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The affect of urea on the kinetics of local unfolding processes in chymotrypsin inhibitor 2

Matteus Lindgren *, Per-Olof Westlund

Department of Chemistry, Umeå University, S-901 87 Umeå, Sweden

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ABSTRACT

The dynamics of chymotrypsin inhibitor 2 (CI2) in water, as well as in 10 M urea, have been studied by Molecular Dynamics simulations. The analysis aims at investigating how local protein processes are affected by urea and how the perturbation by urea on the local level manifests itself in the kinetics of the global unfolding.

The results show that the effect of urea on local processes depends upon the type of process at hand. An isolated two-residue contact on the surface of CI2 has a decreased frequency of rupture in the urea solvent. This is in contrast to the increased frequency of rupture of the hydrogen bonds in secondary structure elements in the urea solvent. It is proposed that the increase in the unfolding rates of complex protein processes is based upon the retardation of the refolding rate of small scale, isolated processes.

1. Introduction

The equilibrium between a protein in the native state and its unfolded state is a delicate balance between protein intra-molecular interactions and protein–solvent interactions. It is well known that a native protein can be destabilized and eventually unfolded by the addition of urea in high concentrations. However, despite the extensive use of urea in protein research, the mechanism by which urea destabilizes proteins is not fully understood. Based upon the results of thermodynamic experiments [1–8], two proposed mechanisms have gained the most attention: either urea preferably interacts with the hydrophobic protein residues, thereby decreasing the hydrophobic effect, or urea interacts with the peptide bonds and destabilizes the protein by an enthalpic driving force. These mechanisms, as well as the others that have been proposed, differ in the thermodynamic driving forces of denaturation, but it is very likely that they also differ in the dynamics of the protein as well as differ in the unfolding pathway.

In stopped-flow experiments [9], it is clearly seen that urea causes a shift in the protein folding equilibrium by increasing the unfolding rate, while at the same time decreasing the folding rate. The effect of urea on the protein folding and unfolding kinetics is very large. The folding free energy in water is approximately −30 kJ/mol for CI2[15]. This means that the folding rate in water is 180,000 times faster than the unfolding rate. At high concentrations of urea, the ratio is commonly inverted, i.e. the unfolding is 180,000 times faster than the folding rate. This large perturbation on the global protein kinetics must, on a molecular basis, start with very local interactions that manifest themselves in a large effect on the global level. However, the actual perturbation of urea on the kinetics of local protein processes is not yet known.

The absence of information regarding how urea affects local protein kinetics might be due to the lack of relevant experimental techniques that can study the effect. However, one technique that has been used is amide hydrogen exchange NMR, see for example Refs. [10–15]. In such experiments, the protein is immersed in D_2O and the rate of exchange of the amide hydrogens with deuterium is followed by the decay of the corresponding ^1H–^{15}N HSQC peaks. Since the amide hydrogen must have solvent contact in order to be replaced by deuterium, the exchange of amide hydrogens that does not have solvent contact in the native structure can only occur by local fluctuations or by the global unfolding. The mechanism of exchange can be described by the following scheme:

$$NH_{\text{closed}} \underset{k_{\text{close}}}{\overset{k_{\text{open}}}{\rightleftharpoons}} NH_{\text{open}} \xrightarrow{k_{\text{int}}} ND$$

where the rate constants for the opening and closing of the amide to solvent contact are k_{open} and k_{close}, respectively. While the amide is in the open state, the rate of exchange is determined by the intrinsic rate constant k_{int}. The relative magnitudes of the rate constants are highly pH dependent and two limits have been identified [16]. In the most common EX2 limit, the intrinsic exchange rate, k_{int}, is limiting and the overall exchange rate k_{ex} can then be written as:

$$k_{\text{ex}} = \frac{k_{\text{open}}}{k_{\text{close}}} k_{\text{int}} = K_{\text{open}} k_{\text{int}}$$

* Corresponding author.
E-mail addresses: matteus.lindgren@chem.umu.se (M. Lindgren), per-olof.westlund@chem.umu.se (P.-O. Westlund).

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where K_{open} is the equilibrium constant of the conformational transition between the open and closed protein structures. Since the intrinsic rate of exchange k_{int} is slow, on the order of minutes [17], even fast local protein processes can be observed, if not K_{open} is large. The decay of the signal belonging to surface residues will often be fast since K_{open} is large for such processes. In contrast, the opening and closing transitions of the residues in the core of the protein will be governed by the global unfolding and K_{open} will therefore be small for a stable protein in water, giving slow exchange rates. It is noted in an amide hydrogen exchange study of CI2 [15] that the surface residues, as well as the residues at the edges of the β-strands, exchange too rapidly to be measured under the used experimental conditions. It was also found that mutations that lower the global stability of CI2, left the exchange rate of most residues in CI2 unaffected. This means that most residues exchange by local unfolding processes, despite that CI2 has been found to follow two-state unfolding dynamics [18]. It has been shown [19] that the change in the solvent accessible surface area (ΔSASA) of the protein that occurs during the global unfolding is proportional to the *m*-value of the protein, where $m = -\frac{\partial \Delta G^0}{\partial [U]}$. This proportionality was observed to apply for the process of global protein unfolding, not for local processes. Still, the proportionality is sometimes assumed to be applicable for local processes, such as the fluctuations of surface residues in hydrogen exchange studies. When guanidinium chloride (GdmCl) was added to CI2 solutions, *m*-values could be extracted for different hydrogen exchange processes [14]. The *m*-values were interpreted in terms of the ΔSASA of the exchange process, for the residues showing both fast and slow exchange rates. In other hydrogen exchange studies [11,13], it was assumed that the exchange rates of local fluctuations do not depend upon the urea concentration, due to the small ΔSASA of these processes. This assumption simplified the theoretical model that was used to interpret their data.

