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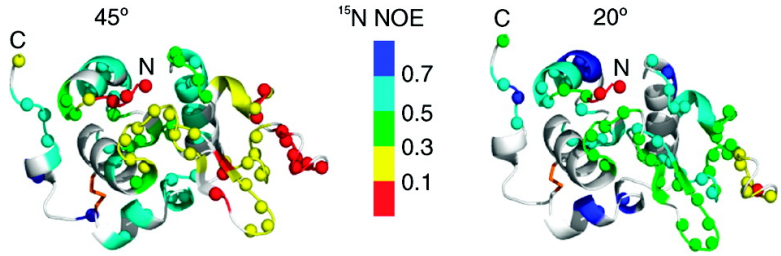


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# Probing the Effect of Temperature on the Backbone Dynamics of the Human α-Lactalbumin Molten Globule

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***Abstract:*** Molten globules are compact, partially folded proteins postulated to be general intermediates in protein folding. Human α-lactalbumin (α-LA) is a two-domain $Ca^{2+}$-binding protein that partially unfolds at low pH to form a molten globule. NMR spectra of molten globules are characterized by broadened resonances due to conformational fluctuations on microsecond to millisecond time scales. These species are often studied at high temperature where NMR resonances are observed to sharpen. The effect of higher temperatures on fast time-scale backbone dynamics of molten globules has not been investigated previously. Here, 1D $^{15}N$ direct-detection and 2D indirect-detection $^{1}H-^{15}N$ heteronuclear NOE experiments have been used to probe fast time-scale dynamics at low and high temperatures for three disulfide-bond variants of human α-LA that form molten globules. Disulfide bonds are found to have a significant effect on backbone dynamics within the β-domain of the molten globule; within the α-domain, dynamics are not significantly influenced by these bonds. At 20 °C, backbone mobility is significantly decreased in both domains of the molten globule compared to the mobility at 40−50 °C. Heteronuclear NOE values determined at 20 °C for the α-domain are closely similar to those observed for native α-LA, indicating that the α-LA molten globule has even more native-like character than suggested by studies conducted at higher temperature. Our results highlight the importance of considering the temperature dependence of the molten globule ensemble when making comparisons between experimental data obtained under different conditions.

## Introduction

Characterization of the structure and dynamics of intermediate species is an important challenge in understanding the mechanism of protein folding. Molten globules are compact, partially folded proteins that have native-like secondary structure but lack rigid side-chain packing interactions.[1−3]They are suggested to be general intermediates in protein folding. It has been shown previously that for a number of proteins, including apomyoglobin and α-lactalbumin (α-LA), a close similarity exists between molten globules observed at equilibrium under mildly denaturing conditions and those formed during the early stages of refolding.[4−10] Detailed characterization of these stable equilibrium molten globules has provided important insights into the transient species observed during kinetic refolding experiments.[8−17]

Human α-LA is a 14 kDa, two-domain (α + β), $Ca^{2+}$-binding protein containing four disulfide bonds (Figure 1).[18] In the native state, one domain is largely helical (the α-domain) and the other has a significant content of β-sheet, a long loop, and a $3_{10}$ helix (the β-domain). Two disulfide bonds, C6−C120 and C28−C111, are located in the α-domain, the third, C61−C77, is in the β-domain, and the fourth, C73−C91, links the two domains. The $Ca^{2+}$ is bound in a loop between the two domains and is important for stabilizing the native structure.[18,19] At low pH, α-LA loses the $Ca^{2+}$ and forms a partially folded molten globule.[2,20] A number of studies have shown that the overall architecture of this molten globule has extensive native-like

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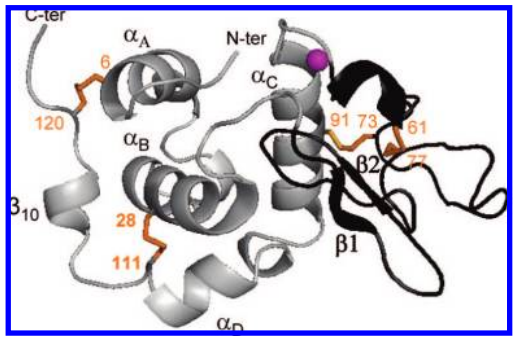


***Figure 1.*** Ribbon representation of the X-ray structure of native α-LA.[18] The α-domain (comprising residues 1−39 and 82−123) and $\beta$-domain (residues 40−81) are colored in gray and black, respectively. The $Ca^{2+}$ ion is indicated as a sphere in magenta. The four disulfide bonds are colored in orange with the C28−C111 disulfide shown in bold. The A, B, C, D, and C-terminal $3_{10}$ helices and the first two $\beta$-domain $\beta$-strands are labeled.

character, particularly in the α-domain where native-like helices are arranged in a native-like topology; the $\beta$-domain appears to be less ordered and lacks the propensity for a native-like fold.[11,12,16,21−30]

The lack of rigid side-chain packing interactions in molten globules results in a heterogeneous structural ensemble that is not amenable to structure determination using conventional X-ray and NMR methods. The NMR spectra of classical molten globules, such as α-LA, are characterized by poorly resolved and broadened peaks. The severe line broadening arises from constrained conformational fluctuations on the microsecond to millisecond time scales.[16,30−32] The $^1H-^{15}N$ HSQC spectrum of human α-LA at pH 2 and 20 °C contains sharp peaks from only 3 of the 121 possible backbone amides;[12] under these conditions direct characterization of the α-LA molten globule is not possible. However, an indirect approach using stepwise unfolding in urea, monitored by 2D HSQC, has provided important insights.[12,33−35] The number of sharp peaks in $^1H-^{15}N$ HSQC spectra is observed to increase, in a noncooperative manner, as the concentration of urea is increased; these peaks arise from regions of the protein that are unfolded, while the broadened peaks correspond to regions that retain structure in the molten globule. Using this approach, the stability of different regions of the α-LA molten-globule structure to urea has been mapped. A highly stable core composed of the A-, B-, D-, and C-terminal $3_{10}$ helices has been identified.[12] A study of an α-LA variant, in which all eight cysteines have been replaced by alanine (all-Ala α-LA), has demonstrated that the overall architecture of the molten globule is determined by the polypeptide sequence itself and not as a result of cross-linking by disulfide bonds.[33] However, the disulfide bonds do contribute to the stability of the molten globule in urea. Removal of the C61−C77 and C73−C91 disulfides leads to significant destabilization of the $\beta$-domain, and cross peaks from most of these residues are observed in the absence of urea.[33,35] In addition, the C28−C111 disulfide bond has been shown to contribute significantly to the stability of the α-domain of the molten globule.[25,35]

