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PROCEEDINGS OF THE 3RD INTERNATIONAL CONFERENCE ON MOLECULAR SIMULATION

Exposure of thiol groups in the heat-induced denaturation of β -lactoglobulin

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Protein aggregates can be stabilised by disulphide bridges. The whey protein β -lactoglobulin (β -lac) contains a disulphide bridge and a free cysteine that are shielded from the solvent by an α -helix. These groups are important in the thiol–disulphide exchange that occurs during aggregation and gelation of β -lac. Replica exchange molecular dynamics simulations show that the exposure mechanism is very different for the two buried groups. While melting of the α -helix enhances exposure of the free cysteine, it does not for the buried bridge. These findings shed light on the molecular mechanism of the first step of β -lac denaturation and aggregation.

Keywords: proteins; replica exchange molecular dynamics; aggregation

1. Introduction

Aggregation of globular proteins is of great interest, both from a fundamental and from an application point of view. For instance, whey proteins (a fraction of bovine milk) are commonly used in the food industry as thickening agents. [1] In addition, whey proteins can find application as components of edible protein films. As such, their aggregation and gelation properties are of particular interest. Notwithstanding extensive experimental research, a fundamental understanding of the underlying mechanisms of whey protein aggregation is lacking. [2] This is partly due to the fact that, in addition to the pH, salt and protein concentration, the aggregate and gel properties depend on the temperature used for the initial aggregation and the time the proteins are kept at that temperature, as well as the mechanism used to obtain a gel (e.g. change in pH, salt concentration or drying). [3]

As the complete system of all whey proteins is quite complex, it is more useful to concentrate on one model system. When proteins are heated they tend to lose their secondary and tertiary native structure. Exposure of buried hydrophobic groups makes these denatured proteins prone to aggregation and gelation. Such aggregation can be stabilised by the presence of free cysteines and/or disulphide bridges in the protein, allowing the formation of intermolecular covalent bonds by a thiol–disulphide exchange reaction. The formation of these bonds strengthens aggregates, gels and films, and under some conditions is the primary aggregation process. [4] Not all thiol and disulphide groups are immediately accessible at room temperature, and they become only available for thiol–

disulphide exchange during heating. Here we are concerned with the first step in this process: the exposure of thiol groups and disulphide bridges.

As a model system we take the bovine β -lactoglobulin (β -lac) protein, which is the most abundant protein in the whey fraction. β -lac is a 162 residue globular protein consisting of a central calyx, formed by two β -sheets, which can bind small hydrophobic molecules. [5] β -lac forms a dimer at room temperature and neutral pH. β -lac contains a free thiol group and two disulphide bridges, making two β -lac molecules capable of undergoing thiol–disulphide exchange. One of the disulphide bonds (C66–160) is exposed and anchors the C-terminus on the calyx. The other disulphide bridge (C106–C119) connects two β -strands of the calyx. The free thiol group (C121) and the second disulphide bridge (C106–C119) are buried, and shielded from the solvent by an α -helix [6] (see Figure 1). This helix interacts with the β -sheet through hydrophobic contacts. The buried groups, in particular the free thiol group, must become solvent exposed before thiol–disulphide exchange (SH–SS) can occur. This heat-induced denaturation is a crucial step in the aggregation process. The denaturation temperature of β -lac lies around 70°C, [7] but the exact temperature is hard to determine due to the concurrent aggregation process.

At first sight it would seem that melting of the α -helix would cause exposure of the buried thiol group and disulphide bridge. While some experimental studies show little change in the secondary structure of β -lac during heating, CD spectra including the 170–200 nm region show a significant change in the helical content. The total

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Figure 1. (Colour online) Crystal structure 1BSY,[6] 162 residues. The free cysteine (C121) is shown in red, the buried bridge (C106–C119) in orange and the exposed bridge (C66–C160) in purple.

helicity drops from 10% to 3% between 60 and 70°C. The β -sheet content is more constant, and drops from 51% to 41% between 40 and 60°C, which might be partially due to the dissociation of the dimer.[8]