Urea-induced denaturation has been extensively studied by Molecular Dynamics (MD) simulations [20–34,40]. Many of these studies have characterized the global unfolding of proteins in urea solution, but to the best of our knowledge no MD study has yet focused on how urea affects the kinetics of local processes. This is surprising since MD simulations are well suited for such a study. However, Stumpe and Grubmüller noted in their simulations of the Cold Shock protein [40] that an apolar contact was ruptured several times in both water and urea solvent, but in water it reformed more quickly than in urea. They therefore proposed that urea stabilizes initial unfolding events rather than triggers them. This means that a urea solvent would stabilize local parts of the protein that already has unfolded by thermal motions rather than actively unfolding the protein.

In this paper, we study the effect of urea on the kinetics of local protein processes by using MD simulations. Three different types of local processes in CI2 were chosen to be analyzed: the rotation of the amine group in lysine, the dynamics of an intra-protein electrostatic bond and the dynamics of backbone hydrogen bonds. These three processes were chosen since they can all be defined as local but they differ with regard to scale, ΔSASA and solvent accessibility. They also occur on time scales suitable for study by MD simulations. The aim of the paper is to answer the following questions:

- What types of local protein processes can be kinetically affected by urea?
- The global unfolding rate of a protein in water increases when urea is added. The origin of the effect on the global rate must be the effect of urea on the kinetics of local processes. Is the increase in the global unfolding rate accomplished by an increase in the unfolding rates on the local level or is the main effect a decrease of the local refolding rates?
- Is the proportionality between ΔSASA and the *m*-value [19] also valid for local unfolding processes? If not, what other factors might influence the magnitude of the effect of urea on the free energy of a local protein process?

2. MD simulation protocol

Chymotrypsin inhibitor 2 (CI2) pdb structure 1ypc.pdb was simulated in explicit solvent (water or 10 M urea) by using Gromacs version 4.0.4 [35] and the G53a6 force field [36]. SPC/E [37] water was used together with the urea model from Smith et al. [38] to solvate CI2 in 6.6 nm rhombic dodecahedron boxes. Charge neutrality was maintained by adding two chloride ions. The water system contained 6367 water molecules. The 10 M urea system contained 3671 water molecules and 968 urea molecules. The time step was set at 2 fs. The systems were energy minimized by the steepest descent algorithm for 1000 steps, followed by 1 ns of equilibration of the system while applying positional restraints on the protein. The box volumes were adjusted at the start of the simulations by applying an external pressure of 1 atm using the Parrinello–Rahman algorithm with a time constant of 1 ps and a compressibility of 4.5E−5 bar^{-1}. After 1 ns the pressure control was turned off after a period of stable box volumes. Temperature control through the Nose–Hoover algorithm, with a time constant of 2 ps, was applied during the entire simulations. The resulting temperature was stable without large fluctuations. Van der Waals interactions were handled by a twin-range cutoff method with *rlist* and *rvdw* parameters set at 1.0 nm and 1.4 nm respectively. The neighbor lists were updated every 5 steps. Electrostatic interactions were handled by PME, with the parameters *rcoulomb* = 1.0, *fourierspacing* = 0.14, *pme_order* = 6. Water molecules were kept rigid using the SETTLE algorithm while bonds between hydrogen and heavier elements were kept fixed on urea and CI2. For some of the simulations, positional restraints were applied to the α-carbons. The force constants were given the Gromacs default value of 1000 kJ mol^{-1} nm^{-2}. The lengths of the room temperature simulations were ~150 ns. The lengths of the high-temperature simulations were 536 ns and 756 ns for W-350 and W2-350 respectively, 680 ns and 714 ns for U-350 and U2-350 respectively and 828 ns and 714 ns for W-325 and U-325 respectively. Coordinates for analysis were saved every ps. The analyses have been performed using Gromacs and VMD [39].

CI2 consists of a small 3_{10} helix followed by a 10 residue α-helix at the front and behind these, the two main beta strands that are connected by a region which contains the active site. When the α-helix is packed against the beta strands β1 and β2, a small hydrophobic core is created. A visualization of CI2 is displayed in Fig. 1.

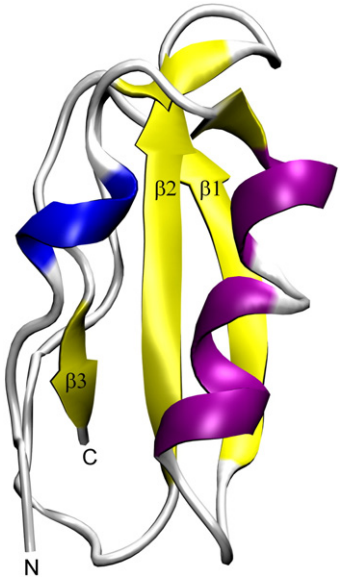

Fig. 1. Chymotrypsin inhibitor 2.