An alternative approach for the detailed characterization of molten globules using NMR has been the use of higher temperatures. Peaks are observed to sharpen as the temperature is increased; this can be attributed to modifications of the complex microsecond to millisecond dynamic properties of the molten globule.[13,31,36,37] A relatively well-resolved HSQC spectrum is obtained for the pH 4.1 molten globule of apomyoglobin at 50 °C, and this has been exploited to allow a detailed characterization of this partially folded protein.[13,14,36] $^1H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, and $^{13}C'$ chemical shifts have been used to identify residual α-helical structure for the native A, G, and H helices and the C-terminal part of the B helix in apomyoglobin; these helices are proposed to form a native-like structured core.[13,14] $^{15}N$ $T_1$ and $T_2$ relaxation times and the $^1H-^{15}N$ steady-state heteronuclear NOE have been used to correlate backbone dynamics with these structural propensities. The $^1H-^{15}N$ NOE is most sensitive to picosecond to nanosecond time-scale motions of the backbone and provides a good measure of fast time-scale dynamics. $^1H-^{15}N$ NOE values of ∼0.7, approaching those obtained for the native protein (0.8−0.9), are observed for the structured A, B, G, and H helices, indicating only limited fast time-scale mobility consistent with a well-packed hydrophobic core.[13,14] Lower $^1H-^{15}N$ NOE values, of −0.3 to 0.5, are observed for the residues between the B and G helices, indicating a significantly higher degree of flexibility.[13,14]

High temperature has also been used to improve the quality of $^1H-^{15}N$ HSQC spectra of the human α-LA molten globule.[37] The number of peaks observed in HSQC spectra increases from 3 to ∼100 as the temperature is raised from 20 to 50 °C. However, peaks from some residues in the B helix, in the C-terminal part of the protein, and adjacent to cysteines were still too broad to detect at 50 °C. The presence of disulfide bonds in α-LA leads to complex, slower time-scale dynamics at 50 °C that are not observed for apomyoglobin, which does not have disulfide bonds. Nevertheless, analysis of the $^1H^{\alpha}$, $^1H^N$, and $^{15}N$ secondary chemical shifts obtained at 50 °C led to identification of regions of native-like helix in the α-domain and of nonnative helical or turn propensity in the $\beta$-domain of the α-LA molten globule.[37]

Here, we extended the high-temperature studies of the molten globule to the [28−111] α-LA variant, which contains only the α-domain C28−C111 disulfide bond, and all-Ala α-LA variant,

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which lacks all four disulfide bonds. The decrease in the number of disulfide bonds in these proteins results in a lessening of exchange broadening at higher temperature, permitting a larger number of peaks to be observed in HSQC spectra. To complement the structural characterization of the molten globule using chemical shift data, we exploited the improved sensitivity available with a cryoprobe to analyze fast time-scale dynamics by measuring $^{1}H-^{15}N$ steady-state heteronuclear NOE data for these α-LA variants.

Higher temperature is known to modify the slower microsecond to millisecond time-scale dynamics of molten globules leading to an increase in $T_2$ values.[31,36,37] However, the effect of higher temperature on the faster picosecond time-scale dynamics of molten-globule states has not been investigated previously. Here, we used 1D $^{15}N$ direct-detection NMR for the 4SS α-LA variant and standard 2D indirect-detected $^{1}H-^{15}N$ steady-state heteronuclear NOE methods for all-Ala and [28−111] α-LA to probe fast time-scale backbone dynamics at 20 °C. We find that at lower temperature the α-LA molten globule has even more native-like character than might be concluded from experiments carried out at higher temperature.

## Materials and Methods

**Sample Preparation and Resonance Assignment.** $^{15}N$-labeled recombinant 4SS, [28−111], and all-Ala α-LA were expressed and purified as described previously.[11,25] Resonance assignments for the pH 2 molten globules of [28−111] and all-Ala α-LA at 20 °C and of 4SS α-LA at 20 and 50 °C have been reported previously.[12,33,35,37] $^{15}N$-edited 3D gradient-enhanced TOCSY-HSQC and NOESY-HSQC spectra[38−40] with mixing times of 46 and 200 ms, respectively, were used to assign resonances in the spectra of [28−111] and all-Ala α-LA at 45 °C as described previously.[12]

**2D $^{1}H-^{15}N$ Steady-State Heteronuclear NOE Experiments.** NMR samples contained ∼0.3−0.5 mM protein at pH 2 in 95% $H_2O$/5% $D_2O$. Experiments were performed on a Bruker Avance 500 MHz spectrometer with a Cryoplatform, equipped with a TCI CryoProbe. 2D $^{1}H-^{15}N$ steady-state heteronuclear NOE experiments[41] were collected in an interleaved fashion with and without $^{1}H$ saturation for a period of 3 s. One hundred twenty eight complex $t_1$ increments of 512 complex data points were collected. Sweep widths of 5411.255 and 1028.595 Hz were used in the $^{1}H$ ($F_2$) and $^{15}N$ ($F_1$) dimensions, respectively, for molten globule samples at pH 2. For native α-LA at pH 7, sweep widths of 7042.25 and 1420.455 Hz were used in $F_2$ and $F_1$, respectively. A total of 64−88 scans were collected for each $t_1$ increment. Heteronuclear NOE values were calculated as the ratio of the peak heights with and without $^{1}H$ saturation. Errors in the NOE values were calculated using spectral noise; errors in the range of 0.01−0.05 and 0.01−0.1 were obtained at 40−50 and at 20 °C, respectively.

**1D $^{15}N$ Direct-Detection NMR Experiments.** The NMR samples contained 1.5 mL of 1 mM $^{15}N$-labeled 4SS α-LA, at either pH 7 (native) or pH 2 (molten globule), dissolved in 95% $H_2O$/5% $D_2O$ in a 10 mm Shigemi NMR tube. Directly detected $^{15}N$ 1D NMR spectra were collected using a home-built 500 MHz spectrometer equipped with a 10 mm Bruker broadband probe. Steady-state 1D $^{15}N$ heteronuclear NOE experiments were collected with and without $^{1}H$ saturation for a period of 3 s. Broadband $^{1}H$ decoupling was applied during data acquisition. A sweep width of 12500 Hz, 2K complex data points, and a $^{15}N$ frequency of 50.67 MHz were used. A total of 34 816 and 28 800 scans were collected for the experiments at 20 and 50 °C, respectively. Heteronuclear NOE values as a function of the $^{15}N$ chemical shift were calculated as the ratio of the intensity with and without $^{1}H$ saturation at each data point with intensity above a noise threshold. A $^{15}N$ spectrum was simulated using the amino acid sequence of human α-LA and sequence-corrected $^{15}N$ random-coil chemical shifts.[42]