Mass spectrometry experiments can provide information about the distribution of inter-molecular bonds. MALDI-TOF MS experiments of tryptic digests of aggregated β -lac show which peptide fragments are connected to each other. [9,10] Unfortunately, because C106, C119 and C121 are in the same tryptic digest, these residues cannot be distinguished. C160 appears in more combinations of peptide dimers and trimers than C66, which suggests that it is more easily available for thiol–disulphide exchange. Indeed, C160 is only two residues away from the C-terminus and breaking the native C66–C160 disulphide bond results in a flexible C-terminal tail. The experiments also find a different composition of covalently linked tryptic digests depending on the salt concentration and on the temperature used to initiate the aggregation. This suggests that the availability of the different cysteine residues depends on the level of denaturation of the protein.

In this work we employ molecular dynamics (MD) simulations to provide specific atomistic insight into the denaturation process, and the exposure of the thiol groups in particular. During the thiol–disulphide exchange, free thiol groups and disulphide bridges must be able to approach each other. Ideally one would like to simulate the denaturation, the diffusion of the thiol groups and their subsequent reaction, but in practice, this is computationally unfeasible using all-atom straightforward MD because denaturation is a rare event on the molecular time scale. Biased sampling methods can be used to enhance simulations, but they usually require prior knowledge of

a proper reaction coordinate to describe the process of interest. In contrast, replica exchange molecular dynamics (REMD) offers a way to speed up simulations without any bias, by using high temperature replicas to overcome energy barriers.[11]

β -lac has been studied recently with all atom MD simulations. For instance, Euston compared several accelerated methods to study the heat-induced denaturation of β -lac, and found that REMD simulations of 10 ns were insufficiently long to find substantial unfolding.[12] Stănciuc et al. [13] used both MD simulations and spectroscopic methods to study the denaturation of β -lac. They found that both tryptophan residues W19 and W61 are more accessible at higher temperatures. Here, we study the exposure of buried thiol groups using REMD on a single β -lac protein in explicit solvent. We show the results of two REMD runs starting from the native structure, one with the GROMOS and one with the AMBER forcefield. In addition, we show results for a REMD run starting from a partially denatured structure using the AMBER forcefield. After a discussion on the global denaturation features, we focus on the exposure of the buried thiol groups during denaturation. Finally, we investigate the importance of the protonation state of the free thiol group by performing conventional MD simulations for that system.

2. Methods

2.1. MD simulations

While β -lac is a dimer at room temperature and neutral pH, at 55–60°C the protein exists mostly in the monomeric state.[2,14] Therefore, we can improve the computational efficiency by simulating only one monomer. The protein studied is full-length bovine β -lac, genetic variant A (PDB code 1BSY [6]), with standard protonation (total protein charge -9). The protein was solvated in a rhombic dodecahedral periodic box and nine Na^+ counter ions were added to obtain a neutral system. After conjugate gradient minimisation the water was equilibrated in a 10 ps NPT simulation with position restraints on the protein. The whole system was then equilibrated at 1 bar and 298 K during a 1 ns NPT simulation. This procedure was followed for two different forcefields. The GROMOS96 43a1 forcefield was used with the SPC water model.[15] In these simulations the box diameter was 6.76 nm after equilibration and 6358 water molecules were present. The TIP3P water model was used with the Amber99SB forcefield for the protein.[16] In the simulations with this forcefield, the system consisted of 6357 water molecules and the box diameter was 6.75 nm.

Simulations were carried out using GROMACS version 4.0,[17] the AMBER forcefield was ported into GROMACS with the AMBER ports.[18] The bonds between protein atoms were constrained with LINCS [19]

and the bonds between water atoms were constrained with SETTLE,[20] making it possible to use a 2-fs time step. Long-range electrostatics were treated with fast particle-mesh Ewald.[21,22] The velocity-rescale thermostat was used [23] to control the temperature ($\tau = 0.2$ ps), and Parrinello–Rahman pressure coupling [24] was used in the NPT equilibration steps.