3. Results

3.1. Rotation of the amine group in lysine

We start by studying the effect of urea on a small scale structural transition. The amine group in lysine has three energy minima with respect to rotation around the C–N bond. The energy barriers between these minima are very low, causing transitions to occur on a picosecond timescale. We chose to study the dynamics of lysine since it is seen in the MD simulations that the majority of the lysine side chains in CI2 extend far out to the solvent. The movement of the amine group should therefore mainly be caused by interactions with the solvent rather than with the rest of the protein. The rotation of the amine group in lysine is also an example of a process that does not change the protein SASA. The transitions among the three energy minima can be probed by the dihedral angle of the atoms C_δ–C_ε–N–H, see Fig. 2.

Simulations of CI2 in water and in 10 M urea at a temperature of 298 K were performed. Harmonic positional restraints were applied to the α-carbons but the side chains were not restrained. This ensures that the proteins have the same overall structure, which facilitates comparison between the two simulations, but the side chains are left free to interact with the solvent. The dihedral angle of all 6 lysine residues in CI2 was calculated from both the CI2–water simulation as well as the CI2–10 M urea simulation. A distribution function of one lysine dihedral angle that was obtained from the CI2-water simulation is displayed in Fig. 3.

It can be noticed that the energy barriers create three clear distribution maxima at the applied temperature but that the barriers are low enough to give a relatively high minimum distribution density. The residence times in a certain minima, i.e. the times between transitions among the minima, were calculated and averaged over all three minima for all lysine residues. Distribution functions of the residence times could then be calculated and compared between CI2 in water and in urea for the purpose of analyzing the perturbation of urea on the dynamics of the transitions among the energy minima. The results are displayed in Fig. 4.

There is no clear difference between the distribution functions of the residence time with respect to if the solvent is water or 10 M urea. The average residence times are found to be the same for the two solvents. Not surprisingly, urea does not seem to affect a small scale process such as rotation of an amine group.

3.2. Dynamics of an intra-protein electrostatic bond

We continue with a study of the effect of urea on a larger scale transition, which require a higher degree of cooperativity among the protein atoms and results in a small change in the protein SASA. The same simulations as in Section 3.1 were used with positional restraints applied to the α-carbons in order to keep the same backbone structure in both solvents. The restraints also serve another purpose in this analysis. Protein structural transitions occurring on different sides of the protein cannot affect each other *via* the network of intra-protein bonds when the backbone is restrained. The restraints therefore ensure that the cause of the dynamics of the studied protein residues is local in origin. In addition, solvent-induced perturbations acting directly on the studied protein residues will increase in importance since intra-protein interactions are restricted. In the native state of CI2, the sulfur of methionine, residue 1, is weakly attached to the amine group of lysine, residue 24, see Fig. 5.

The bond, here designated S1–N24, is electrostatic in nature due to the charge of the sulfur: −0.482 and of the amine group: +0.873. At a temperature of 298 K, the bond is broken and reformed several times during the simulation time of 150 ns, both in water and in the urea solution. The bond is interesting to analyze since the rupture of this contact opens up CI2 for further solvation of the α-helix. Visualizations of the simulations show that this is the starting point of the global unfolding. In addition, both residues involved in the bond have much solvent contact and when the movement of the backbone is restrained, the rupture of the bond should be dominated by local protein–solvent contacts. The distance between the sulfur in residue 1 and the nitrogen in residue 24, $r_{S1-N24}(t)$, may therefore be indicative of local urea solvent–protein side chain interactions. The distance $r_{S1-N24}(t)$ was calculated from all frames in the simulations of CI2 in

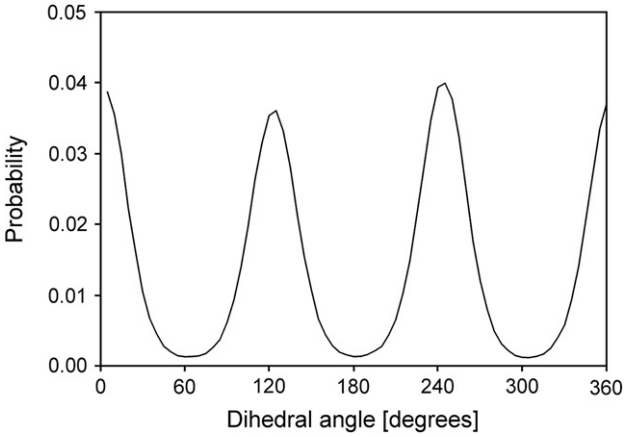

Fig. 3. Distribution function of a lysine dihedral angle during 10 ns.

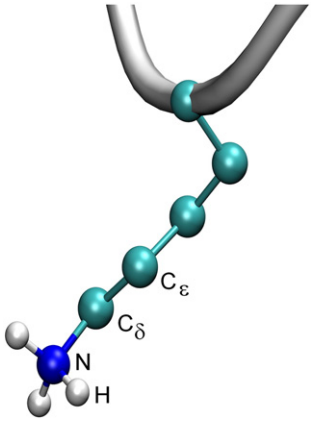

Fig. 2. A lysine residue with the labeled atoms included in the dihedral angle.