## Results

**Effect of Temperature on [28−111] and All-Ala α-LA.** HSQC spectra of [28−111] and all-Ala α-LA at pH 2 were collected as a function of temperature; an increase in temperature from 20 to 45 °C leads to a progressive appearance of peaks in the HSQC spectra (Supporting Information Figure 1), in line with our previous observation for 4SS α-LA.[37] We showed previously that the dramatic increase in the number of HSQC peaks observed for 4SS α-LA at 50 °C is accompanied by only a ∼25% loss in helical structure, as assessed by far-UV circular dichroism (CD), indicating that the majority of peaks observed in the HSQC spectrum at 50 °C arise from compact, structured regions of the molten globule rather than completely unfolded regions.[37] Far-UV CD measurements have been used to monitor the decrease in helical content with increasing temperature for [28−111] and all-Ala α-LA (Supporting Information Figure 2); helical structure is lost at slightly lower temperatures than for 4SS α-LA. Approximately 75−80% of the helical content observed at 20 °C is retained for 4SS, [28−111], and all-Ala α-LA at 50, 45, and 40 °C, respectively. Therefore, these temperatures were chosen for more detailed analysis of the α-LA variants.

**Secondary Structure Propensities from $^{1}H^{\alpha}$ Chemical Shifts.** Deviations of $^{1}H^{\alpha}$ chemical shifts from sequence-corrected random-coil shifts[43] were used to evaluate secondary structure propensity in the α-LA variants (Figure 2). Most residues display upfield shifts of $^{1}H^{\alpha}$ with a clustering of large deviations of more than −0.1 ppm, indicative of helical propensity, occurring mainly in the α-domain. The large negative deviations observed for [28−111] and all-Ala α-LA from K5 to D16 suggest a more extended A helix than observed in 4SS α-LA, where large negative deviations are only seen from L8 to D16. This is consistent with studies suggesting that removal of the strained C6−C120 disulfide bond leads to a lengthening of this helix.[21,24,25,35,44,45] The large upfield shifts observed for E25 to M30 in all-Ala α-LA indicate helical conformation. In native α-LA, the B helix extends to S34. $^{1}H^{\alpha}$ upfield shifts of less than 0.1 ppm are observed for F31 to S34, indicating that the B helix is shortened in this molten globule at 40 °C. This is consistent with the observation that M30 is the last B-helix residue with a protected amide in H/D exchange studies of the 4SS α-LA molten globule.[11,46] Large upfield shifts are also observed for residues in the C-terminal part of the α-domain at positions corresponding to the C helix (residues 85−99), D helix

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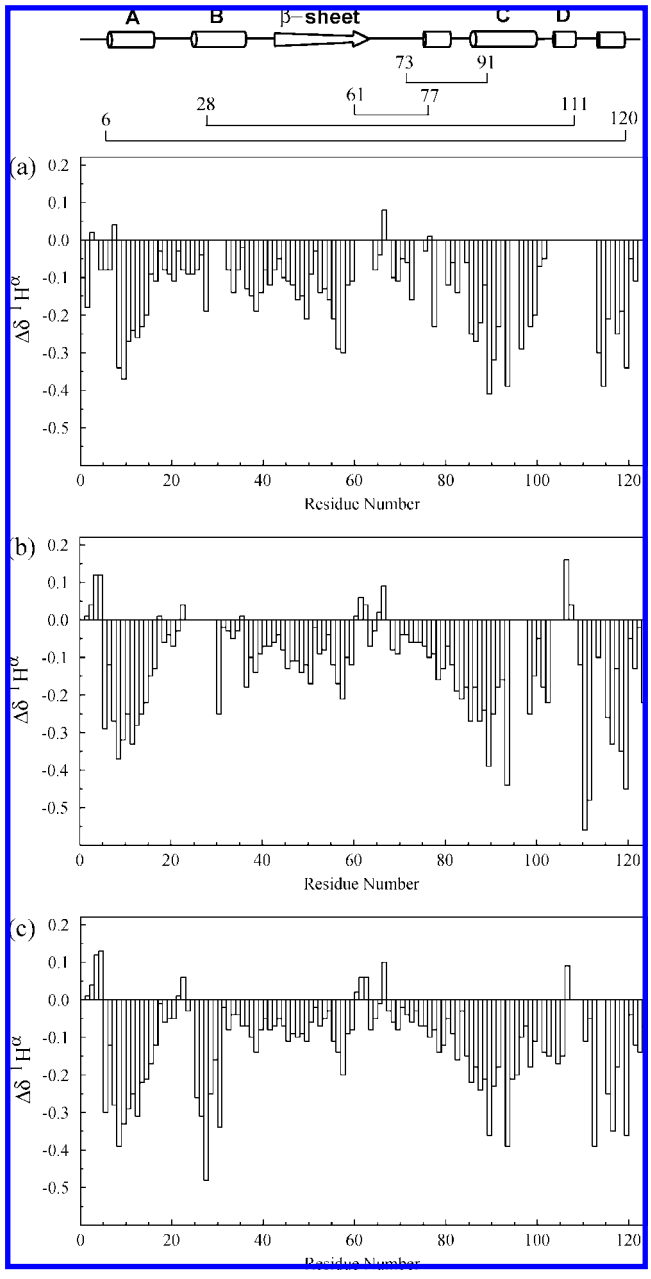


***Figure 2.*** Analysis of $^1H^\alpha$ chemical shifts observed in the human α-LA molten globule at pH 2. Deviations of the observed $^1H^\alpha$ chemical shifts from sequence-corrected random-coil shifts ($\Delta\delta^1H^\alpha = \delta^1H^\alpha_{observed} - \delta^1H^\alpha_{random\_coil}$) are shown for (a) 4SS α-LA at 50 °C, (b) [28−111] α-LA at 45 °C, and (c) all-Ala α-LA at 40 °C. Negative chemical shift differences correspond to an upfield shift of the resonance relative to the sequence-corrected random-coil shift.[43] $^1H^\alpha$ shifts were measured for 90, 105, and 113 residues in 4SS, [28−111], and all-Ala α-LA, respectively; no bar is shown if the $^1H^\alpha$ resonance was not assigned. The secondary structure found in native α-LA and the disulfide bonds found in 4SS α-LA are shown.[18]

(residues 100−104 and 105−111), and C-terminal $3_{10}$ helix (residues 115−119) in all three variants. We conclude that at temperatures of 40−50 °C the structural ensembles for all three α-LA molten globules contain a significant amount of native-like helical structure in the α-domain.

