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Simulation of urea-induced protein unfolding: A lesson from bovine β-lactoglobulin

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ABSTRACT

To investigate the molecular mechanisms involved in the very initial stages of protein unfolding, we carried out one long (1 μs) simulation of bovine β-lactoglobulin (BLG) together with three (500 ns) supporting MD runs, in which the unfolding conditions were produced by adding the osmolyte urea to the simulated systems and/or by increasing the thermal energy raising the temperature from 300 to 350 K. BLG was chosen, since it is a well-characterized model protein, for which structural and folding properties have been widely investigated by X-ray and NMR. MD trajectories were analyzed not only in terms of standard progress variables, such as backbone H-bonds, gyration radius width, secondary structure elements, but also through the scrutiny of interactions and dynamical behavior of specific key residues previously pointed out and investigated by NMR and belonging to a well known hydrophobic cluster.

MD trajectories simulated in different unfolding conditions suggest that urea destabilizes BLG structure weakening protein::protein hydrophobic interactions and the hydrogen bond network. The early unfolding events, better observed at higher temperature, affect both secondary and tertiary structure of the protein.

1. Introduction

Molecular dynamics (MD) is a useful approach for understanding molecular behavior at an atomic resolution. The continued increase in available computational power, and the availability of newer and more accurate force fields, are opening the way for new applications of this computational tool, *e.g.* in the field of structural biology [1]. In spite of the above improvements, protein folding is a process too slow and too complex to be accurately described via MD. Monitoring unfolding, both with experimental and with theoretical procedures, however, may provide a shortcut for investigating the key patterns for protein stability [2], allowing crucial interactions stabilizing the folded form to be identified. High temperatures or high concentrations of some osmolytes, *e.g.* urea or guanidinium chloride, allow for, and enhance the rate of, protein unfolding. While urea is non-ionic, guanidinium chloride solutions contain a large number of charges, making the setup of a realistic force field difficult [3]. In the last few years a number of papers in which the mechanism of protein unfolding induced by urea has been investigated by MD simulations have appeared in the literature [2,4–12]. Different simulation setups have been used, for example using classical MD or replica exchange MD, and with a wide range of temperatures and simulated times. It is however difficult to compare the results produced by different groups and, sometimes, to discriminate between the effects of the osmolyte and of the temperature.

During this investigation we run MD simulations of long duration (up to 1 μs) for a model protein, bovine β-lactoglobulin (BLG), in urea. The aim of this work was to test whether MD could sample at least the initial stages of protein unfolding. BLG is a 162 amino acid β-barrel protein belonging to the lipocalin family [13], consisting of nine antiparallel β-sheets and one terminal α-helical segment as determined by X-ray at neutral pH [14,15] and by NMR at acidic pH [16–19]. The folding properties of BLG have been widely investigated through different spectroscopic techniques such as circular dichroism, fluorescence and NMR [20–25] and by

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Table 1
Description of the systems submitted to MD simulations.

Protein	Solvent	Molecules	Box (before MD)	Temperature	Simulated time
BLG	Water	9261 water, 6 Na^+	7.5 nm × 6.5 nm × 6.5 nm	300 K	500 ns
BLG	10 M urea	6070 water, 1717 urea, 6 Na^+	6.9 nm × 6.9 nm × 6.9 nm	300 K	1000 ns
BLG	Water	9261 water, 6 Na^+	7.5 nm × 6.5 nm × 6.5 nm	350 K	500 ns
BLG	10 M urea	6070 water, 1717 urea, 6 Na^+	6.9 nm × 6.9 nm × 6.9 nm	350 K	500 ns

electrophoresis [26,27]. BLG stability appears to depend on pH and ionic strength of the medium, and to be higher at acidic than at neutral pH [26,27]. NMR studies, which evaluated the pattern of H-bond protection factors [21,25] and of the H–H nuclear Overhauser effects (NOE) [18], highlighted the relevance of the region composed of the A, F, G, and H strands, containing amino acids that form a buried cluster (core β-sheet) responsible for BLG folding and stability.

