange spectra to obtain the number of amide protons present as a function of time.

NMR Diffusion Measurements. These were carried out using the PG-SLED sequence (*40*). A small amount of 1,4-dioxane was added to each sample as an internal standard (*40*, *41*). A total of 20 spectra were collected with the gradient strengths varying linearly from 5 to 100%. From the ratio of the rate of decay of protein and dioxane resonances as a function of the gradient strength, the hydrodynamic radius of the protein is determined assuming a hydrodynamic radius of 2.16 Å for dioxane. The errors for the measured values are estimated to be in the range ±(0.1−0.3) Å on the basis of repeat measurements on each sample.

Enzymatic Digestion and LC−MS Analysis. Nativelike and misfolded BLA isolated from the 120 min heated sample by HPLC were digested with thermolysin as described by Chang and Li (*42*), and the resulting peptide mix was analyzed by LC−electrospray MS essentially as described by Moreno et al. (*43*) using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, U.K.).

Intact MALDI-TOF Mass Spectrometry. Samples were analyzed using an UltraFlex-MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Coventry, U.K.). Samples were prepared by mixing each sample with a saturated solution of matrix in the ratio 1:1. The matrix solution was made by dissolving sinapinic acid in 30% acetonitrile/0.05% trifluoroacetic acid to saturation. 0.5 μL of this combined mixture was spotted onto a polished stainless steel target and allowed to crystallize prior to analysis in the MALDI-TOF spectrometer using a nitrogen laser.

RESULTS

Biochemical Analysis of Thermally Treated BLA. Heating dilute solutions (0.05 mM) of holo BLA to 80 °C, 10 deg above the main thermal transition for this protein (*44*), for up to 60 min had little effect on the molecular weight of the protein, as determined by size-exclusion chromatography (SEC) (Figure 2A). However, prolonged heating for 120 min resulted in broadening of the M_r 14 200 peak and formation of a small M_r 26 700 peak equivalent to dimerized material together with a smear running with a M_r of 33,000−73000 corresponding to larger oligomers. SDS−PAGE of these native and heated samples under nonreducing conditions (Figure 2B tracks 7−11) showed little change up to 60 min. However, the 120 min heated monomeric BLA showed some evidence of modification with a weak band of higher mass in addition to the native M_r 14 200 polypeptide (track 9) while the "dimer" and oligomeric fractions showed evidence of aggregation (tracks 10, 11). Analysis of these samples under reducing conditions (tracks 2−6) showed that while the 60 min heated sample appeared identical to the unheated material, the 120 min monomer contained some discrete lower molecular weight polypeptides which were more prominent in the "dimer" fraction, the oligomeric fractions containing no intact M_r 14,200 BLA polypeptide but comprising only small peptides of M_r <6000. Thus, prolonged heating resulted in some peptide bond hydrolysis although the resulting peptides had formed into larger disulfide-linked structures. Deamidation of Asn and Gln residues (with the amide bond of the former being more labile) following heating has been postulated for BLA, as heating at 100 °C can result in release of ammonia (*47*) and has been proven for other proteins (*45*, *46*). 2D PAGE analysis of the heated monomer fractions gave predominantly a single spot with a p*I* identical to that of the native protein (~4.7), indicating that under the conditions employed in this study deamidation did not take place (data not shown).

The 120 min heated monomer gave a broad peak on SEC, indicative of its comprising of multiple species. When subjected to reverse-phase HPLC analysis (Figure 2C) it was found to comprise a major polypeptide with a retention time corresponding to the native monomer (peak 1) accompanied by a minor peak of slightly longer retention time (peak 1b).