Residues in the $\beta$-domain of the α-LA variants have $^1H^\alpha$ shift differences that are significantly smaller than those observed for the α-domain (Figure 2). No large positive shift deviations characteristic of $\beta$-sheet conformation are observed. The chemical shift deviations in the second half of the $\beta$-domain, residues 60−77, are small (<0.1 ppm) and indicative of random-coil conformation. In contrast, a significant number of residues between 40 and 60 have upfield shifts of >0.1 ppm of $^1H^\alpha$, characteristic of helical or turn propensity. The observed shifts for 4SS α-LA are significantly larger (up to ∼0.3 ppm) than the shifts for [28−111] and all-Ala α-LA (up to ∼0.2 ppm). Thus, the $\beta$-domain disulfide bonds (C61−C77 and C73−C91) contribute to stabilization of structure in this region. In the 4SS and [28−111] α-LA molten globules consecutive upfield shifts of greater than 0.1 ppm, indicative of helical or turn propensity, are observed for residues 45−49 and 55−59 (Figure 2a and 2b). In native α-LA, residues 41−43, 48−50, and 55−56 make up the three strands of an antiparallel $\beta$-sheet (Figure 1);[18] this structure is clearly not retained in these molten globules at higher temperatures. This analysis confirms the bipartite nature of the α-LA molten globule structural ensemble; significant native-like helical structure is found in the α-domain, while the $\beta$-domain lacks native-like structure but may contain regions with some propensity for non-native helical or turn structure.

**Backbone Dynamics at High Temperature.** The $^1H-^{15}N$ steady-state heteronuclear NOE has been measured for the molten globules of 4SS α-LA (at 50 °C), [28−111] α-LA (at 45 °C), and all-Ala α-LA (at 40 °C) at pH 2 and for native α-LA (at 40 °C) at pH 7 in the presence of $Ca^{2+}$; this is plotted as a function of sequence for each protein in Figure 3a. With the exception of the C-terminal residues (122 and 123), native α-LA gives uniformly high NOE values at 40 °C (0.73 ± 0.04), indicating that in its native state α-LA has a rigid backbone (Figure 4a). No significant difference is observed between the α-domain (0.72 ± 0.04) and the $\beta$-domain (0.73 ± 0.05). For 4SS α-LA at pH 2, NOE values ranging from −2.7 to 0.71 are observed, indicating a range of fast time-scale backbone dynamics (Figure 3a). Negative NOE values are observed for residues 1−5 in the 4SS α-LA molten globule, in contrast to values of 0.58−0.74 for native α-LA. This indicates a significant difference in backbone dynamics at the N-terminus of the sequence for the two states (Figure 4a and 4b). In native α-LA, residues 1−3 are involved in a short antiparallel $\beta$-sheet structure with residues 36−38 (Figure 1).[18] In the molten globule this $\beta$-sheet is not present; in addition to the negative NOE values for residues 1−3, low NOE values of 0.27, 0.22, and 0.07 are observed for residues 36, 37, and 38, respectively; these residues also have upfield-shifted $^1H^\alpha$ characteristic of helical or turn propensity rather than extended $\beta$-strand structure (Figure 2a). In the absence of this long-range $\beta$-sheet interaction, the N-terminal residues preceding C6 are unstructured and very mobile at pH 2.

For residues 6−123 of 4SS α-LA at pH 2, NOE values ranging from 0.07 to 0.71 are observed at 50 °C (Figures 3a and 4b). Generally higher values are observed in the α-domain than in the $\beta$-domain. In the N-terminal part of the α-domain a decrease in NOE values is observed between the A and B helices. This is consistent with the trends observed in the $^1H^\alpha$ chemical shift data, particularly for all-Ala α-LA (Figure 2c). Significant upfield shifts characterize the A and B helix regions of the molten globule, while chemical shifts closer to random-coil values are observed for the AB loop. The NOE values observed in the α-domain are generally lower than those observed in native α-LA, indicating larger amplitude fast time-scale backbone dynamics in the molten globule. The pattern of NOE values observed in the $\beta$-domain of 4SS α-LA reflects the location of cysteine residues; high values are observed adjacent to C61 (0.66 for K62), C73 (0.47 for I72), and C77