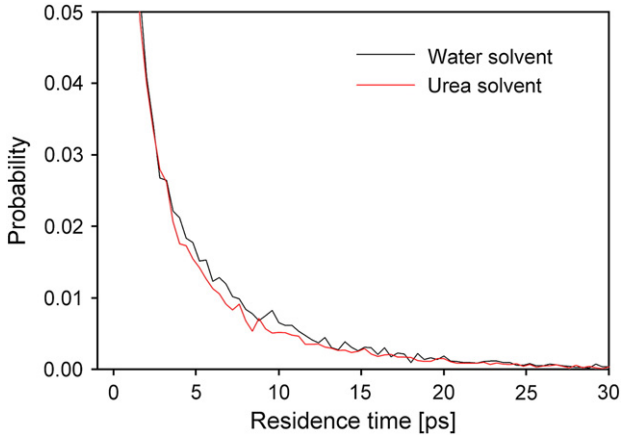

Fig. 4. Distribution function of the residence time in a certain lysine dihedral angle energy minima.

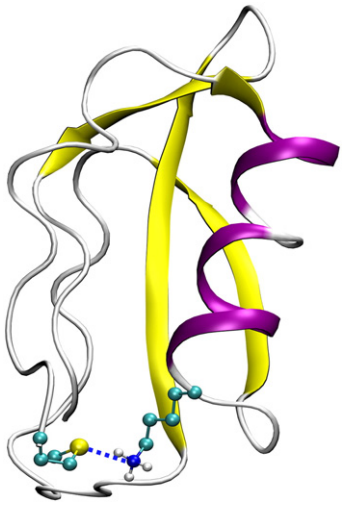

Fig. 5. The S1–N24 bond between residues 1 and 24.

both water and in 10 M urea. A running average of 250 ps was performed on the data in order to remove very fast fluctuations while keeping the distinct opening and closing of the structure. Based upon the distribution function of r_{S1-N24}, we decided that when r_{S1-N24} increased from<4 Å to>5 Å, the bond was defined as broken. When r_{S1-N24} decreased from>5 Å to<4 Å, the bond was defined as reformed. The distance $r_{S1-N24}(t)$, extracted from 50 ns of the simulations, is displayed in Fig. 6. In total, 150 ns of simulation time were analyzed for each solvent. The fluctuations of $r_{S1-N24}(t)$ can be compared between the protein–water simulation (upper part of Fig. 6) and the protein–10 M urea simulation (lower part of Fig. 6). The rupture and the reformation of the bond occur very fast in both solvents but the protein seems to stay in the two states for a longer time in the urea solvent. This was typical for the whole trajectory.

The mean life times that the protein spent in the open state (with the bond broken) and in the closed state (with the bond intact) were

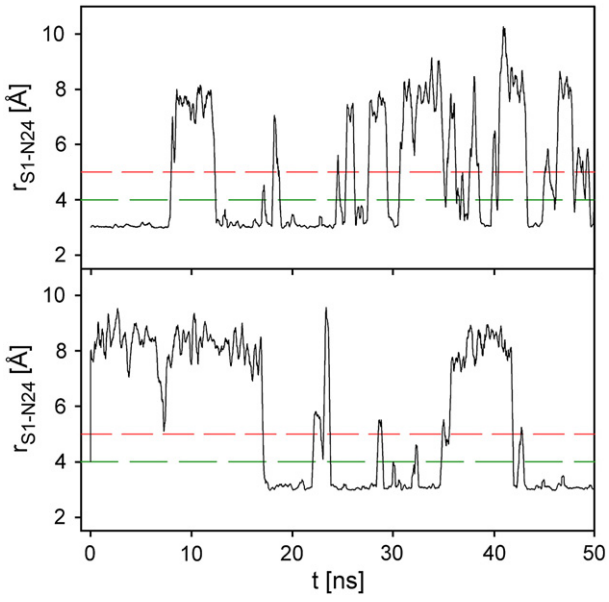

Fig. 6. The distance $r_{S1-N24}(t)$ during 50 ns. Upper part) CI2 in water. Lower part) CI2 in 10M urea. The green and the red lines mark the distances of 4 Å and 5 Å where the bond was defined as reformed or broken, respectively.

Table 1
Parameter values and their standard deviations for the S1–N24 bond.

Solvent	k_{open} [μs]$^{-1}$	k_{close} [μs]$^{-1}$	K_{open}	ΔG^0_{open}[kJ / mol]
Water	630 ± 90	530 ± 70	1.2 ± 0.2	-0.4 ± 0.5
10 M urea	220 ± 40	200 ± 80	1.1 ± 0.5	-0.3 ± 1.2

calculated for the two solvents. The rate constant for rupture of the bond, k_{open}, was defined as the inverse of the mean time spent in the closed state. Similarly, the rate constant for reformation of the bond, k_{close}, was defined as the inverse of the mean time spent in the open state. The change in Gibbs free energy of the bond rupture process (Closed state → Open state) was calculated from the rate constants as:

$$\Delta G^0_{open} = -RT \ln K_{open} = -RT \ln \frac{k_{open}}{k_{close}}$$

The results are displayed in Table 1.

The affect of urea on the kinetics of the S1–N24 bond is large as seen in Table 1. Both the unfolding rate and the refolding rate are reduced by a factor of 2–3 in urea as compared to in water. In contrast, the equilibrium constant and the change in Gibbs free energy of the process,ΔG^0_{open}, are unaffected by urea.