REMD aims to sample the canonical distribution of systems in which the dynamics is hampered by the presence of high free energy barriers.[11] A fixed number of replicas are started at different temperatures. Replicas at higher temperatures will be able to overcome energy barriers more easily, and reach denatured or exposed states sooner. Replicas are regularly allowed to exchange temperatures through a Monte Carlo move that maintains the Boltzmann distribution.

We performed three different REMD simulations with 64 replicas each. Two runs started from the native structure, with the GROMOS and AMBER forcefields, labelled GRO_N and AMB_N, respectively. A third run employing also the AMBER forcefield was initiated from a high temperature denatured structure and labelled AMB_D. All three REMD simulations were run for 40 ns per replica. The REMD was performed in the canonical NVT ensemble using the velocity-rescale thermostat.[23] Replicas were allowed to exchange every 2 ps. The replica exchange was executed by a perl wrapper script, allowing for all pair exchange. A geometrical temperature distribution leads to an overall exchange acceptance around 20%. As we are interested in the denaturation process at relatively high temperatures, we selected a high temperature range. In the GRO_N simulation, the temperature ranged from 330 to 607 K, with an overall exchange of 23.55%. For both AMB_N and AMB_D runs, the temperatures ranged from 330 to 614 K, resulting in an exchange of 24.21% and 24.25%, respectively.

For the conventional MD simulations with a deprotonated C121, the same system was used as for the AMBER REMD run. As the deprotonation gives an additional negative charge, one extra Na⁺ ion was added, to neutralise the system.

2.2. Analysis

To investigate conformational changes of the protein, we monitored several order parameters during the simulations. The root mean square deviation (RMSD) based on C_α's was calculated with respect to the equilibrated structure at room temperature for the same forcefield. Besides the RMSD for the whole protein, denoted rmsd_{Cα}, we computed the RMSD for the α-helix and the calyx, respectively, labelled rmsd_{ahx} and rmsd_{calyx}. The α-helix was defined as residues 130–142. The calyx was defined as residues 16–21, 40–50, 52–62 and 66–77 (β-sheet 1) and 22–27, 80–84, 90–98, 101–110 and 117–125

(β-sheet 2). To quantify exposure of the thiol groups, we computed the minimum distances of the cysteine residues to the solvent, defined as the shortest distance from the sulphur atom of the cysteine(s) to any water molecule. The three distances of interest are the minimum solvent distance for the buried free cysteine C121, dmin_{bf}, the minimum solvent distance for the buried bridge C106–C119, dmin_{bb}, and the minimum solvent distance for the exposed bridge C66–C160 dmin_{eb}. In addition, we establish the number of water molecules, nw_{bf}, within 7 Å of the sulphur atom of the buried free cysteine C121.

The MD trajectories were converted into 2D population histograms $P_i(q^{(1)}, q^{(2)})$, for various pairs of order parameters, $q^{(1)}, q^{(2)}$. The first 8 ns was considered equilibration and was not taken into account. The Landau free-energy landscape for replica i follows from the normalised population histogram by applying

$$F_i(q^{(1)}, q^{(2)}) = -k_B T_i \ln P_i(q^{(1)}, q^{(2)}), \quad (1)$$

where F_i is the (Landau) free energy at temperature T_i and k_B is Boltzmann's constant. By applying the following equation for P_i , we make use of all replica information[25]:

$$\begin{aligned} P_i(q^{(1)}, q^{(2)}) &= \sum_{j=1, j \neq i}^N (1 - \min[1, e^{\Delta\beta_{ij}\Delta U_{ij}}]) \delta(q_i^{(1)} - q^{(1)}) \delta(q_i^{(2)} - q^{(2)}) \\ &+ \sum_{j=1, j \neq i}^N \min[1, e^{\Delta\beta_{ij}\Delta U_{ij}}] \delta(q_j^{(1)} - q^{(1)}) \delta(q_j^{(2)} - q^{(2)}) \end{aligned} \quad (2)$$

in which i and j loop over all N replicas, $\Delta\beta_{ij} = \beta_i - \beta_j$ is the reciprocal temperature difference between replicas i and j ($\beta = 1/k_B T$) and $\Delta U_{ij} = U_i - U_j$ is the potential energy difference.