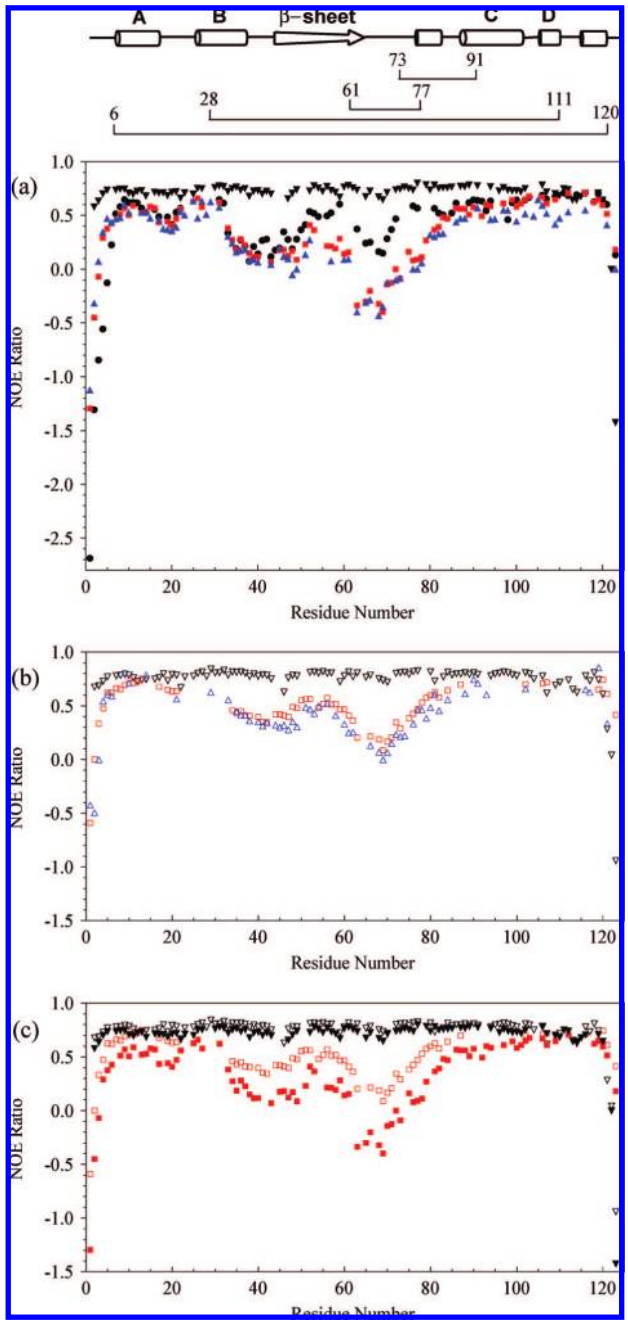


***Figure 3.*** $^{1}H-^{15}N$ heteronuclear NOE values measured for α-LA. Data for 4SS α-LA are shown in black, for [28-111] α-LA in red, and for all-Ala α-LA in blue. (a) NOE values measured for native 4SS α-LA at pH 7 and 40 °C (▼), 4SS α-LA at pH 2 and 50 °C (●), [28−111] α-LA at pH 2 and 45 °C (red ■), and all-Ala α-LA at pH 2 and 40 °C (blue ▲). (b) NOE values measured for native 4SS α -LA at pH 7 and 20 °C (▽), [28−111] α -LA at pH 2 and 20 °C (red □), and all-Ala α -LA at pH 2 and 20 °C (blue △). (c) NOE values measured for native 4SS α-LA at pH 7 at 20 (▽) and 40 °C (▼) and [28−111] α -LA at 20 (red □) and 45 °C (red ■). The secondary structure found in native α-LA and the disulfide bonds found in 4SS α-LA are shown.[18] Errors in the NOE values range from 0.01 to 0.05 at 40−50 °C and from 0.01 to 0.1 at 20 °C. The size of the symbols used gives an estimate of the experimental errors in the NOE measurement.

(0.59 for S76) (Figures 3a and 4b). The lowest values are observed within the long segments between C28 and C61 (0.07 for T38) and between C61 and C73 (0.15 for S69) (Figures 3a and 4b). In apomyoglobin at pH 4.1, NOE values as low as −0.3 are observed for the less structured region between the B and G helices. Apomyoglobin does not contain disulfide bonds; therefore, backbone mobility is less restricted.[13]

It has been shown previously that the high stability in urea of residues in the β-domain of the 4SS α-LA molten globule is related to the presence of the C61−C77 and C73−C91 disulfide bonds.[12,33,35] It can be seen in Figure 3a that removal of these disulfide bonds in [28−111] and all-Ala α-LA also has a dramatic affect on the backbone dynamics in the β-domain, particularly for residues 63−73 in the second half of the domain. NOE values as low as −0.4 are observed for residues 68−69, indicating a significant increase in the amplitude of fast time-scale backbone dynamics in comparison to 4SS α-LA (Figures 4b and 4c). These values for [28−111] and all-Ala α-LA are closer to those observed for apomyoglobin.[13,14] However, in the α-LA molten globule, the NOE values do not decrease steadily between residues 34 and 70 as they do within the unstructured region between the B and G helices in the apomyoglobin molten globule. Instead, higher NOE values are observed for residues 52−53 in [28−111] and all-Ala α-LA, indicating more restricted backbone dynamics around these residues, which are located in the center of the β-domain. Elevated NOE values are also observed in 4SS α-LA, although they are masked, to an extent, by the proximity of C61. This increase in NOE values around residues 52−53 is similar to that observed near to the β-domain cysteine residues in 4SS α-LA and may reflect a similar 'long-range tethering' interaction which reduces backbone mobility. This restriction in mobility correlates well with the upfield-shifted $^{1}H^{\alpha}$ in this region of the β-domain (Figure 2), which indicate a propensity for helical or turn conformation in the molten globule ensemble.[37] L52 and F53 have bulky hydrophobic side chains which may favor nonrandom structure. However, the results clearly show that the decreased mobility observed for these residues is not due simply to their hydrophobic nature; hydrophobic residues L59, W60, V66, I72, and I75, which are also located in the β-domain, have significantly lower NOE values than L52 and F53. In native α-LA, L52 and F53 have contacts with L8, M30, I85, A92, and W104, which are located in the A, B, C, and D helices in the α-domain.[18] It is tempting to speculate that restricted backbone dynamics for L52 and F53 result from long-range native-like interactions with hydrophobic residues in the α-domain which persist in the molten globule ensemble.

Removal of three or four of the disulfide bonds has little effect on fast time-scale dynamics in the α-domain (Figure 3a). In fact, removal of the C6−C120 disulfide appears to lead to a decrease in dynamics at the N-terminus; this is consistent with an elongation of the A helix (see above).[24,25,35,37,44] Within the α-domain very similar NOE values are observed for 4SS and [28−111] α-LA, indicating that the C61−C77 and C73−C91 disulfide bonds do not have an affect on dynamics within the α-domain. For all-Ala α-LA slightly reduced NOE values are observed particularly in the C-terminal part of the α-domain (82−123); this reflects a small effect arising from removal of the C28−C111 disulfide bond.

**Backbone Dynamics of 4SS α-LA at Low Temperature.** Measurement of fast time-scale backbone dynamics at lower temperature is more difficult due to significant line broadening, and as a result, such data have not been reported previously for the α-LA or apomyoglobin molten globules. The standard 2D methods for such measurements use indirect detection of $^{15}N$ and require relatively narrow $^{1}H^{N}$ resonances. Even with a cryoprobe, NOE values for only 5 residues can be measured for the 4SS α-LA molten globule at 20 °C (−1.27, −0.55,

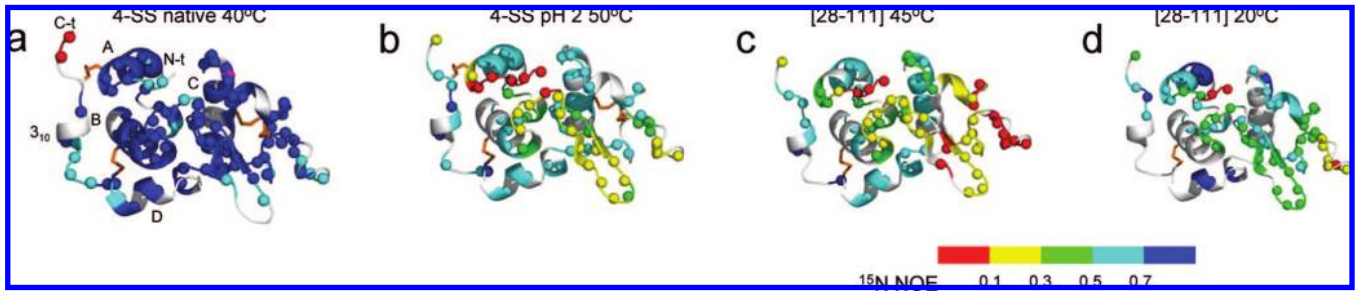


***Figure 4.*** Representation of the fast-time scale dynamics in the native and molten globule states of human α-lactalbumin. The experimentally measured $^{1}H-^{15}N$ heteronuclear NOE values are color coded on the ribbon diagram of the native structure, and the $^{15}N$ atoms are shown as color-coded spheres. Heteronuclear NOE values are shown for (a) native α-LA at pH 7 and 40 °C, (b) the 4SS α-LA molten globule at pH 2 and 50 °C, and the [28−111] α-LA molten globule at pH 2 and (c) 45 and (d) 20 °C. Residues for which the measurement of the NOE value was not possible due to broadening, overlap, or missing assignment are indicated with a white ribbon.