In this paper, the computational results are discussed in comparison with the experimental data to draw conclusions on the mechanism of action of urea in unfolding processes and on the efficiency of MD simulations at sampling the onset of the unfolding phenomenon.

2. Materials and methods

2.1. Molecular dynamics simulations

The BLG crystal structure, 1B8E chain A [28], was obtained from the RCSB Protein Data Bank and solvated with (w/) or without (w/o) urea. The 10 M urea box was prepared starting from a pdb file from Rocco et al. [8]. Briefly, we set up a 6 nm × 6 nm × 6 nm cubic box containing 160 molecules of urea and 480 of water. The system was subjected to energy minimization with the steepest descent algorithm down to a maximum gradient of 2000 kJ/mol × nm^{-1}, and simulated for 1 ns, annealing from 300 to 0 K under an isotropic pressure of 100 bar. The system was then relaxed for 1 ns at standard pressure, heating from 0 to 300 K, and simulated for another 1 ns at 300 K. Na^+ ions were added to each system for electroneutrality. The NAMD (v 2.6) program with the CHARMm22 force field was used, and the urea topology was taken from Duffy et al. [29], and Caballero-Herrera and Nilsson [30]. Two systems were prepared: BLG in 10 M urea, and BLG in pure water; the TIP3P water model was used in all the simulations. The system in 10 M urea was simulated for 1 μs at 300 K and for 500 ns at 350 K and the system in pure water was simulated for 500 ns both at 300 and at 350 K. The energy of each system was minimized with 2000 steps of the steepest-descent algorithm, after which MD simulations were performed with a timestep of 2 fs. The trajectories produced were subsequently analyzed with the GROMACS [31] and Wordom [32] tools. The NPT ensemble was simulated by a combination of the Nosé-Hoover Langevin piston pressure control, and Langevin dynamics for the control of the temperature. Particle Mesh Ewald (PME) electrostatics was applied with a PMEGridSpacing of 1 Å, as recommended. Cut-offs for H-bonds were set as follows: distance between donor and acceptor atom less than 3.5 Å and angle formed by acceptor–donor–hydrogen less than 30°.

3. Results and discussion

3.1. Monitoring unfolding via classical progress coordinates

Long time-scale simulations in explicit solvent were run for BLG under native and denaturing conditions, produced by increasing the temperature up to 350 K and/or adding urea. In particular, we carried out a 1 μs classical MD simulation in 10 M urea at 300 K, and three supporting 0.5 μs classical MD simulations w/and w/o 10 M urea at 300 and 350 K. A summary of the conditions used for the MD runs is reported in Table 1.

Usually the unfolding process is followed by monitoring selected progress variables such as the number of backbone H-bonds, the gyration radius, the number of structured amino acids (*i.e.* organized in secondary structure), and the native contacts of the protein [3,8,33,34]. A number of these progress variables have been demonstrated to be useful for monitoring/describing through classical MD simulations the urea-induced unfolding process of barnase [33] and chymotrypsin inhibitor 2 [34]. In our previous papers [3,8], we also evaluated these variables for the MD simulation of peptostreptococcal protein L in urea or guanidinium chloride solutions, and observed statistically significant variations for all of them during the unfolding process. Fig. 1 plots two of these variables (number of backbone H-bonds in panel A, and width of gyration radius in panel B) for all the MD simulations carried out on BLG. The analysis of the results relative to the main MD simulation, run in 10 M urea at 300 K (black line), shows that, despite the long time-scale, no significant unfolding event is detected by the selected progress variables. Much more pronounced differences are seen for most observables when increasing the temperature from 300 to 350 K, both in the absence (blue line) and in the presence (red line) of 10 M urea (Fig. 1, panels A and B). However, at high temperature, additional effects on protein structure stability are clearly observed in the presence of urea, as highlighted by the divergence of the plots (blue and red line) in the second part of the MD simulation, from 250 to 500 ns.