3. Results and discussion

3.1. REMD simulations

We performed three different REMD simulations for the β-lac system, labelled GRO_N, AMB_N and AMB_D. The GRO_N and AMB_N simulations started from the native structure and employed the GROMOS and Amber forces fields, respectively. The AMB_D simulation also employed the AMBER forcefield but started from a high temperature structure from the AMB_N simulation with a completely melted α-helix.

Experimentally, exposure of the thiol groups occurs somewhere between 65 and 105°C.[2] The rate at which the exposure occurs is strongly temperature dependent. We therefore chose to be close to the upper limit of the denaturation window, and concentrate our discussion on a temperature around 375 K, rather than at low

Figure 2. (Colour online) Free energy landscapes showing global denaturation features. On the x-axis the RMSD of the C_{α} atoms of the calyx is shown, $\text{rmsd}_{\text{calyx}}$. The y-axis is the rmsd of the C_{α} atoms of the helix, rmsd_{ahx} . One contour line corresponds to $1 k_B T$. The star denotes the starting structure of the REMD run, the letters correspond to the snapshots in Figure 3. The energy landscapes are shown for all three runs, GRO_N , AMB_N and AMB_D , at 376 K (top row) and 498 K (bottom row).

temperature. We note that the forcefields are predominantly parameterised at room temperature, and usually predict a higher denaturation temperature than found experimentally.

Before we focus on the precise extent of exposure of the thiol groups, we will first discuss the global REMD results. Figure 2 shows free energy landscapes projected in the $\text{rmsd}_{\text{ahx}} - \text{rmsd}_{\text{calyx}}$ plane for all three REMD runs. The top row shows the landscapes at 375 K and the bottom row landscapes at 500 K. A comparison of Figure 2(a) and (b) reveals that the protein is stable for larger temperature ranges in the AMBER forcefield. In fact, the high temperature AMB_N landscape shown in Figure 2(e) is more similar to the low temperature GRO_N result shown in Figure 2(a) as both show sampling extending towards $\text{rmsd}_{\text{calyx}} \approx 0.4 \text{ nm}$, and a higher $\text{rmsd}_{\text{ahx}} > 0.2 \text{ nm}$. This indicates that employing the GROMOS forcefield yields more flexible protein structures than the AMBER forcefield. Nevertheless we observe the same trends for both forcefields.

At the end of 40 ns simulations, the REMD runs with different starting structures, AMB_N and AMB_D , had not yet converged (compare Figure 2(b) and (c)). This behaviour is well known for REMD.[26,27] Still, we can still draw some qualitative conclusions.

In the AMB_N runs, the α -helix did not unfold at temperatures around 376 K, and rmsd_{ahx} stays below 0.2 nm

(Figure 2(b)). Moreover, in the AMB_D simulation, the α -helix actually refolded until $\text{rmsd}_{\text{ahx}} < 0.1 \text{ nm}$. The native state itself was not reached, as seen by, e.g. the larger $\text{rmsd}_{\text{calyx}}$ in AMB_D than in AMB_N simulation. A snapshot of a structure from AMB_D that comes close to the native state can be seen in Figure 3(e). The lower rmsd_{ahx} goes hand in hand with a lower $\text{rmsd}_{\text{calyx}}$ (below 0.15 nm).

3.2. Exposure of the buried cysteines

While a simulation of only one β -lac monomer cannot directly predict the availability of the cysteine residues for thiol–disulphide exchange, we can investigate how the cysteines are exposed to the solvent during denaturation. To quantify the exposure of the buried cysteines (C106–C119 and C121), we use the minimum distance d_{min} of the solvent to the sulphur atom as an order parameter. This order parameter is more convenient than the number of water molecules within a certain radius as it is continuous and more sensitive to changes in the environment.