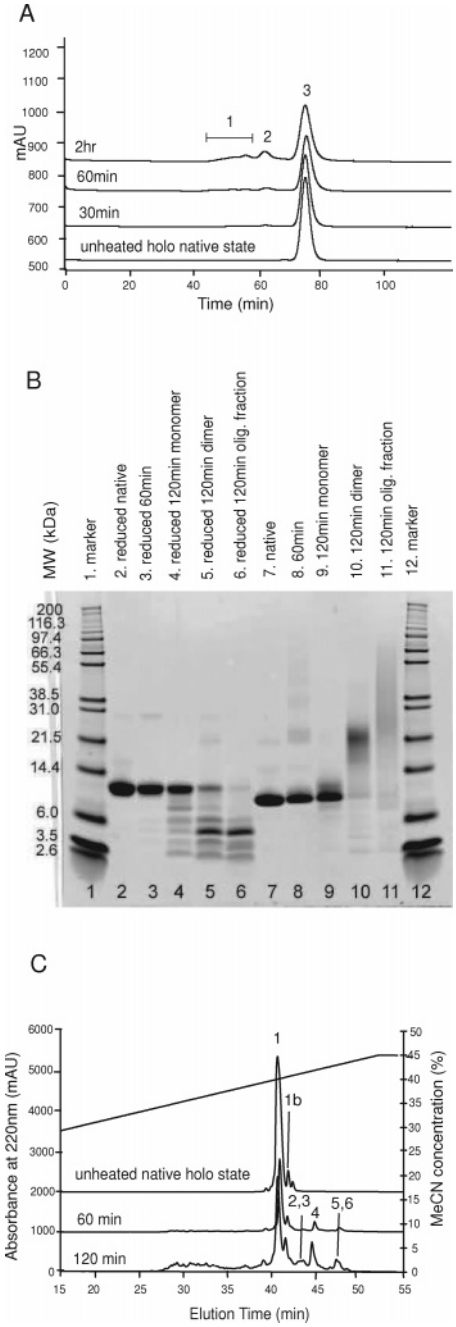

FIGURE 2: (A) Size-exclusion chromatography elution profiles for native and heat-treated BLA. BLA (0.05 M) was heated in phosphate buffer pH 7 containing 0.1 M $CaCl_2$ at 80 °C for 30, 60, and 120 min. The positions of oligomeric (1), "dimeric" (2), and monomeric (3) species are indicated. (B) SDS−PAGE analysis of heated BLA samples prior to (lanes 7−11) and after (lanes 2−6) reduction. Lanes are as follows: native BLA, 2, 7; BLA heated to 80 °C 60 min, 3, 8; SEC fractions from BLA heated for 120 min as oligomeric fraction 6, 11; "dimer" 5, 10; monomer 4, 9. (C) HPLC fractionation of native BLA, and heated 60 and 120 min monomeric BLA. Peaks 1, 1b, "native" BLA; peaks 2−6, misfolded BLA monomer.

A similar pattern has been observed by others (*48*). Both peaks gave identical masses by MALDI-TOF MS of 14 176 Da corresponding to intact BLA. This was accompanied by a system of peaks (peaks 2−6) of longer retention time indicative of new forms of BLA with different hydrophobicities, and suggests formation of misfolded monomeric species. In this work we refer to a sample containing these various misfolded monomer species as the "misfolded monomer".

Table 1: Percentage of Nativelike BLA and Misfolded Forms Present in the Heated BLA Samples[a]

	HPLC analysis of heated BLA monomer			
sample	% nativelike	% misfolded	near-UV CD estimate of % nativelike	surface hydrophobicity as measured by ANS binding S_0 (a.u.)
unheated	100	0	100	1.5
30 min heated	89	11	∼82	7.7
60 min heated	70	30	∼61	18.8
120 min heated isolated monomer	51	49	∼46	26.8

[a] The data are obtained from analytical-HPLC. The estimated percentage of native protein from near-UV CD spectroscopy and the surface hydrophobicities as measured by ANS binding is also given.

Quantification of the native and the misfolded forms from these chromatograms (Table 1) showed the progressive increase in the proportion of misfolded monomer during heating such that after 120 min at 80 °C it constituted around 50% of the monomeric BLA fraction. Native PAGE analysis of the 120 min monomer isolated by preparative SEC also showed the appearance of higher mobility bands corresponding to non-native monomeric species at the expense of the native monomer (data not shown).

Surface Activity of Thermally Treated BLA. The effect of heating on the surface properties of BLA was investigated as a function of surface tension (γ) at the air:water interface. The results (Figure 3A) are shown in terms of surface pressure (surface tension of buffer − surface tension of sample) as a function of time. Heat treatment improved the surface activity in all cases. The most pronounced increase in surface activity was observed for the misfolded fractions isolated from the 120 min monomer sample (which contain mainly peaks 4, 5, and 6 as shown in the HPLC profile in Figure 2C). The dimer and oligomeric fractions resulting from prolonged heating also had enhanced surface properties when compared with the unheated holo native state (Figure 3A), but the enhancement was more pronounced for the monomer and dimer. Both the rates at which the protein adsorbed to the surface and the final surface pressure reached were very similar for the monomer and dimer, although greater for the former.

Thermally Induced Changes in Secondary Structure of BLA. Insight into the thermally induced conformational changes was obtained by using fluorescence spectroscopy to probe changes in the environment of the Trp residues (Figure 3B). Heating for 30 min had little effect on the fluorescence spectrum, but after 60 min λ_{max} shifted from 332 to 345 nm and was accompanied by an increase in intensity of ∼30%. These changes are similar to the red shift observed for BLA when it adopts the pH 2 molten globule and results from movement of the Trp residues into a more solvent-exposed environment compared with native BLA (*49*). The monomer and dimer formed by heating for 120 min showed a shift in λ_{max} to 352 nm with an increase in intensity of ∼54−61% compared with native BLA. In contrast the oligomeric fraction showed a shift in λ_{max} to 350 nm but only a modest increase in fluorescence intensity of ∼10% compared with native BLA (Figure 3B). These data indicate that overall the Trp residues in the monomeric