The comparison of secondary structure content (Fig. 1, panel C), which is strongly related to the presence and persistence of a H-bond network among backbone atoms (Fig. 1, panel A), gives consistent results, and notable changes can be observed only when urea acts on a system which is evolving under high temperature conditions. It seems that the temperature is the crucial factor needed to make urea effects evident within the simulated timeframe. The MD data therefore point to urea acting through the perturbation of the protein intramolecular hydrogen bonds, which reflects on the reduction of secondary structure as it can be clearly observed by comparing the two simulations at 350 K w/and w/o urea 10 M (Fig. 1, panels A and C, red *vs.* blue lines).In particular, the MD simulation carried out in 10 M urea at 350 K shows a noticeable reduction in the total number of structured amino acids (approx. 35%), which can be ascribed to the decrease in the number of residues in β-sheet (from 70 to 51, Fig. 1E) and the consequent increase in the number of residues in random coil (from 26 to 47, Fig. 1F). The α-helix is hardly affected and appears quite stable in all the tested denaturing conditions (panel D). Similar results, pointing out the stability of α-helices and the sensitivity of β-strands to high urea concentrations, were already reported and thoroughly discussed for chymotrypsin inhibitor 2 in Bennion and Daggett [34], and for peptostreptococcal protein L in Rocco et al. [8].

A comment should be added on the spikes detectable in the gyration radius of BLG simulation in water at 350 K (Fig. 1, panel B). Specific timeframes isolated in correspondence with these spikes show the loss of tertiary structure both at the N-terminus and at the C-terminus (Fig. 2). This evidence suggests that high temperature seems to be able to start the protein unfolding by decreasing both