−0.14, 0.49, and 0.48 for K1, Q2, F3, T38, and S47, respectively). For this reason, an alternative approach, direct detection of the $^{15}N$ spectrum, has been used to probe the backbone dynamics of 4SS α-LA at 20 °C. Although the sensitivity of the directly detected $^{15}N$ spectrum is low, the experiments are feasible with a room-temperature 10 mm broadband probe.[47−49]

The 1D $^{15}N$ spectrum of the native (pH 7 with $Ca^{2+}$) and molten globule (pH 2) states of 4SS α-LA at 20 °C are shown in Figure 5a and 5b, respectively. For the fully folded native state, a well-resolved $^{15}N$ spectrum characterized by narrow resonances is observed. In contrast, the $^{15}N$ spectrum of the molten globule shows relatively poor resolution and significantly broader resonances. Nevertheless, the spectrum contains peaks from many more residues than the group of five observed in the 2D HSQC. A spectrum simulated using the sequence-corrected $^{15}N$ random coil shifts of Braun et al.[42] is shown in Figure 5c. The range of experimental and simulated chemical shifts is very similar (∼129 to ∼108 ppm), but the major envelope of peaks in the experimental spectrum is shifted upfield compared to the simulated spectrum; this is consistent with the presence of significant helical structure in the 4SS α-LA molten globule ensemble.[50]

The 1D $^{15}N$ spectra of 4SS α-LA at 20 °C without and with saturation of the $^{1}H$ spectrum are shown in Figure 5d; the ratio of peak intensities of these two spectra gives a measure of the $^{1}H-^{15}N$ heteronuclear NOE. The sharp peak at 124.5 ppm is assigned to K1, and the negative peak observed in the spectrum collected with $^{1}H$ saturation is consistent with the NOE value measured using standard 2D methods. Negative NOE values are also observed for Q2 at 122.0 ppm and for the Asn/Gln side chain $^{15}NH_2$ at ∼112 ppm. For the remainder of the spectrum positive NOE values are observed; these are plotted as a function of chemical shift in Figure 5e. The peaks at ∼129 ppm are assigned to E7 and A22 on the basis of the high-temperature HSQC spectrum.[37] The average NOE value at this chemical shift is 0.70 ± 0.10. In order to validate the comparison of NOE values obtained by 1D $^{15}N$ at 20 °C and standard 2D methods at 50 °C, the directly detected $^{15}N$ spectra were also collected at 50 °C (Figure 5f). For E7 and A22, the NOE value of 0.47 ± 0.10, obtained from 1D $^{15}N$ spectra at 50 °C, is in good agreement with the value of 0.54 ± 0.04, obtained by 2D at 50 °C. Thus, at low temperature the backbone dynamics for these residues are more restricted and the observed NOE value is closer to the average value of 0.73 ± 0.08 observed for these residues in the native state of α-LA at 20 °C (Figure 3b). Peaks observed at ∼108 ppm arise from the six glycine residues (G17, G19, G20, G35, G51, and G100), and these give an average NOE value of 0.74 ± 0.08 (Figure 5e). At 50 °C these residues show lower values of 0.44 ± 0.15 (by 2D) and 0.34 ± 0.10 (by 1D $^{15}N$, Figure 5g), while for the native state an average value of 0.78 ± 0.03 is observed at 20 °C (Figure 3b). Again, the NOE values observed for the molten globule at 20 °C are higher than those at 50 °C and very similar to the native-state NOE values. In the region between 121 and 117 ppm in the 1D $^{15}N$ spectrum an average NOE value of 0.69 ± 0.04 is observed at 20 °C (Figure 5e). At 50 °C values of 0.52 ± 0.17 (by 2D) and 0.48 ± 0.08 (by 1D $^{15}N$, Figure 5g) are observed for residues with these $^{15}N$ chemical shifts. For the native protein a value of 0.77 ± 0.05 is observed (Figure 3b). Therefore, it appears that for 4SS α-LA the fast time-scale backbone dynamics of the molten globule at 20 °C are very similar to the native-state dynamics, both in the α- and $\beta$-domains. The increase in temperature from 20 to 50 °C not only leads to a sharpening of peaks due to increases in $T_2$ values, resulting from faster interconversion between structures in the molten globule ensemble, but also leads to a significant increase in the amplitude of fast time-scale dynamics that are probed by the $^{1}H-^{15}N$ heteronuclear NOE experiment.

**Backbone Dynamics of [28−111] and All-Ala α-LA at Low Temperature.** The HSQC spectra of [28−111] and all-Ala α-LA at 20 °C contain a significant number of peaks[33,35] (Supporting Information Figure 1), and residue-specific information can be obtained using the standard 2D $^{1}H-^{15}N$ heteronuclear NOE experiment. The NOE values at 20 °C are plotted as a function of sequence and compared with the values for native α-LA at 20 °C in Figure 3b. The observed NOE values for α-domain residues are similar to those observed for native α-LA. In the $\beta$-domain, lower NOE values are observed at 20 °C compared to the native state. However, the NOE values at 20 °C are significantly higher than the values at 40 or 45 °C, indicating a significant reduction in the amplitude of fast time-scale dynamics in the $\beta$-domain at 20 °C (Figure 4c,d). The NOE data at 20 °C show the same trends described above for the higher temperature