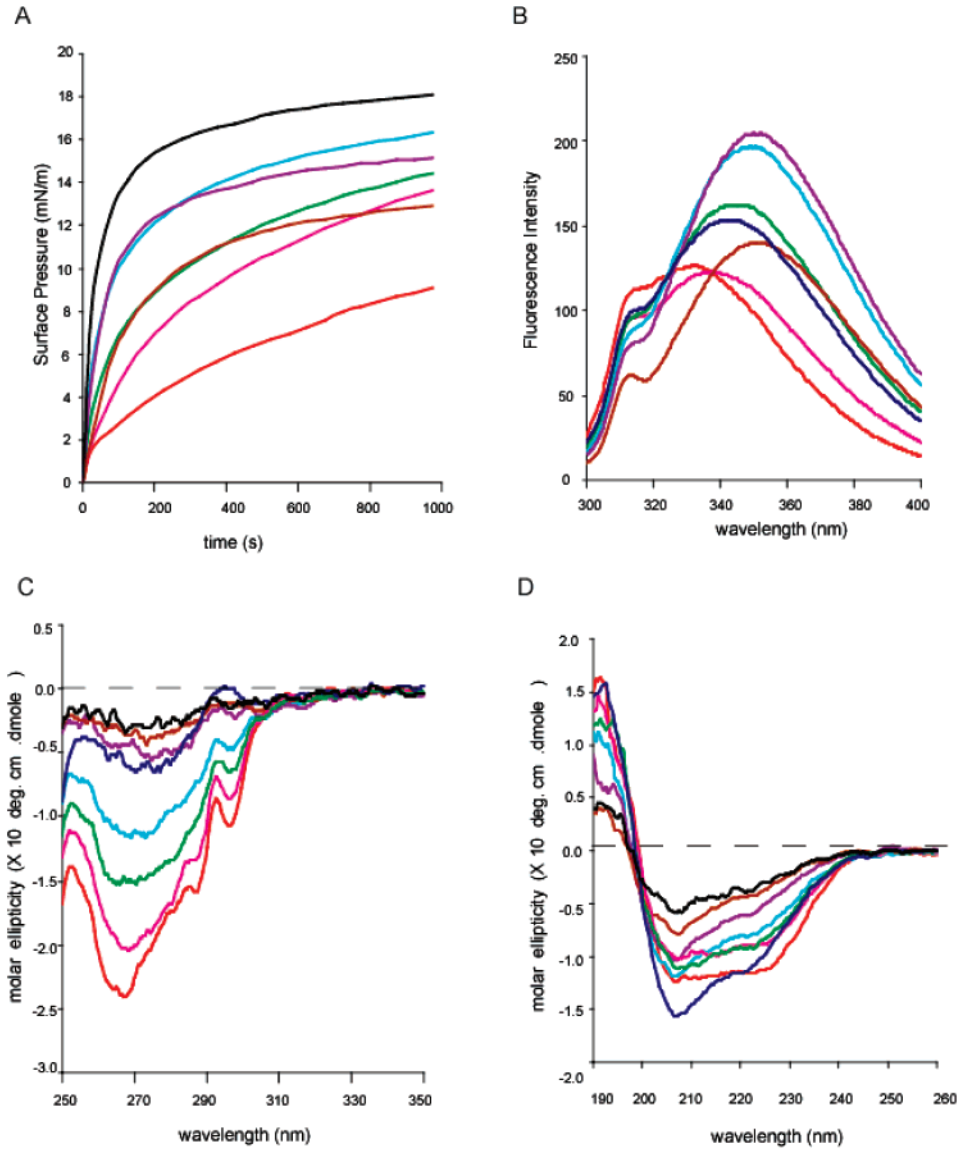

FIGURE 3: The colors used in panels A−D are as follows. BLA heated at 80 °C for 30 min (pink); 60 min (green); SEC fractions of BLA heated for 120 min, monomer (turquoise), dimer (violet), and oligomeric fractions (brown); HPLC fractionated 120 min misfolded fraction (black); unheated holo native BLA (red). In panels B−D the pH 2 molten globule is also shown (blue). (A) Surface pressure plotted as a function of adsorption time. (B) Fluorescence emission spectra. All measurements were made at 25 °C. The protein concentration was kept constant for all the samples, and the fluorescence intensity shown has not been normalized. Samples contained 0.5 μM BLA in 1 mM pH 7 phosphate buffer and 1.0 μM $CaCl_2$. (C) Near-UV and (D) far-UV CD spectra. All measurements were made at room temperature. 25 μM protein in 0.05 M pH 7 phosphate buffer containing 0.05 mM $CaCl_2$ was used for far-UV CD measurement. 50 μM protein in 0.1 M pH 7 phosphate buffer containing 0.1 mM $CaCl_2$ was used for near-UV CD measurements.

and dimeric heated samples were in a more exposed environment than in the native state of BLA.

The impact of heating on the environment of aromatic side chains, which reflects the tertiary structural contacts, was followed using near-UV CD (Figure 3C). The native holo state gave a characteristic spectrum with a broad Tyr minimum around 270 nm and a local Trp minimum around 297 nm. In the heated samples the trough intensities at 270 nm and 297 nm decreased in proportion to the extent of heating and were used to estimate the percentage of the molecules in the samples remaining in the native state (*49*). These estimates were in reasonable agreement with those obtained using HPLC analysis to separate the native and misfolded forms (Table 1). The misfolded monomer, "dimer", and oligomeric fractions formed after heating for 120 min gave near UV spectra with very little molar ellipticity, similar to that of the pH 2 molten globule indicating the reduction in persistent tertiary structure.

The far-UV CD intensity at 222 nm is an indicator of helical secondary structure. As observed previously (*52*) unheated holo native BLA displays a double minimum at around 222 and 208 nm, characteristic of its α-helical structure, the pH 2 molten globule state having essentially the same spectrum and proportion of α-helix (Figure 3D). Heating resulted in a slight decrease in trough intensity compared to the spectrum of the unheated holo native state, indicating a loss of α-helical secondary structure. The misfolded monomer formed after 120 min heating had only around 42% of the native α-helical secondary structure remaining; the "dimer" and oligomeric fractions also had reduced molar ellipticity at 222 nm although the spectra were indicative of some residual α-helical secondary structure.