To determine whether a thiol group is exposed or not, we take as a reference the solvation of the exposed disulphide bond (C66–C160). Therefore, we compute the probability histogram for the minimum distance of the solvent of the exposed disulphide bridge (C66–C160), $d_{\text{min}_{\text{eb}}}$. For both the GROMOS and AMBER forcefield, the probability distribution of this order parameter is

Figure 3. (Colour online) Snapshots from the three REMD runs. Water is shown when within 7 Å of the buried cysteine groups. The caption labels correspond to the labels in Figure 2. (a) GRO_N 375 K, a structure close to the native state, (b) + (c) GRO_N 375 K, structures with a melted α -helix and distorted calyx, (d) GRO_N 500 K, a structure with a melted α -helix and distorted calyx, (e) AMB_D 376 K, a structure close to the native state and (f) AMB_D 498 K a structure with a melted α -helix.

peaked at $dmin_{eb} = 0.3$ nm. Hence, we can consider a minimum distance around this value to indicate an exposed cysteine. In Figures 4–7, this value is highlighted by a solid line.

Figure 4 shows free energy landscapes for the GRO_N and AMB_N runs as a function of the minimum distance of the buried bridge to the solvent, $dmin_{bb}$, the minimum distance of the free thiol group to the solvent, $dmin_{bf}$ and the RMSD of the helix, $rmsd_{ahx}$. Focusing first on the GRO_N results, Figure 4(a), the deepest minimum corresponds to a buried state for both the C106–C119 disulphide bridge and free cysteine groups ($dmin_{bb}$ and $dmin_{bf}$ both around 0.6–0.8 nm), with an intact α -helix ($rmsd_{ahx} < 0.2$ nm). This basin extends towards low $dmin$ values, and according to the contour lines, exposure is

Figure 4. (Colour online) Free energy landscapes of the REMD simulations starting from the native structure as a function of the minimum distance of the sulphur from the buried free thiol or the buried bridge to the solvent ($dmin_{bf}$ and $dmin_{bb}$) and the RMSD of the α -helix ($rmsd_{ahx}$). One contour line corresponds to 1 $k_B T$. The purple line corresponds to the equilibrium value for the exposed disulphide bridge ($dmin_{eb} = 0.3$ nm). (a) GRO_N 375 K. (b) AMB_N 376 K.

slightly more accessible for the free cysteine than for the buried bridge. There is some unfolding of the α -helix, as can be seen from the second minimum at $rmsd_{ahx} \approx 0.3$ nm. Also here the minimum is in the buried state (both $dmin_{bb}$ and $dmin_{bf} \approx 0.6$ –0.8 nm) and while there is some exposure of the free cysteine (around a $dmin_{bf} \approx 0.3$ nm, at a $rmsd_{ahx} = 0.3$ nm), no exposure of the buried bridge can be seen as there is no sampling around $dmin_{bb} \approx 0.3$ nm.

Figure 5. (Colour online) Free energy landscapes of the REMD simulations starting from the native structure as a function of the minimum distance of the sulphur from the buried free thiol or the buried bridge to the solvent ($dmin_{bf}$ and $dmin_{bb}$) and the RMSD of the α -helix ($rmsd_{ahx}$). One contour line corresponds to $1 k_B T$. The purple line corresponds to the equilibrium value for the exposed disulphide bridge ($dmin_{eb} = 0.3$ nm). (a) GRO_N 500 K and (b) AMB_N 498 K.

The AMB_N run, shown in Figure 4(b), did not sample structures above $rmsd_{ahx} = 0.16$ nm. For these low RMSD values, the buried bridge is more frequently exposed than the free cysteine C121. There is, however, a distinct minimum where C121 is exposed ($0.2 < dmin_{bf} < 0.4$ nm). The upper right corner of Figure 4(b) shows that the buried cysteines are hardly simultaneously

Figure 6. (Colour online) Free energy landscape at 376 K of the AMB_D run as a function of the minimum distance of the sulphur from the buried free thiol or the buried bridge to the solvent ($dmin_{bf}$ and $dmin_{bb}$) and the RMSD of the α -helix ($rmsd_{ahx}$). One contour line corresponds to $1 k_B T$. The purple line corresponds to the equilibrium value for the exposed disulphide bridge ($dmin_{eb} = 0.3$ nm). The stars denote the starting structure, and the letters correspond to the structures in Figure 8.

exposed, similar to the results of the GROMOS run. In this landscape there are three distinct energy minima, one where only C121 is exposed ($0.2 < dmin_{bf} < 0.4$ nm), one where only C106–C119 is exposed ($0.2 < dmin_{bb} < 0.4$ nm) and one where neither is exposed ($dmin_{bf} \approx 0.65$ nm, $dmin_{bb} \approx 0.45$ nm).