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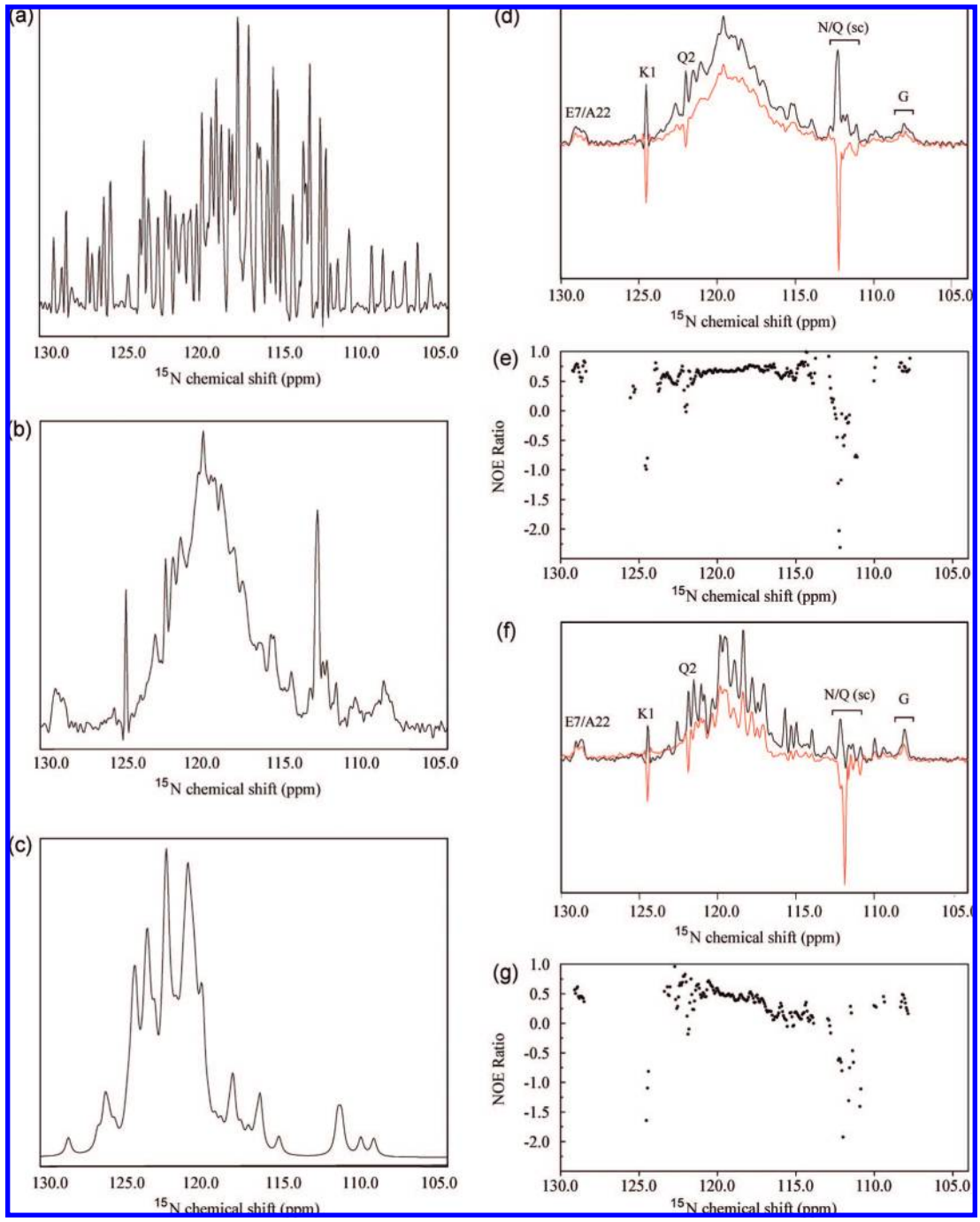


***Figure 5.*** 50 MHz $^{15}$N directly detected 1D NMR spectra of 4SS α-LA. $^{15}$N NOE spectra (without $^{1}$H saturation) for 4SS α-LA at (a) pH 7 (native state) and (b) pH 2 (molten globule) both at 20 °C. (c) Simulated $^{15}$N spectrum of backbone resonances of 4SS α-LA at pH 2 calculated using sequence-corrected random-coil shifts.[42] $^{15}$N NOE spectra collected for 4SS α-LA at (d) 20 and (f) 50 °C without (black) and with (red) saturation of the $^{1}$H spectrum. The ratio of intensities at each data point with and without $^{1}$H saturation at (e) 20 and (g) 50 °C is plotted as a function of the $^{15}$N chemical shift. $^{15}$N resonances corresponding to K1, Q2, E7/A22, the six glycines (G), and the N/Q side chains are labeled.

data; in particular, a restriction of mobility is observed around residues 52−53.

## Discussion

**Temperature Dependence of Backbone Dynamics in α-LA.** A significant difference in the backbone dynamics of the α-LA molten globule is observed at low (20 °C) and higher temperature (40−50 °C). This is clearly demonstrated in the comparison of the NOE values for [28−111] α-LA at 20 and 45 °C (Figures 3c, 4c, and 4d). The amplitude of fast time-scale dynamics in the molten globule at 20 °C is significantly smaller than that observed at 45 °C, particularly in the $\beta$-domain. For comparison, the NOE values for native 4SS α-LA at 20 and 40 °C are shown in Figure 3c. The average NOE values of 0.77 ± 0.05 and 0.73 ± 0.04 at 20 and 40 °C, respectively, are very similar, indicating that the fast time-scale dynamics of the native state of α-LA do not change significantly with temperature. This difference in the temperature dependence of the dynamics of the molten globule and native states is likely to reflect differences in their thermal unfolding properties. Native α-LA

displays cooperative thermal unfolding with a transition midpoint above 50 °C. It appears that the structure and dynamics of native α-LA do not change significantly at temperatures below this thermal unfolding transition. By contrast, the α-LA molten globule does not unfold in a cooperative manner; helical secondary structure monitored by CD decreases in a linear fashion between 20 and 70 °C (Supporting Information Figure 2).[37] The temperature dependence of the fast time-scale dynamics of the molten globule reflects this noncooperative behavior.

The α-LA molten globule cannot be described by a single, well-defined structure; instead, it is represented by an ensemble of interconverting structures. Temperature-dependent changes in fast time-scale backbone dynamics and helical content monitored by CD suggest that this ensemble also changes with temperature. At 20 °C, the native-like ellipticity measured at 222 nm and the protection of amides in helices from hydrogen/deuterium exchange indicate that well-defined, stable helices exist in nearly all structures in the ensemble.[11] This is reflected in the native-like backbone dynamics observed at 20 °C. The gradual loss of helical CD signal as temperature is increased does not correspond to the unfolding of a specific region of helix; analysis of chemical shifts indicates that at 40−50 °C helical propensity is maintained in all the native helices of the α-domain (Figure 2). Instead, the loss in helicity is likely to reflect increasing fluctuations within the helices, resulting in a gradual decrease in their regularity and rigidity as temperature is increased. This 'loosening' of backbone structure is accompanied by an increase in the amplitude of the fast time-scale dynamics of the backbone amide groups.