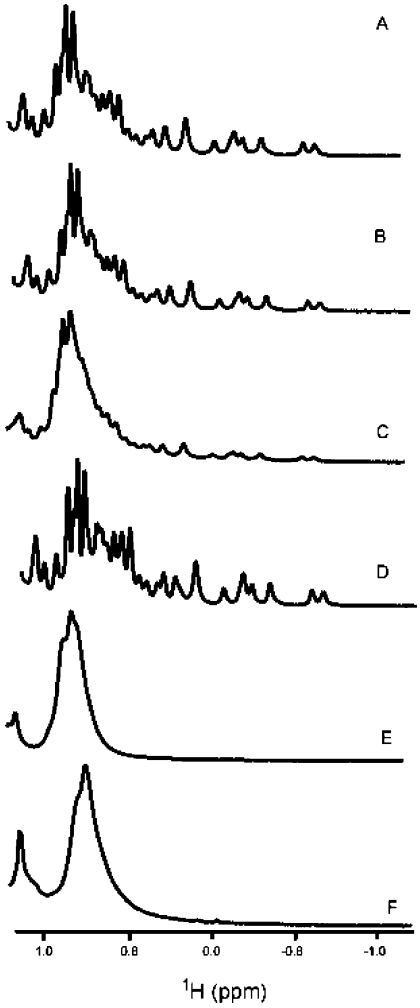

FIGURE 4: Methyl region from ^{1}H NMR spectra of heat-treated solutions of BLA. The spectra shown are for samples of BLA as follows: BLA heated at 80 °C for 30 min (A), 60 min (B), and SEC isolated monomer after heating for 120 min (C); unheated holo native state (D); pH 2 molten globule state (E); HPLC fractioned monomer misfolded BLA post 120 min heating (F). Spectra A−D and F are at pH 7; all the spectra have been recorded at 20 °C.

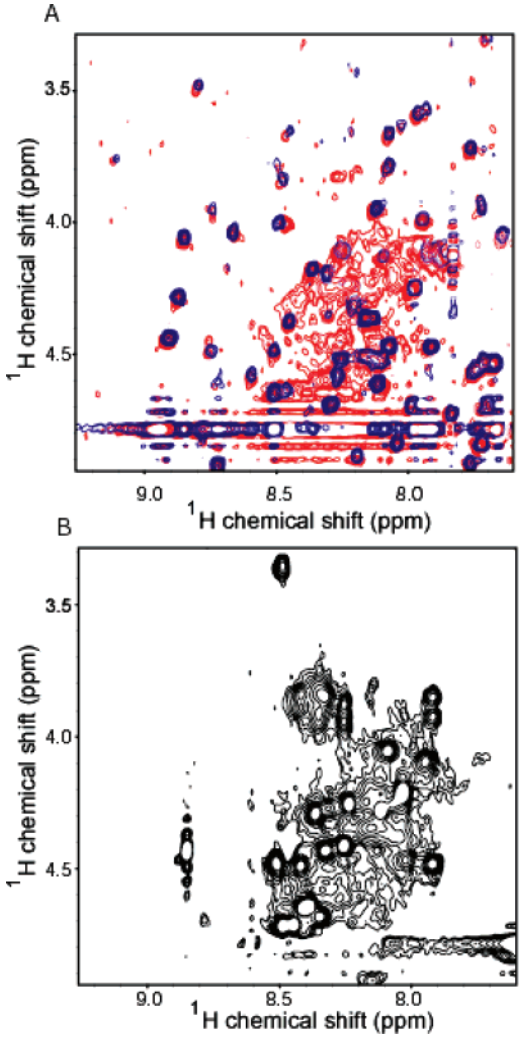

FIGURE 5: Part of the fingerprint region of 2D TOCSY at pH 7 and 20 °C in 95% H_2O/5%^2H_2O of (A) the SEC isolated 120 min 80 °C heated monomer (red) overlaid with the spectrum of the unheated holo native state (blue) of BLA and (B) the pH 2 molten globule state of BLA. The spectra were collected at 20 °C.

Thermally Induced Partially Folded BLA. BLA heated for 30 and 60 min and the monomer isolated by SEC after heating for 120 min gave NMR spectra which showed resonances with considerable chemical shift dispersion, typical of a native folded protein (Figure 4A−C). However, when the large methyl envelope (1−0.5 ppm) is compared for the heated samples with the unheated holo native state (Figure 4D), it is evident that the fine structure is reduced in all the heated samples, though to different degrees. These spectral data therefore suggest that all three heated samples contain some proportion of protein which has lost the native fold.

The spectrum for the 120 min monomer has some very broad signals, with line widths similar to those observed in spectra of the pH 2 molten globule state (Figure 4E) of BLA. Figure 5A shows the fingerprint region of a 2D TOCSY spectrum of this sample overlaid on a spectrum of the unheated holo native state. Peaks corresponding to those of the native state of BLA are present in the 120 min monomer spectrum, overlapping very well. This confirms that at least a fraction of the sample retains the native structure despite the prolonged heating. Detailed comparison of the NH chemical shifts and Hα chemical shifts in the two spectra did not reveal any significant differences (<0.03 ppm for Hα, <0.05 ppm for NH). Hence, the protein that refolds back into the native state in the 120 min monomer is very similar to the native BLA. In addition to the native peaks, a broad set of peaks with a distinct lack of chemical shift dispersion were observed which are similar to the peaks seen in the spectrum of the pH 2 molten globule state of BLA (Figure 5B). The broadening in the molten globule state spectrum is recognized to arise from interconversion between the conformers populated in the partially folded ensemble on the millisecond to microsecond time scale (*53*).