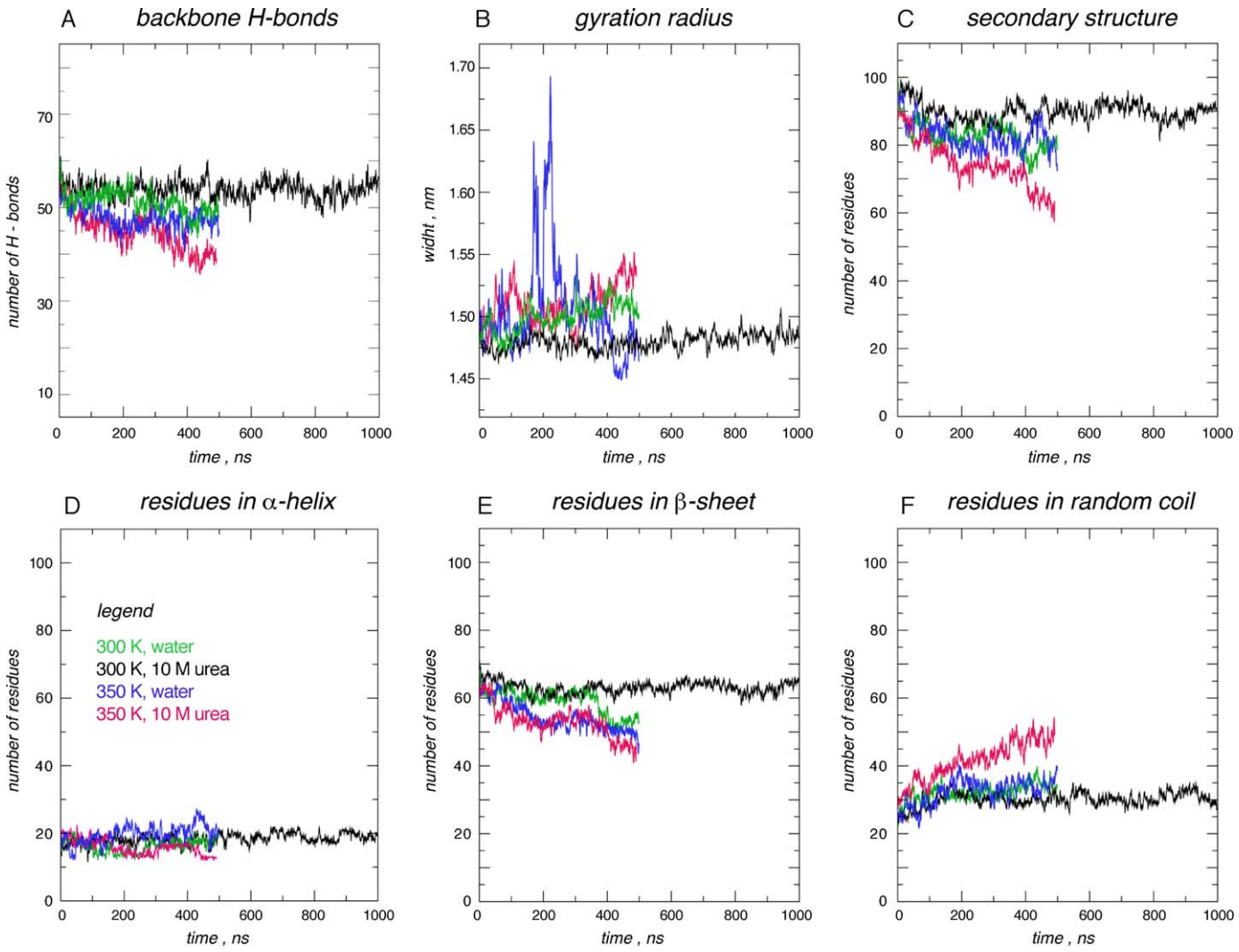

Fig. 1. Progress coordinates for BLG unfolding in four MD simulations. Color code: green, water at 300 K; black, 10 M urea at 300 K; blue, water at 350 K; red, 10 M urea at 350 K. Secondary structure elements are classified according to the program DSSP [39].

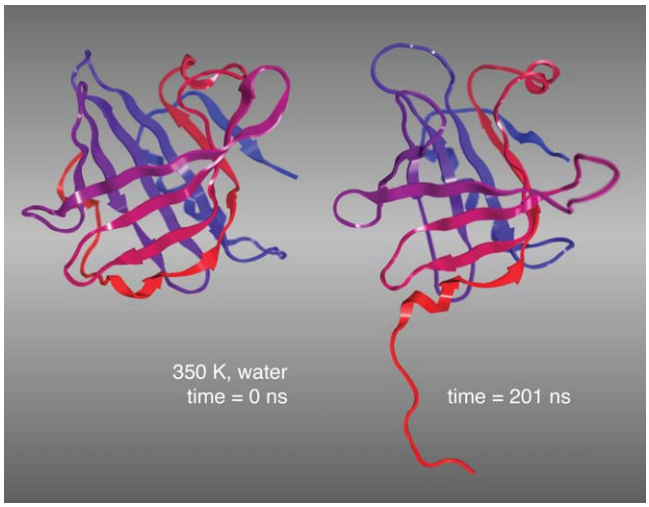

Fig. 2. BLG structure during classic MD simulation in water at 350 K. Left, time 0 ns; right, time 201 ns.

secondary (Fig. 1, panels C–F) and tertiary structure (Fig. 1, panel B) [11].

3.2. Monitoring unfolding via H-bond existence matrices

Hydrogen bonds stability along MD simulation is a subtle marker of unfolding, especially when the attention is focused on the behavior of specific H-bonds that experimental works have recognized as relevant for BLG stability and folding. Amide protons can exchange when they are solvent-exposed and not involved in stable H-bonds. Studying the refolding kinetics of BLG via H/D exchange pulse labelling coupled with heteronuclear NMR, Forge et al. measured the H/D exchange protection factors of the BLG native state at pH 3 and 318 K [25]. From their list, we sub-classified the protons in two groups – those with a low (<1000) and those with a high protection factor (>3000) – and monitored their behavior in all the conditions we have simulated. We score as positive (*i.e.* stable, showing rare H-bond opening events) the H-bonds present for at least 80% of the MD timeframes, whereas we consider as negative (*i.e.* unstable, showing frequent H-bond opening events) the H-bonds present in less than the 20% of the MD timeframes. The bonds corresponding to neither criterion (positive or negative) are marked with blanks; the corresponding data can be found in Table 2.