Our results highlight the importance of considering the temperature dependence of the molten globule ensemble when making comparisons between experimental data obtained under different conditions. For example, in this study we concluded on the basis of $^1H^\alpha$ chemical shifts and heteronuclear NOE values at 50 °C that stable $\beta$-sheet structure does not exist in the equilibrium pH 2 molten globule of 4SS α-LA. Fourier-transform infrared (FT-IR) studies of the bovine 4SS α-LA molten globule indicate the presence of small amounts of native-like and non-native $\beta$-sheet.[15] This observation is not necessarily inconsistent with our conclusions because the FT-IR studies were carried out at 3 °C. Our results show significant backbone rigidity at 20 °C, particularly in the 4SS α-LA molten globule where fast time-scale dynamics in both the α- and $\beta$- domains are very similar to native-state dynamics. Therefore, the molten globule ensemble at 3 °C may very well contain a significant population of molecules with some stable $\beta$-sheet structure.

**Comparison with Previous Studies.** $^{19}$F NMR has been used previously to probe Phe and Trp side-chain accessibility in the 4SS α-LA molten globule.[30] F3, F53, F80, W60, W104, and W118 were found to have side-chain accessibilities of 61%, 24.5%, 17.0%, 30.4%, 0%, and 5.7% respectively. The high side-chain accessibility for F3 is consistent with the negative backbone NOE values measured at 20 and 50 °C in 4SS α-LA, indicating that both the side chain and the backbone of F3 are relatively unstructured. The very low accessibilities for W104 and W118 are consistent with the highly restricted backbone dynamics for the C-terminal region of the α-domain. The accessibilities reported for F53, W60, and F80 in the $\beta$-domain also correlate with the backbone NOE values; for example, W60, the most accessible of these residues, is also found to have the lowest backbone NOE value of the three residues, while F80, the least accessible residue, has the highest NOE value (Figure 3a). Bai et al. concluded from analysis of $^{19}$F linewidths that side-chain dynamics in the molten globule are heterogeneous.[30] This is consistent with the observation in the present study of a range of fast time-scale backbone dynamics for the α-LA molten globule.

Previous computational studies using molecular dynamics (MD) simulations and Monte Carlo sampling have attempted to describe the structural ensemble of the 4SS α-LA molten globule.[51−54] In these studies, considerable native-like $\beta$-sheet structure is retained in the structural ensembles.[52−54] We concluded that stable $\beta$-sheet structure does not exist in the molten globules of 4SS, [28−111], and all-Ala α-LA at high temperature (40−50 °C). The NOE values observed for [28−111] and all-Ala α-LA at 20 °C also suggest the absence of stable $\beta$-sheet structure at lower temperature. For 4SS α-LA at 20 °C, NOE values for individual residues have not been measured but the overall average value of $0.69 \pm 0.04$ indicates more restricted fast time-scale dynamics throughout the molecule. These results indicate that the $\beta$-domain is not completely unfolded in the 4SS α-LA molten globule at 20 °C and could be consistent with some $\beta$-sheet structure. The structure observed in MD and Monte Carlo simulations is characterized by changes in the register of the strands and interconversion between native and non-native hydrogen bond patterns.[53,54] This fluctuating structure could lead to chemical shift averaging which might remove the characteristic downfield $^1H^\alpha$ shifts usually associated with $\beta$-sheet. It is interesting to note that a non-native helix spanning residues 56−60 is present in ∼60% of the MD ensemble;[53] this is consistent with our observation of upfield-shifted $^1H^\alpha$ for residues 56−59 (Figure 2a).

A Monte Carlo sampling procedure has been used in conjunction with the residue-specific urea-unfolding data for 4SS and all-Ala α-LA[12,33] to determine the ensemble of structures populated in the molten globule.[54] In these ensembles the contacts between residues 1−3 and 36−38 are retained even in high urea concentrations. The reduced heteronuclear NOE values and $^1H^\alpha$ chemical shifts reported here indicate that the small $\beta$-sheet involving these residues does not persist in the molten globules of 4SS and all-Ala α-LA. This discrepancy may arise from the assumption in the Monte Carlo sampling procedure that residues that are 'structured' in the molten globule interact only through native contacts.[54]

## Conclusions

Using 1D $^{15}$N direct detection, an approach rarely used for proteins,[47−49] and standard 2D methods we collected $^1H-^{15}N$ heteronuclear NOE data for the α-LA molten globule at both low and high temperatures. We show that 1D $^{15}$N direct detection provides useful information when resonances are too broad to be detected by conventional 2D methods. The heteronuclear NOE data presented here provide a detailed picture of the fast time-scale backbone dynamics of the α-LA molten globule and will provide useful experimental constraints for future Monte Carlo simulations of the molten globule ensemble.[54]

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The α-domain is found to have significantly more restricted backbone dynamics than the β-domain, an observation that is consistent with the bipartite structure of the molten globule described previously.[21] However, our results reveal that the β-domain does not behave as an unstructured chain as has been postulated;[21] significantly restricted dynamics are observed for residues located in the first half of the β-domain (40−60) even in the absence of disulfide bonds. In particular, residues in the vicinity of L52 and F53 have restricted dynamics, which may be related to a propensity for non-native helical or turn structure in the molten globule ensemble.

The amplitude of fast time-scale backbone dynamics of the α-LA molten globule at low temperature (20 °C) is significantly smaller than that observed at higher temperatures (40−50 °C). At 20 °C, backbone dynamics within the α-domain of the molten globule and native states of α-LA are strikingly similar. Thus, the molten globule at 20 °C appears to have even more native-like character than might be concluded from experiments carried out at elevated temperature where better quality spectra are obtained. The classical molten globule is defined as a compact structure with native-like secondary structure but lacking rigid side-chain packing. Our observations for α-LA at 20 °C suggest that this definition can be extended to include native-like backbone dynamics in large parts of the structure. Thus, the backbone is not particularly 'molten' in the molten globule. On the other hand, side-chain chemical shifts measured for the α-LA molten globule are very close to random-coil values;[55] methyl $^{1}$H chemical shifts of 40 threonine, alanine, leucine, and isoleucine residues in all-Ala α-LA show variations of only ∼0.04 ppm from random-coil values, indicating that these groups are not found in unique environments in the molten globule. Thus, significant side-chain motions are likely to be largely responsible for the fluctuating nature of the molten globule ensemble. In the future, studies aimed at probing the dynamics of methyl groups and other hydrophobic side chains are likely to provide new insights into the structural ensemble of the molten globule state.

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**Supporting Information Available:** HSQC spectra for [28−111] and all-Ala α-LA molten globules at pH 2 and 20, 30, and 40 °C; figure of the temperature dependence of helicity in 4SS, [28−111], and all-Ala α-LA determined by circular dichroism. This material is available free of charge via the Internet at http://pubs.acs.org.

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