Analysis of the downfield region of the 1D spectra (Figure 6i), which has little resonance overlap, was used to clarify the changes occurring in the heated samples. This region of a 1D spectrum of the pH 2 molten globule of BLA shows only one broad resonance originating from the tryptophan side chain indole NH groups; its position is close to the random coil chemical shift value for these protons. The downfield region of the spectrum of the native state of BLA shows a number of well-resolved amide and aromatic resonances. The spectrum of the 120 min monomer is similar

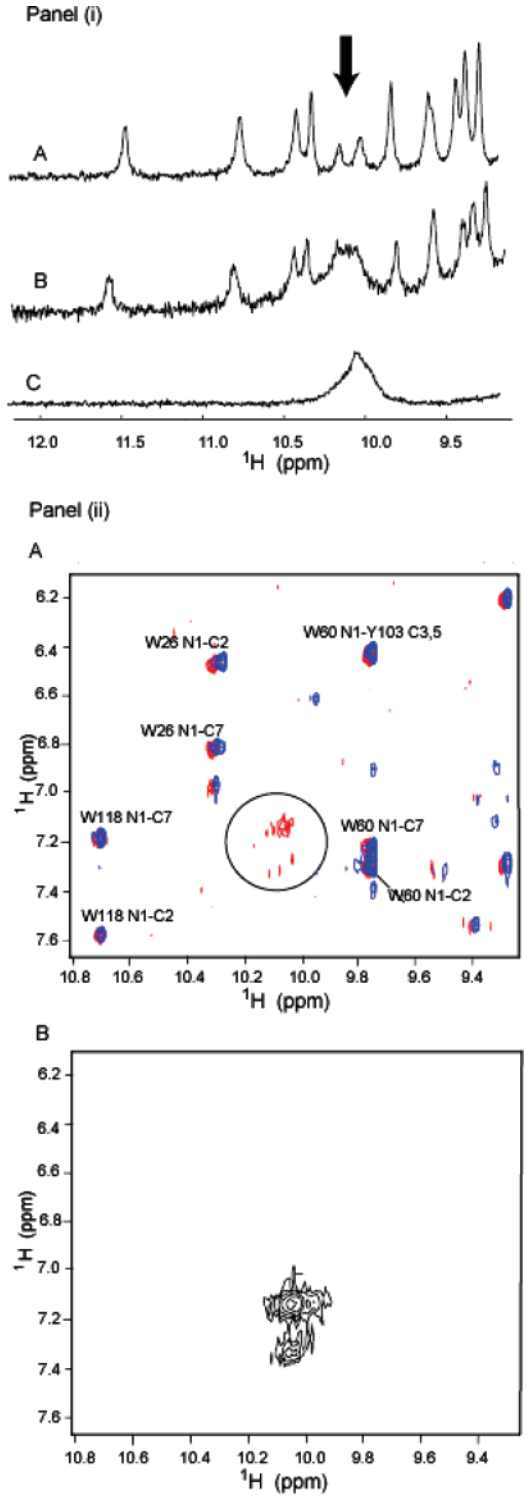

FIGURE 6: The downfield region of NMR spectra of BLA samples at pH 7 and 30 °C in 95% H_2O/5% ^2H_2O. Panel i: 1D spectrum of the SEC isolated 120 min 80 °C heated monomer (B) compared with same spectral region of unheated holo BLA (A) and the pH 2 molten globule state of BLA (C). Positions where spectral changes occurring in the heated sample can be readily resolved are arrowed. Panel ii: An expanded area of the downfield region of the 2D NOESY spectra of (A) the SEC isolated 120 min 80 °C heated (red) overlaid with spectrum for the unheated holo native state (blue) and (B) the BLA pH 2 molten globule state. The assignments of the NOEs involving the Trp indole NH groups in the native state of BLA are labeled. The encircled peaks in panel A indicate the position of new peaks appearing in the heated sample. The NOESY spectra were collected at 30 °C in order to give better spectral resolution.

to a combination of the resonances seen in the spectra of the native and pH 2 molten globule states of BLA. Comparison of the 2D NOESY spectra of the same samples confirms this (Figure 6ii). Thus, the 2D spectrum of the 120 min monomer contains both nativelike cross-peaks and those from tryptophan indole NH groups in an unfolded environment. These data show that the 120 min monomer contains a mixture of native folded BLA and partially folded species similar, in at least some respects, to the BLA molten globule, with a smaller proportion of these being present in the samples heated for only 60 min.

The spectrum (at pH 7 in the presence of Ca^{2+}) of the misfolded monomer isolated from the 120 min monomer is shown in Figure 4F. This is very similar to that of the pH 2 molten globule, and does not contain any nativelike peaks. (The 2 upfield shifted peaks visible at 0.13 ppm and 0.02 ppm do not correspond to any native peaks. These peaks are visible even in a spectrum recorded at pH 2, 9 M urea.) 2D NOESY and TOCSY spectra for this misfolded monomer fraction show mainly very broad peaks indicating that the broad peaks seen in the 120 min monomer arise from this misfolded fraction.