When we look at higher temperatures, the α -helix does unfold, and the free energy landscapes look different. In Figure 5 we observe again a large difference between the GROMOS and the AMBER forcefields, and the high temperature AMBER landscape. Figure 5(b) looks again more comparable to the 375 K GROMOS one (Figure 4(a)).

To obtain more insight into the effect of the melting of the α -helix, we inspect the AMB_D results which do show unfolding of the helix. Figure 6 shows energy landscapes at 376 K from this run. Based on the $rmsd_{ahx}$ parameter, we can distinguish three regions, $rmsd_{ahx} > 0.25$ nm, $0.1 < rmsd_{ahx} < 0.25$ nm and $rmsd_{ahx} < 0.1$ nm. For the exposure of C121 (lower right corner of Figure 6), a high $rmsd_{ahx}$ clearly enhances exposure, as the broad energy minimum lies on the purple (exposed) line, roughly between a $dmin_{bf}$ of 0.2 nm and 0.4 nm. The snapshot in Figure 8(c) shows a typical configuration, in which C121 is clearly exposed. There is also a very small additional minimum located at label B, the snapshot corresponding to this minimum can be seen in Figure 8(b). In the region

Figure 7. (Colour online) Free energy landscape at 579 K of the AMB_D run as a function of the minimum distance of the sulphur from the buried free thiol and the buried bridge to the solvent ($dmin_{bf}$ and $dmin_{bb}$). One contour line corresponds to 1 $k_B T$. The purple line corresponds to the equilibrium value for the exposed disulphide bridge ($dmin_{eb} = 0.3$ nm).

where the α -helix is moderately deformed, with an $rmsd_{ahx} \approx 0.1 - 0.25$ nm, there is still some exposure, but the energy minimum is a buried state (a minimum distance to the solvent of 0.6–0.8 nm). During refolding of the helix, the free thiol group stayed buried, as the exposed area around label A was only sampled after the region $rmsd_{ahx} < 0.1$ and $0.6 < dmin_{bf} < 0.8$ was sampled. This suggests that the solvated state A at low RMSD values is close to the native state, which is supported by the fact that this region is also sampled in the AMB_N run (Figure 4(b)).

The free energy landscape for the buried disulphide bridge (upper left landscape) shows a different pattern. Here the energy minimum for the unfolded α -helix is a state in which the buried bridge is still shielded from solvent (see structures B and C in Figure 8(b) and (c), respectively). While there is no exposure in region $0.1 < rmsd_{ahx} < 0.2$ nm, below 0.1 nm, surprisingly, water can again come close to the buried disulphide bridge (label A), although there is no distinct energy minimum.

Another remarkable observation is that at lower temperatures (around 375 K for the GROMOS and up to 498 K for the AMBER forcefield), the free cysteine and the buried bridge are not simultaneously exposed (see Figures 4 and 5(b)). In Figure 6, structures in which this occurs are sampled; however, there is no distinct energy minimum as can be seen at higher temperatures (Figures 5(a) and 7).