Table 2
H-bonds of the protons associated with high protection factors[a], monitored during the MD simulations. A gray background highlights H-bonds not stable at 300 K in water. Bold text points out H-bonds stable in all the tested conditions. For a description of Phe82 behavior, please, see Section 3.2.

Residue	300 K water	300 K urea	350 K water	350 K urea
Val15 (helix)	+	+	+	+
Ala16			–	
Trp19 (βA)	+	+	+	
Tyr20 (βA)	+	+	+	+
Ala23 (βA)				
Met24 (βA)				
Ala25 (βA)	+	+	+	+
Val43 (βB)			–	
Leu54 (βC)	+			
Ile56 (βC)	+	+	+	+
Leu57 (βC)	+	+	+	+
Gln59 (βC)	+	+	+	+
Ala67 (βD)		+	–	–
Val81 (βE)	–	–	–	–
Phe82 (βE)	(+)	+	+	+
Ile84 (βE)	+	+	+	+
Lys91 (βF)	+	+	–	
Val92 (βF)	+	+	+	+
Leu93 (βF)	+	+	+	+
Val94 (βF)				
Leu95 (βF)	–	–	–	–
Tyr102 (βG)	–	–	–	–
Leu103 (βG)	+	+	+	+
Leu104 (βG)	+	+		+
Phe105 (βG)	+	+	+	+
Cys106 (βG)	+	+	+	+
Met107 (βG)	+	+	+	+
Glu108	+	+	+	+
Gln 115	–	–	–	–
Val118 (βH)				
Cys119 (βH)	+	+	+	
Gln120 (βH)	+	+	+	+
Cys121 (βH)	+	+	+	+
Leu122 (βH)	+	+	+	+
Val123 (βH)	+	+	+	+
Phe136 (helix)	+	+	+	+
Ala139 (helix)	+	+	+	+
Leu140 (helix)	+	+	+	+

[a] Data from Forge et al. [25].

Table 3
H-bonds of the protons associated with low protection factors[a], monitored during the MD simulations at 300 K in water. Bold text points out H-bonds unstable in all the tested conditions.

Residue	300 K water
Gln 13 (helix)	–
Lys 14 (helix)	–
Gly17 (βA)	+
Ser 21 (βA)	–
Ser27 (βA)	
Leu 31 (helix)	–
Ala34	–
Ala37	–
Tyr42 (βB)	–
Glu45 (βB)	
Lys47 (βB)	+
Thr49 (βB)	+
Gly52 (βC)	–
Glu 55 (βC)	–
Trp61	
Glu62	
Glu65	–
Ile 78	–
Lys83 (βE)	+
Glu89	–
Asp96 (βF)	–
Asp98	
Tyr99	
Lys 100	–
Glu 112	–
Leu117	
Glu127	–
Asp137 (helix)	+
Lys 141 (helix)	–
Met 145	–
Ile147 (βI)	
Leu149 (βI)	+

[a] Data from Forge et al. [25].

It is worth mentioning that 70% of the hydrogen atoms classified by Forge et al. [25] as associated with high protection factors (>3000) are recognized as involved in stable H-bonds during the long MD simulation run at 300 K in water at neutral pH. The amide hydrogen of Phe82 is involved in a stable H-bond in all the denaturing conditions, but not in the MD simulation at 300 K in water in which it is present for approx. 70% of the analyzed timeframes. For this reason, Phe82 is specifically tagged in Table 2.

Focusing only on H-bonds with high protection factor and stable from the beginning of the simulation in water at 300 K (rows without gray background in Table 2), we identify five residues affected by high temperature and/or urea (rows without bold font): Trp19, Leu54, Lys91, Leu104 and Cys119. Among these, the H-bonds involving Trp19 and Cys119 are affected only by urea at high temperature. The H-bond involving Leu54 is affected by both urea and temperature, whereas those involving Lys91 and Leu104 are affected by high temperature.

Table 3 scores the behavior during our MD simulations for all the 32 H-bonds of the protons associated with the lowest protection factors (<1000) according to Forge et al. [25]. From MD data analysis, 81% of these protons are recognized as transiently involved in H-bonds.