NMR Spectroscopic Hydrogen-Exchange Study. Information on the dynamical characteristics of protein structures can be obtained from studies of hydrogen-exchange protection (*54*). It is possible that some of the molten globule like species present in the heat-treated samples of BLA are in equilibrium with the native state folded protein. To test this, hydrogen-exchange studies have been performed using NMR to characterize the exchange rates of individual amide protons. The pH 2 molten globule of BLA is known to have very little hydrogen-exchange protection when compared with the native state (*14*). At pH 7, the exchange would be even faster, due to the effect of pH on the intrinsic exchange rates of peptides and proteins (*39*). Therefore, if even a small population of protein having a partially folded conformation is present in equilibrium with the native protein in the heat-treated samples, this would lead to a rapid loss of hydrogen-exchange protection for the nativelike protein.

Hydrogen-exchange experiments were carried out on BLA heated for 30 and 60 min, the 120 min monomer, and unheated holo native state sample. The analysis used only the resonances of the native protein in the spectra. The hydrogen-exchange rates seen for the native folded protein in all three heated samples were very similar to those seen for the unheated holo native state protein. As the signal-to-noise ratios of the native state peaks in the spectra of the 120 min monomer were weak, further detailed analysis was undertaken using the 60 min sample. Protection factors were calculated from the experimental rates obtained, and were found to be closely similar to those for the unheated holo native state (Figure 7A,B). It is therefore clear that the native folded form and the partially folded species present in the heat-treated sample are not in equilibrium with each other.

It is not possible to obtain residue specific hydrogen-exchange information for the misfolded monomer isolated from the 120 min monomer as there are no resolved peaks in the 2D spectra. However, the bulk exchange behavior of labile hydrogen atoms can be measured by NMR. As the loss of hydrogen-exchange protection in the low pH molten globule state of BLA is known to be very fast (*14*), it can be expected that the loss of protection at pH 7 of a molten