3.3. Backbone hydrogen bond dynamics

Six new simulations without positional restraints were performed for the study of backbone hydrogen bonds: 2 replicate simulations of CI2 in water at a temperature of 350 K named W-350 and W2-350, 2 replicate simulations of CI2 in 10 M urea at 350 K named U-350 and U2-350 as well as 2 simulations at the lower temperature of 325 K, one in water named W-325 and one in urea named U-325. The elevated temperatures were needed in order to increase the kinetics of the local processes we intend to study, so that proper sampling could be reached within the practical limit of 1 μs simulation length. The high temperatures will destabilize the protein as well, but the effect of urea could clearly be seen in these simulations. CI2 unfolded almost completely in the urea solution at a temperature of 350 K. The apparent key events of the protein unfolding seen in these simulations are displayed in Figs. 7. and 8. The unfolding was not as extensive in water at the same temperature, see the graphs of C_α-RMSD in Fig. 9. All simulations had the same protein start structure (1ypc.pdb) and the C_α-RMSD graphs were calculated by fitting the alpha-carbons to this start structure. In the simulation at 325 K, the protein stayed very close to the start structure in the water solvent. Minor protein structural change occurred in the urea solvent at 325 K, as seen in the graph of C_α-RMSD in Fig. 10.

The protein structures were similar between all six simulations up to 15 ns, at which time they start to diverge. Particularly the simulations in the urea solution show small scale unfolding events of the protein after this time period. It is interesting that an effect of urea on the protein structure can be seen already after 15 ns, even though the global unfolding takes place on a much longer time scale. What caused the protein structures to diverge after 15 ns? It is possible that the dynamics of the proteins in water and in urea differ for times t<15 ns and that this difference cause the proteins in urea to unfold after 15 ns. The atomic fluctuation amplitudes can be analyzed in terms of Root Mean Square Fluctuations (RMSF), calculated as:

$$RMSF(i) = \sqrt{\frac{1}{T}\sum_{t=1}^{T} ||\mathbf{r}_i(t) - \mathbf{r}_i(0)||^2}$$

where i is the atom under study, T is the simulation length and $\mathbf{r}_i$ is the atomic position vector. We calculated the RMSF of the individual backbone atoms during the first 15 ns of all simulations where all protein structures are similar to the start structure. The mean RMSF

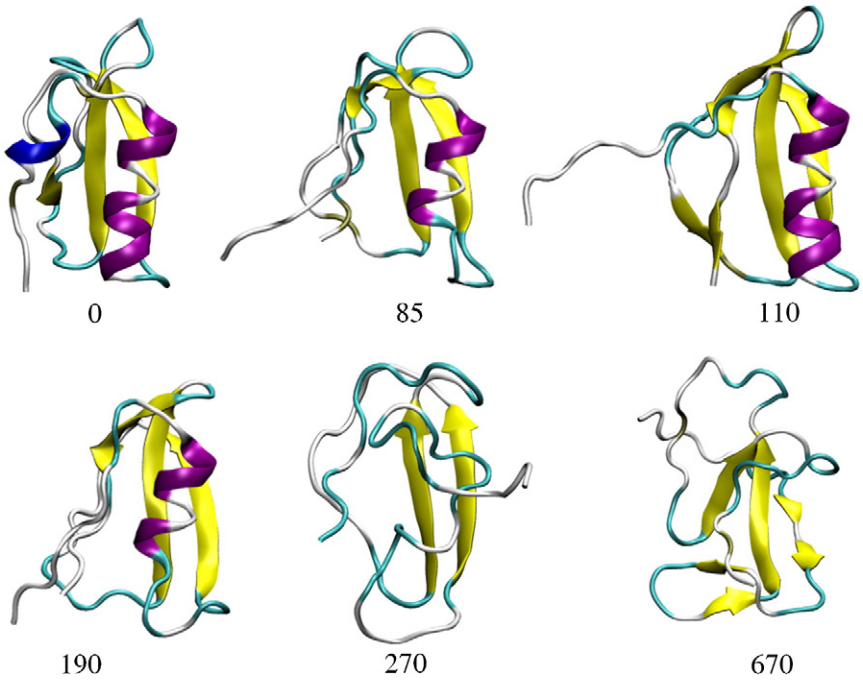

Fig. 7. Protein structures at certain times [ns] in the simulation *U*-350.

values of the whole backbone are displayed in Fig. 11. The results do not reveal any clear effect of urea on the backbone RMSF of the folded protein since no trend in the data is apparent. Apparently, the fluctuation amplitudes of the backbone atoms are not higher in 10 M urea than in water despite that CI2 has a much higher tendency to unfold in the urea solution.

We proceed by performing an analysis of the kinetics of the backbone hydrogen bonds. The hydrogen bond lengths ($r_{H-O}(t)$) in the α-helix, the β-sheet and the C- and N-terminal region were