The good correlation of the simulated and experimental data prompted us to investigate the effect of urea and temperature on the stable H-bonds. Some residues (60%) revealed a remarkable stability in all the experimental conditions (Table 2), suggesting they can play a relevant role in the BLG folding mechanism. These data, summarized on a topological representation of β-lactoglobulin β-barrel (Fig. 3, panel A), show that residues belonging to E, F, G, H, and A strands form a stable interaction network, which is not observed between A, B, C, and D strands. The role of the H-bonds between A and H strands seems to be joining and keeping structurally organized the N- and C-terminal domains of BLG.

The H-bonds that are not stable in all the investigated denaturing conditions appear to be more sensitive to high temperature rather than to high urea concentration (four H-bonds are destabilized by the increase of temperature, while one H-bond is lost upon urea addition at low temperature), in agreement with data reported in Fig. 1. In general, the H-bonds that partially loose their stability in at least one of the denaturing conditions are located at the border of β-strands and never involve residues belonging to the GH interface. The stability of this region was also identified by cold denaturation studies of BLG, where a residual structure was observed at the level of GH strands, possibly stabilized by the disulphide bond connecting C106 and C119 side-chains [35]. These results are in good agreement with NMR data pointing to the role of the GH β-hairpin as a folding initiation site [36].

This comparison thus demonstrates that MD is able to correctly sample some of the fast processes in protein structural reorganization that in an experimental setup are currently monitored by spectroscopic techniques.

3.3. Monitoring unfolding via H–H interatomic distances

The existence of a hydrophobic cluster across the lipocalin protein family had been assessed on the basis of structural alignment and NMR measurements [18]. The BLG buried cluster was found to be stable at pH 2 not only at room temperature but also at

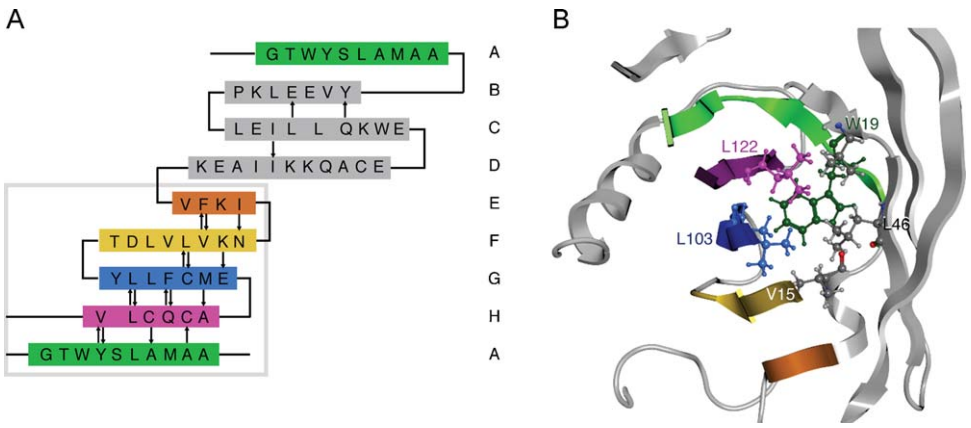

Fig. 3. (A) Network of most stable H-bonds in BLG. Stable backbone H-bonds are represented as arrows. The strands forming the most stable BLG beta-sheet are boxed and highlighted in color. (B) Ball and stick representation of residues (Val15, Trp19, Val43, Leu46, Leu103, Leu122) involved in stable hydrophobic interactions in all the tested conditions. The selected residues and strands are color-coded as in panel A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