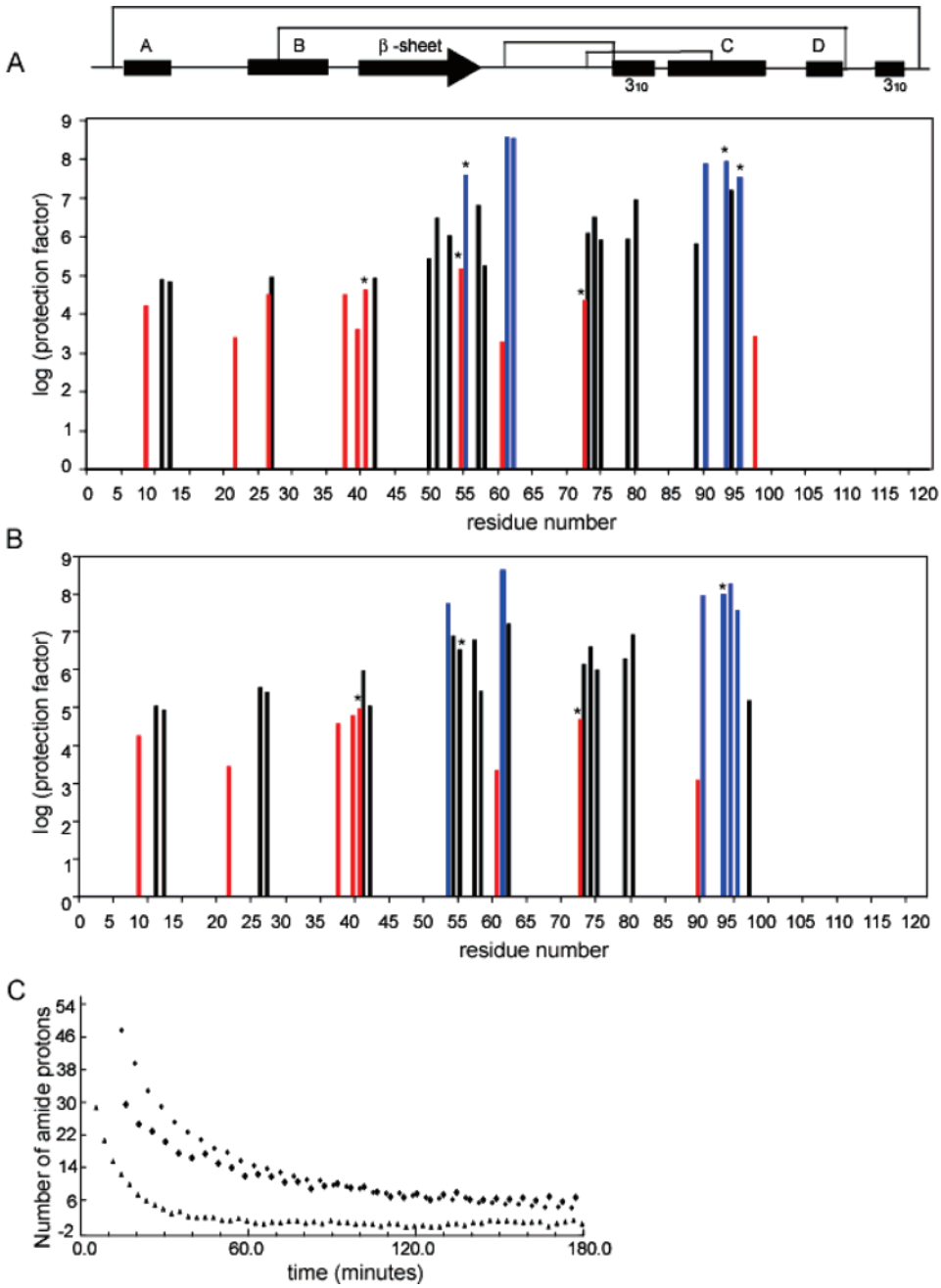

FIGURE 7: Histograms showing the distribution of protection factors for backbone amide hydrogen exchange at pH 7 and 20 °C in (A) the unheated holo BLA native state and (B) the BLA sample after thermal treatment at 80 °C for 60 min. The protection factors are plotted on a logarithmic scale (log) as a function of residue number. The positions of the regions of secondary structure found in the native structure (labeled A to D for the α-helices, 3_{10} for the 3_{10} helices, and *β*-sheet) and the disulfide bridges are shown schematically above the plots. Blue bars correspond to NHs that exchange too slowly during the time course of the experiment for their rates to be measured. For these residues the values shown are the lower limits of the protection factors. Red bars correspond to NHs seen only up to the third time point in the hydrogen-exchange experiments. The data for these residues cannot be fitted accurately to the rate equation, so an estimate of the experimental rates has been used to obtain the protection factors. An asterisk above a few of the bars (in A, residues 40, 54, 55, 72, 93 and 95; and in B, 40, 55, 72 and 93) indicates the residues whose resonances have very low intensity even in a spectrum collected in 95% H_2O. (C) Time-dependence of the total number of protected amide groups in BLA, pH 2 after dissolution in deuterated solutions, as determined by 1D NMR spectra. The circles and triangles correspond to the molten globule formed by unheated BLA (containing the native disulfide bonds) in ^2H_2O and 10 M urea, respectively. The diamonds correspond to the misfolded fractions isolated from the 120 min monomer sample.

globule like species would be too fast to detect, due to the large increase in intrinsic rates with pH. The NMR spectrum of the misfolded monomer at pH 2 was very similar to that of the unheated protein at pH 2. Therefore, the bulk exchange was carried out at pH 2, which made it possible to compare the data with that obtained for the pH 2 molten globule of the protein with the native disulfide bond pairings. Figure 7C shows the data obtained, together with the hydrogen exchange in 10 M urea of the pH 2 molten globule of an unheated sample. Under these denaturing conditions, BLA is reported to have no significant protection relative to that expected for a random coil state (*12*). The data show that the level of protection in the misfolded monomer isolated from the 120 min monomer sample is lower than for the pH

Table 2: Mass Data from LC−MS[a]

fraction	peak time (min)	MS	S−S bond
1	22.04	1816, 1904	61−77
	27.93	1964, 2051	61−77
2	22.30	1601, 1687	61−73
3	20.10	742, 372	28−111
	22.39	1601, 1687	61−73
4	22.47	1601, 1687	61−73

[a] Disulfide bonds were identified from data from the standard cutter database, and from data provided from Chang and Li(*42*). Fractions (1−4 in Figure 2C) from the 120 min monomer sample were isolated by HPLC, digested with thermolysin and digests analyzed by LC−MS. Fractions 5 and 6 were too weak for LC−MS. Peak time refers to the retention time in HPLC of peptide fragments formed by thermolysin digestion.

2 molten globule of the unheated protein. However, there is much more protection than observed for BLA in a random coil conformation.

Pulse field gradient (PFG) NMR diffusion measurements (*40*, *55*) for the misfolded monomer at pH 2 give a hydrodynamic radius (R_s) of 23.8 (±1) Å (obtained by integrating the isolated His peak seen at 8.6 ppm) compared with 21.6 (±0.2) Å for the pH 2 molten globule of an unheated sample, and 19.4 (±0.7) Å for a native unheated sample. This shows that the misfolded monomer forms on average a more expanded state than the pH 2 molten globule. However the state adopted by the misfolded fractions is more compact than that seen for fully unfolded BLA with native disulfide bridges which has a R_s of ∼28.7 Å (*12*).

Proteomic Analysis of Thermally Induced Misfolded BLA. As suggested earlier, one factor that could be responsible for stabilizing the misfolded 120 min monomer is some type of thermally induced chemical modification or disulfide rearrangement. Thus, an assessment of the disulfide status using thermolysin digestion of the heat-treated samples was undertaken (*42*), since this allows the β-domain disulfides C61−C77 and C73−C91 to be distinguished, unlike trypsin (*56*). Nativelike BLA and selected misfolded monomer fractions (peaks 2, 3, and 4 in Figure 2C) from the 120 min monomer sample were isolated by HPLC and digested with thermolysin, and the digests were analyzed by LC−MS. While only limited analysis was possible due to the small amounts of sample available, a peptide mass corresponding to the non-native disulfide bond 61−73 peptide (*42*) was observed in the misfolded monomer fractions 2, 3, and 4 (Table 2). In addition a peptide mass that matched that of a peptide containing the native disulfide 28−111 was also identified in the misfolded fraction 3.