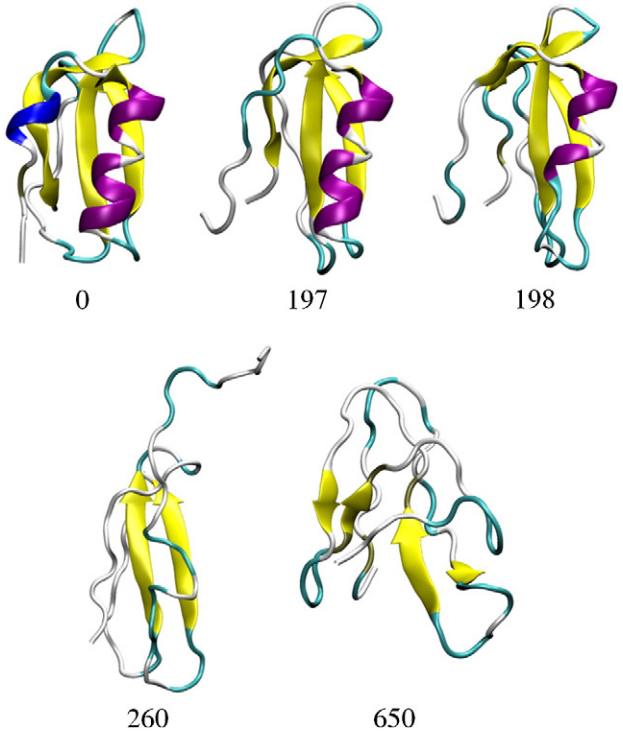

Fig. 8. Protein structures at certain times [ns] in the simulation *U*2-350.

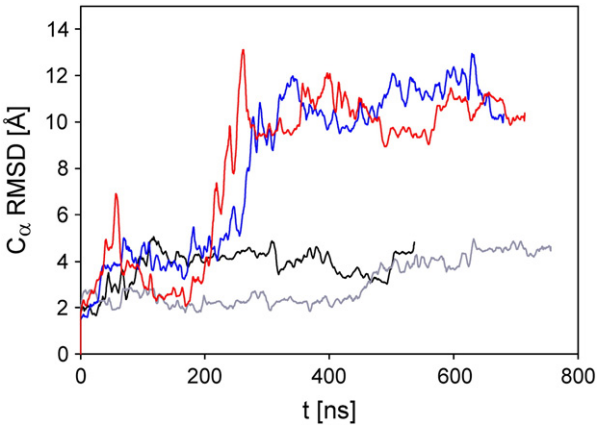

Fig. 9. C_α-RMSD of the simulations W-350 (black), W2-350 (gray), U-350 (blue) and U2-350 (red).

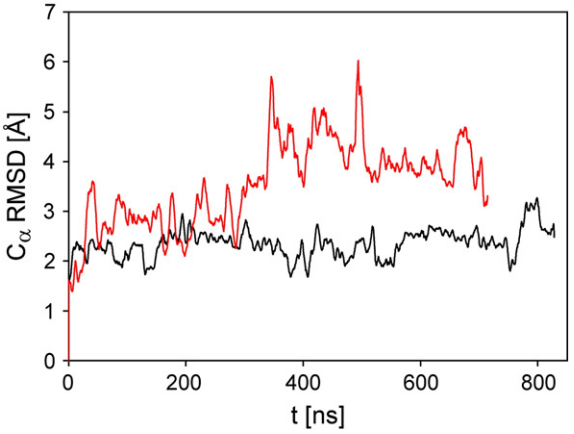

Fig. 10. C_α-RMSD of the simulations W-325 (black) and U-325 (red).

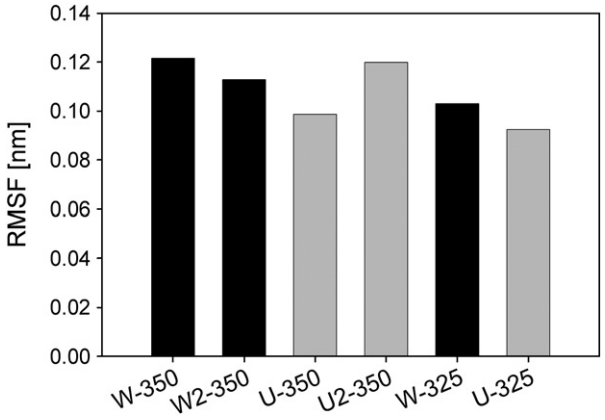

Fig. 11. Backbone RMSF of CI2 in the folded state obtained during the first 15 ns of the simulations.

Table 2
Parameter values and their standard deviations for the hydrogen bonds in the α-helix.

Solvent	Temperature	k_{open} [μs]$^{-1}$	k_{close} [μs]$^{-1}$	K_{open}	ΔG^0_{open} [kJ / mol]
Water	325	3.4 ± 1.0	440 ± 170	0.01 ± 0.004	13 ± 1
10 M urea	325	12 ± 2	180 ± 70	0.06 ± 0.03	7.4 ± 1.2
Water	350	13 ± 2	160 ± 50	0.09 ± 0.03	7.2 ± 1.0
10 M urea	350	29 ± 5	77 ± 30	0.38 ± 0.15	2.8 ± 1.2

Table 3
Parameter values and their standard deviations for the hydrogen bonds in the terminal region.

Solvent	Temperature	k_{open} [μs]$^{-1}$	k_{close} [μs]$^{-1}$	K_{open}	ΔG^0_{open} [kJ / mol]
Water	325	26 ± 4	200 ± 45	0.13 ± 0.04	5.5 ± 0.7
10 M urea	325	100 ± 14	600 ± 100	0.17 ± 0.04	4.9 ± 0.6
Water	350	81 ± 11	150 ± 40	0.55 ± 0.15	1.8 ± 0.8
10 M urea	350	220 ± 26	200 ± 50	1.1 ± 0.3	-0.3 ± 0.8

extracted from all simulations. Based upon the distribution functions of the distance r_{H-O}, the bond was defined to break when $r_{H-O}(t)$ increased from<3.5 Å to>4 Å. The bond was defined as reformed when $r_{H-O}(t)$ decreased from>4 Å to<3.5 Å. No angle restrictions were applied. A running average of 250 ps was used to remove very fast fluctuations from the data. Distinct bond rupture and reformation events could clearly be identified for the α-helix and for the C- and N-terminal region. However, the 2 major β-strands in CI2 were rather stable and the hydrogen bond rupture events of these structures were not very distinct so the data from the β-sheet was not included in the analysis. The hydrogen bonds in the α-helix and in the C- and N-terminal region that were analyzed are visualized in Fig. 12.