These results might explain the difference in the studies of Surroca et al. [10] and Livney et al. [9], which were performed at 68.5 and 85°C, respectively. At higher temperatures, more combinations with the peptide containing C121/C106/C119 were found,[9] including trimers of this peptide, which were not found at lower

Figure 8. (Colour online) Snapshots from the AMB_D run. Water is shown when within 7 Å of the buried cysteine groups. Caption labels correspond to the labels in Figure 6. (a) The α -helix has refolded, and both the buried bridge and the free cysteine are exposed. (b) The helix is mostly denatured and neither of the buried groups is exposed. (c) The helix is denatured and the free cysteine is exposed. (d) The helix is denatured and the buried bridge is exposed.

temperatures.[10] A trimer of the C121/C106/C119 peptides includes at least one peptide with two inter-molecular disulphide bonds. The buried disulphide bond C106–C119 could be broken and forms these two inter-molecular disulphide bonds, but if C121 is exposed as well, the chance of two inter-molecular bonds is higher. We see this state in which both cysteines are exposed only at high temperatures (compare Figures 7 and 6).

While there is evidence for a subtle exposure mechanism for both the buried disulphide bridge and free cysteine which involve the orientation of the residues around the buried groups, by far the most exposure is seen when the α -helix unfolds, especially for the free cysteine. As the free cysteine is more easily exposed than the buried disulphide bridge, the first thiol–disulphide reaction to occur is probably between C121 and C66–C160, which is in agreement with results of Creamer et al. [28].

Figure 9. (Colour online) Four different trajectories of conventional MD simulations with the free cysteine deprotonated (S^-). The solvation occurs within 2 ns at 300 K, and is thus no longer a rare event.

3.3. Deprotonated C121

In addition to REMD simulations, we ran four conventional MD simulations with a deprotonated C121 (S^- instead of SH). In all four different trajectories, solvation of the buried free cysteine occurred within 2 ns at room temperature (Figure 9). In this case, we can use the order parameter nw_{bf} , the number of water molecules within $7 \text{ \AA}$ of the sulphur of C121, as many water molecules come close at once. Figure 10 shows a snapshot of such a solvated structure. The α -helix is clearly still intact. In fact, any distortion of the α -helix happens after the solvation, suggesting it is a result of the solvation instead of the other way around (results not shown). While these results seem to suggest that the protonation state of the free cysteine plays an important role in the aggregation process, it is unclear how to link these results directly to experiments, as the pK_a of the buried cysteine is probably very different from the standard pK_a of a cysteine in solution. Recent work by Eberini et al. [29] suggests that

Figure 10. (Colour online) Snapshot of the free cysteine deprotonated (S^-) system within the first nanosecond. All water molecules within $7 \text{ \AA}$ of the buried cysteines are shown.

the pK_a of C121 could go from 10.56 in the native state to 7.28 in the unfolded state. In this light it seems unlikely that the free cysteine is deprotonated in the native state. Our results do show that there is enough flexibility in the structure of the protein to accommodate solvation of the free cysteine without melting of the helix. A similar state can be seen in the REMD results (for example Figure 8(a)), and it would be interesting to know what the pK_a of the cysteine is in that meta-stable state.

4. Conclusions

We studied the exposure of the buried thiol groups of β -lac during heating using REMD with two different forcefields. While both forcefields give qualitatively comparable results, their temperature dependence is very different. In the AMBER forcefield β -lac is substantially more stable than in the GROMOS forcefield.

The exposure mechanism is different for the buried disulphide bridge (C106–C119) than for the free cysteine (C121). While melting of the α -helix (above RMSD values of 0.2 nm) enhances exposure of the free cysteine, it does the opposite for the buried disulphide bridge. An alternative mechanism of solvent exposure of the free cysteine involves the penetration of water molecules in the area of the thiol group.

When the buried cysteine groups are exposed, they can reach other thiol groups from other molecules leading to covalent aggregation. Our results suggest that C121 is more easily exposed than C106–C119, which makes it likely that in the initial state of aggregation, thiol–disulphide exchange occurs mainly between C121 and the exposed disulphide bridge (C66–C160) of another protein molecule.

Denaturation and aggregation likely influence each other. As it is unfeasible to study the effect of non-native intermolecular disulphide bonds on the denaturation process on an atomic level, a coarse-grained approach is needed. To study the finite size of whey aggregates, one needs to go to even simpler models.[30] Using models aiming at different length and time scales, we can thus obtain insight into the interplay between the denaturation, thiol–disulphide exchange and the aggregation of β -lac.

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