DISCUSSION

This paper reports a detailed characterization of the products formed by prolonged heat treatment of dilute solutions of BLA at 80 °C. The conditions were chosen to ensure that the BLA remained largely monomeric and hence tractable to NMR characterization of the heat-treated states. While there would undoubtedly be aggregation at higher concentrations, it is the monomeric species that are left unaggregated that play the most important role at the interface. When trying to improve surface activity there is always a tradeoff with increased aggregation. Following prolonged heating a small proportion of the BLA underwent hydrolysis with the appearance of a discrete series of disulfide-linked lower molecular weight polypeptide chains, suggesting that certain peptide bonds are more susceptible to hydrolysis than others. There was no evidence of other chemical modification such as deamidation. These hydrolysis products formed larger aggregates corresponding to a "dimer" and higher molecular weight oligomeric species, their size making them intractable to detailed NMR analysis. Consequently we focused on characterizing the monomeric species which comprised the majority of the postheating protein.

A proportion of the protein was found to be essentially unaltered by the heating process using a range of spectroscopic techniques, folding back into the holo native state structure on cooling. Comparison of the chemical shifts of the NMR resonances and hydrogen-exchange behavior of this refolded, heat-treated protein with those of unheated native state BLA has confirmed their very close similarity, despite the considerable length of heating. As the heating time was increased, the proportion of the protein which refolds into the native state decreased, and an increasing amount of protein remains at least partially unfolded even after cooling.

Thermally induced misfolded monomeric species were characterized and were found to resemble to some extent molten globule states. In particular, our CD and NMR data have shown that these species form compact states that have persistent helical secondary structure but disordered tertiary interactions. The NMR spectra of these species showed significantly broadened resonances with a lack of chemical shift dispersion. These characteristics reflect interconversion between conformers within the partially folded ensemble on the millisecond to microsecond time scale. However, the monomer misfolded species have less helical secondary structure and lower levels of hydrogen-exchange protection than seen for the classic pH 2 molten globule state of BLA. The species are on average slightly more expanded (R_s 23.8 Å compared with 21.6 Å for the pH 2 molten globule). These differences presumably arise from the non-native disulfide bridge pairings present in these species. The non-native disulfide bond pairings will also presumably result in a non-native overall fold for the protein.

The thermally induced misfolded forms of BLA described here are irreversibly formed. It is highly likely that this is as a result of thermally induced disulfide-bond shuffling. Species of α-lactalbumin with non-native disulfide bonds have been characterized in a number of previous studies. These include studies of the oxidative folding of the protein (*56*, *61*, *62*), disulfide scrambling experiments (*42*, *63*−*65*), and characterization of three disulfide (*56*, *61*−*63*) and two disulfide (*56*, *61*) bond variants of α-lactalbumin. Disulfide scrambling experiments performed at high temperature have been reported but employed a thiol initiator in the absence of Ca^{2+}. Such conditions resulted in the formation of a predominant BLA species X-αLA-c during the early stages of heating (*42*). This species has two native disulfide bridges (6−120 and 28−111) and two non-native disulfide bridges (61−73 and 77−91). When Ca^{2+} is present during the thermal denaturation, there is a marked reduction in the prevalence of the X-αLA-c species as the binding of Ca^{2+} leads to protection of the two disulfide bridges involving β-domain residues. It is possible for disulfide interchange to take place in the absence of a thiol initiator since heating can result in β-elimination of cystine residues, leading to the production of free thiols. This has been shown for several

proteins, including hen egg-white lysozyme (HEWL), a structural homologue of αLA at pH 4−8 (*45*, *46*, *66*). A preliminary analysis of the S−S shuffled species in the current study indicated that all the misfolded 120 min heated monomeric BLA fractions contained a non-native 61−73 disulfide bond. However, it is likely that there is an ensemble of different species with different combinations of native or non-native disulfide bonds in the misfolded monomeric BLA fractions.

The surface activity of a protein is governed by its surface hydrophobicity and the flexibility of the molecule. The surface hydrophobicity controls the propensity of the molecule to adsorb to the interface while the flexibility helps the molecule to stay there because it can partially unfold upon adsorption to an interface increasing the residence time and the interactions with other proteins in the adsorbed layer (*57*). The non-native monomers are likely to be much more flexible than the native state, and have increased exposure of hydrophobic groups, as is known to be the case for the molten globule state. Measurements of surface hydrophobicity (ANS binding; Table 1) demonstrate the increase in surface hydrophobicity induced by heating. This correlates well with the differences in surface activity of the partially folded forms of BLA seen in Figure 2A where the 30, 60, and 120 min monomer samples all showed consistent increases in surface activity. The surface activity of the dimer and oligomeric fractions from the 120 min heated sample was lower both because diffusion to the surface would have been slower and also because the regions of highest surface hydrophobicity on the molecules would have been buried by the formation of the aggregate. The heating induced formation of misfolded species with a higher surface hydrophobicity, particularly the isolated misfolded 120 min monomer. The disulfide interchange then maintained the misfolded structure playing an important role in improving the surface activity of BLA.

In summary, this work has demonstrated that heating holo BLA for 120 min at 80 °C in pH 7 phosphate buffer in the absence of a thiol initiator results in the formation of a mixture of species. On cooling to room temperature some of the protein refolds into the native state structure. However, misfolded monomeric species and dimeric and oligomeric forms of BLA are also present, with aggregates formed from disulfide bond linked hydrolyzed protein fragments. The misfolded monomeric species have some molten globule like characteristics, but disulfide bond shuffling has occurred and these species contain the non-native 61−73 disulfide bond. These partially folded, disulfide shuffled species are largely responsible for the pronounced improvement in surface activity of the protein at an air:water interface.

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