The number of times that the hydrogen bonds ruptured (opened) and reformed (closed) were counted and the times spent in either the open or the closed state in between these events were logged for each hydrogen bond. Rate constants for the rupture or the reformation of individual hydrogen bonds were calculated and the results were averaged over all hydrogen bonds in either the α-helix or the C–/N-terminal region. No consideration was taken to the correlation among different rupture events for hydrogen bonds that are near each other. The number of times the hydrogen bonds ruptured and reformed varied from 10 for the α-helix in simulation W-325 to 150 for the terminal region in simulation W-350. The resulting kinetic and thermodynamic parameters for the hydrogen bonds that are in the α-helix and the terminal region are summarized in Tables 2 and 3. The rate constants are also displayed in Figs. 13 and 14.

Considering part A of Figs. 13 and 14, it seems clear that the change of solvent from water to 10 M urea increases the rate constants of rupture of hydrogen bonds. However, the result is ambiguous for the rate constants that describe reformation of the bonds. In the α-helix (part B of Fig. 13) the rate of reformation decreases due to the presence of urea but in the terminal region (part B of Fig. 14) the rate of reformation increases in the presence of urea, at both temperatures. These changes in the rate constants cause the stability of the α-helix (as measured by ΔG^0_{open})to decrease in urea, see Table 2). The result for the stability of the terminal region is uncertain. The calculated values of ΔG^0_{open} decrease in the urea solvent at both temperatures but the decrease is not statistically significant on the 95% ($\pm 1.96\sigma$ St. dev.) level, see Table 3. However,

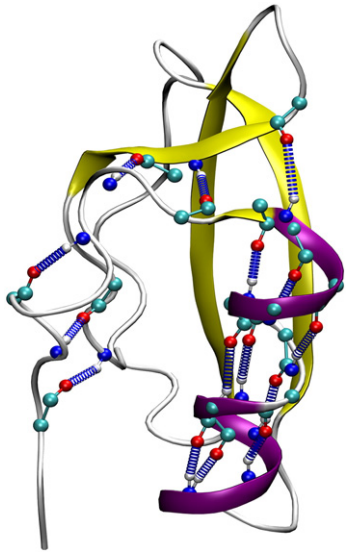

Fig. 12. The hydrogen bonds included in the analysis are shown as springs.

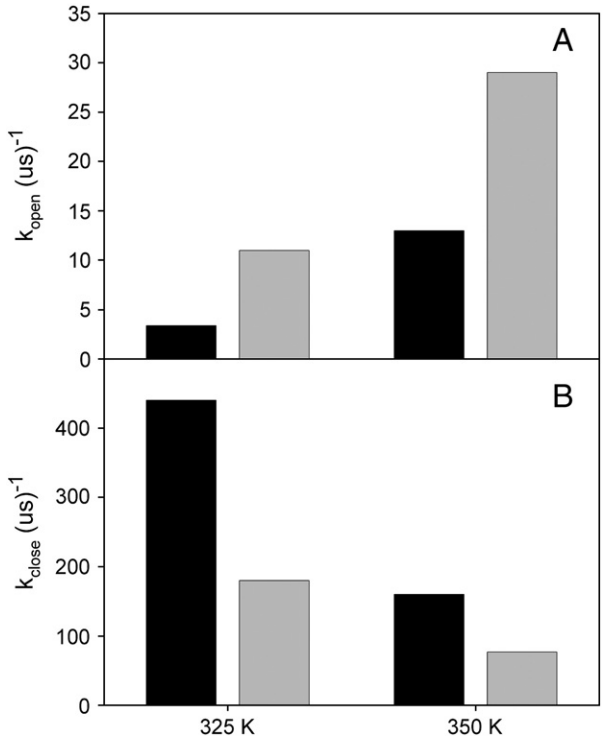

Fig. 13. A) Values of k_{open} for rupture of the hydrogen bonds in the α-helix in water (black) and in 10 M urea (gray). B) Values of k_{close} for reformation of the hydrogen bonds in the α-helix in water (black) and in 10 M urea (gray).