high temperature (323 K). Starting from these observations, among the amino acids identified by Ragona et al. [18] as presenting NOE effects, we selected H–H pairs both with interatomic distances <5 Å in the X-ray data [18] and present (at interatomic distance <5 Å) for at least 50% of the analyzed timeframes in our MD simulation at 300 K in water. According to these criteria, a subset of 25 H–H pairs, out of the 54 H–H pairs listed at pH 2 by Ragona et al. [18], could be identified (Table 4). The hydrophobic network seems to be less extended than under acidic conditions and the observed differences are in good agreement with experimental data pointing to a greater stability of BLG at acidic than at neutral pH [26,27]. Simulations, which are run in an environment that does not correspond to the situation of maximal structural stability, have the advantage of better differentiating the behaviors of the hydrophobic core residues. The interatomic distances, which are stable throughout the simulation in water at 300 K (*i.e.* under non-denaturing conditions), were also monitored during the MD simulations under denaturing conditions. Thirteen H–H short distances result stable over the simulation time (*i.e.* present for at least 50% of the analyzed timeframes) in all the tested conditions, whereas twelve increase to a distance ≥5 Å (for more than 50% of the analyzed timeframes) in at least one of the denaturing conditions. It is interesting to observe that urea addition produces the lengthening of six H–H short distances at 300 K and that temperature increase to 350 K does not further destabilize the hydrophobic cluster. These data suggest that urea is effective in dissolving the core hydrophobic region, in agreement with previous results [11]. Also the urea-induced unfolding of barnase described by Caflisch and Karplus [33] revealed the partial stability of the protein hydrophobic core, which appeared to be the most resistant part of the protein. Furthermore, Bennion and Daggett [34], studying the urea-induced unfolding of chymotrypsin inhibitor 2, reported that the first step of the denaturation of the protein hydrophobic core proceeds through its structural expansion; only in a later step is the core solvated by water, then by urea.

Table 4
Summary of the observed NOE H–H distance at 310 K[a] and of the corresponding distances in MD simulations.[b]

Residues	Proton pair	300 K water	300 K urea	350 K water	350 K urea
Val15–Leu46	H^{β}–$H^{\delta'}$	+	–	–	–
Trp19–Leu122	H^{β}–$H^{\delta'}$	+	+	+	+
Trp19–Leu122	$H^{\beta'}$–$H^{\delta'}$	+	+	+	+
Trp19–Val15	H^{7}–H^{β}	+	+	–	+
Trp19–Val15	H^{7}–$H^{\gamma'}$	+	+	–	+
Trp19–Leu103	H^{6}–H^{β}	+	+	–	+
Trp19–Leu103	H^{5}–H^{β}	+	+	+	+
Trp19–Leu122	H^{4}–H^{β}	+	+	+	+
Trp19–Leu46	H^{2}–$H^{\beta'}$	+	+	+	+
Trp19–Leu46	H^{2}–H^{β}	+	+	+	+
Trp19–Leu46	H^{2}–H^{γ}	+	+	+	+
Trp19–Leu46	H^{2}–H^{δ}	+	+	+	+
Trp19–Val15	H^{1}–H^{β}	+	–	–	–
Trp19–Val15	H^{1}–H^{γ}	+	+	+	+
Trp19–Leu46	H^{1}–$H^{\beta'}$	+	+	+	+
Trp19–Leu46	H^{1}–H^{β}	+	+	+	+
Trp19–Leu46	H^{1}–H^{γ}	+	+	+	+
Val43–Leu122	H^{β}–$H^{\delta'}$	+	+	+	+
Val92–Phe105	H^{γ}–$H^{\beta'}$	+	+	–	+
Val92–Phe105	$H^{\gamma'}$–H^{β}	+	+	–	+
Val94–Phe82	$H^{\gamma'}$–H^{2}	+	–	–	–
Val94–Phe105	$H^{\gamma'}$–$H^{\beta'}$	+	+	–	–
Phe105–Leu122	H^{2}–H^{γ}	+	–	–	–
Phe105–Leu122	H^{3}–H^{γ}	+	–	–	–
Phe105–Leu122	H^{3}–H^{δ}	+	–	–	–

A gray background highlights interactions which are classified as positive in all tested conditions.
[a] Data from Ragona et al. [18].
[b] H–H distance <5 Å for more than the 50% of the analyzed timeframes.

High temperature in the absence of denaturant affects different regions of BLG hydrophobic cluster suggesting that the unfolding process, at least in its initial stages, is quite different depending on the prevailing conditions. It is worth mentioning that BLG hydrophobic interactions that are stable in all the denaturing conditions involve Trp19, the only residue completely conserved in the lipocalin family, which has been recognized as most important for protein stability [37]. A molecular representation of the amino acids interacting with Trp19 is depicted in Fig. 3 panel B.

3.4. Mechanism of urea denaturation

Several experimental and computational papers have aimed at clarifying the denaturing mechanism of urea. Some authors have suggested that urea reduces the number of intra-chain protein::protein H-bonds, substituting to amino acid side chains in the formation of new long-lived bonds [2,4,5,7]; others have proposed instead that urea does not force the change from folded to unfolded conformations, but stabilizes the unfolded forms by shifting to the right the folded ↔ unfolded equilibrium [10]. Two recent papers have suggested that the direct H-bonding of urea to the peptide backbone does not seem to play a dominant role in denaturation [12], but rather is a consequence of the loss of structure, and that the unfolding mechanism can be mainly connected to the weakening of hydrophobic interactions and to the dissolution of the core hydrophobic region, through urea::protein attractive dispersion interactions [11]. In our current and previous [8] simulations we observed the reduction of protein::protein H-bonds, but we could not discriminate whether these events are causes and/or effects of the unfolding process. However for peptostreptococcal protein L [8] two different unfolded structures have been described depending on the presence or absence of urea. The structure obtained at 480 K in urea has a hydrophobic solvent accessible surface significantly higher than in water (respectively, approx. 40 nm^2 *vs.* approx. 27 nm^2). Therefore MD data, collected for peptostreptococcal protein L, and current MD data obtained for BLG strongly support the hypothesis of Stumpe and Grubmuller [9,10], further confirmed by the findings by Zangi et al. [11] and Canchi et al. [12], on the dissolution of the protein hydrophobic core as the main unfolding mechanism mediated by urea.

A very recent report by Lindgren and Westlund [38] confirmed that urea mainly decreases the Lennard–Jones interaction energies between protein and solvent while not affecting the electrostatic component. Specifically, the authors reported that urea could affect similarly the Lennard–Jones energies for both hydrophobic and hydrophilic side chains, and for backbone.

Stumpe and Grubmuller [9] proposed that urea-induced protein unfolding proceeds through an alternated loss of secondary and tertiary structure. The data presented here for BLG support this proposal: indeed urea, at least in the very initial stages of the process, affects both protein secondary (Fig. 1, panels C–F) and tertiary structure, as deduced from the analysis of the hydrophobic interactions (Table 4) between residues belonging the BLG internal cluster, which is responsible for the maintenance of BLG fold.

4. Conclusions

MD unfolding studies performed on BLG have highlighted the importance of evaluating the interactions and dynamical behavior of specific key residues in addition to the analysis of standard progress variables to describe the early events of unfolding. The increase of the system thermal energy by raising the system temperature to 350 K, both in pure water and in the 10 M urea simulation, allowed us to observe the variations in standard progress variables. However, the effect of the increase in temperature for accelerating the sampling of the protein conformational space in MD simulations should be implemented with some care, since it is difficult to discriminate accurately the contribution of urea from that of high temperature. Indeed, the unfolding pathways brought about by the combination of urea and high temperature do not necessarily resemble the ones produced by the presence of urea alone at room temperature. From a careful analysis of the MD data, it can be concluded that urea, in the very first stage of the unfolding process, seems to destabilize BLG structure by weakening protein::protein hydrophobic interactions, affecting the hydrogen bond network, and possibly establishing attractive dispersive interactions with the protein. All these processes become more evident when the effects of urea are studied at higher temperature, allowing a more efficient exploration of the protein conformational space.

Although consistent along both series, simulations in the absence and in the presence of urea, and not in disagreement with literature data, our observations are made and conclusions are drawn on the basis of individual MD simulations for each of the test conditions. While the computational effort connected with these long MD runs prevents the production of a large number of replicates amenable to statistical treatment, we need to write a note of caution about the representativeness of each individual dataset, especially with regard to details in the protein structure. Having in mind this limits and cautions, it seems of interest to systematically explore the overall effects of urea exposure on the structure of a system such as the one corresponding to a medium size protein.

Acknowledgments

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