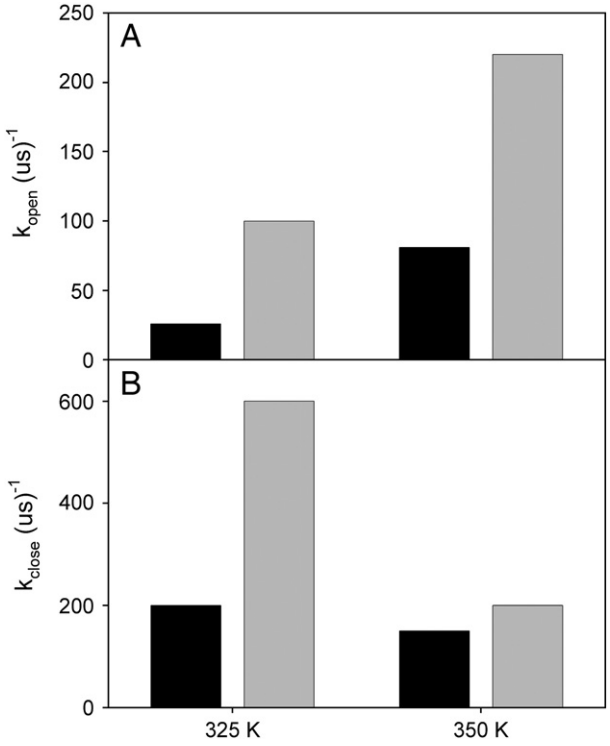

Fig. 14. A) Values of k_{open} for rupture of the hydrogen bonds in the terminal region in water (black) and in 10 M urea (gray). B) Values of k_{close} for reformation of the hydrogen bonds in the terminal region in water (black) and in 10 M urea (gray).

when combining the results of the α-helix with the results of the terminal region, we interpret the data as a decrease in ΔG^0_{open} in the urea solutions.

4. Discussion

The aim of the kinetic analysis of CI2 was to investigate the perturbation of urea on the kinetics of local processes in the protein. Three processes have been studied and analyzed: the rotation of the amine group in lysine, the dynamics of an intra-protein electrostatic bond and the dynamics of backbone hydrogen bonds. These were chosen in part because they represent different types of local processes in a protein. The results show that these three processes are also affected by urea in different ways. We will discuss the results in view of the questions mentioned in the Introduction.

- What types of local protein processes can be kinetically affected by urea?

A local process without any change in SASA is almost unaffected by urea. Local processes that are connected with a change in SASA can be affected in different ways depending on the type of process. The rotation of an amine group was not affected by urea and this process is not connected to a change in SASA. Both the rupture of the electrostatic bond S1–N24 and the rupture of backbone hydrogen bonds are connected with a change in SASA and the kinetics of these processes are affected by urea, but in different ways.

- The global unfolding rate of a protein in water increases when urea is added. The origin of the effect on the global rate must be the effect of urea on local processes. Is the increase in the global unfolding rate accomplished by an increase in the unfolding rates on the local level or is the main effect a decrease of the local refolding rates?

The results show that urea decreases the local refolding rates, instead of increasing the unfolding rates. However, since the global unfolding rate is increased by urea, we see that the effect of urea on the kinetics must depend upon the type and the scale of the unfolding process. Next we describe a model that can explain these observations. If the value of SASA is used as a reaction coordinate, unfolding can be defined as the direction of a structural transition that increases SASA. The (re)-folding is the opposite process, resulting in a decrease in SASA. The unfolding rate of the hydrogen bonded structures in the α-helix and the terminal regions increase in the urea solvent but the frequency of rupture of the S1–N24 bond decreases in urea. Both of these processes can be defined as small and local. In addition, many of the unfolding events of the hydrogen bonds occurred around residues 1 and 24, which is in the same region as the S1–N24 bond. How can urea affect the kinetics of these processes in different ways?

We propose that urea decreases both the unfolding and the refolding rates of isolated unfolding processes, i.e. structural transitions in the protein that occur (almost) independently of other protein structural transitions. An isolated process is likely to be of small scale and to occur on the surface of the protein. The high amount of solvent contact causes the structural transition to be triggered by solvent-induced forces rather than intra-protein forces. All other structural transitions that occur in the protein are not isolated. Instead, they depend upon the present states of the isolated processes. When an isolated process is in the unfolded state, for example when a bond between two side chains have ruptured, nearby residues will be destabilized and unfolding events may be triggered. The unfolding rates of such non-isolated processes may be increased in a urea solvent, in contrast to the unfolding rates of isolated processes. However, the refolding rates will be decreased, as for the isolated processes. Urea-induced unfolding can therefore be said to be hierarchical in nature, with two levels of processes. Urea then affects the kinetics differently at different levels.

The rupture of the S1–N24 bond is an isolated process for the following reasons. The S1–N24 bond is a contact between two side chains on the surface of the protein. The two residues have a high degree of solvent contact. This means that the rupture of the bond is likely to be induced by forces originating directly from the solvent. In addition, positional restraints were applied to the backbone in order to further isolate the bond from other processes in the protein. The probability of non-local forces affecting residues 1 and 24 *via* the network of intramolecular protein bonds will be greatly reduced by the restraints. The unfolding of the S1–N24 bond will therefore occur almost independently of other processes occurring in the protein.

The rupture of a hydrogen bond in our study of the α-helix and the terminal regions is not an isolated process. The studied hydrogen bonded atoms are located in the protein backbone without much solvent contact. Since these simulations were run without any applied restraints on the protein backbone, the hydrogen bonds will be affected by many nearby local protein processes, such as the rupture and the refolding of the S1–N24 bond. The forces responsible for the rupture of the hydrogen bonds will likely not be as local in origin as the forces affecting the S1–N24 bond. The forces will also be mediated *via* the network of protein bonds to a higher degree rather than be caused by direct interactions with the solvent. The rupture of one of the studied hydrogen bonds will therefore depend greatly upon the state of the other processes occurring in the protein.

The unfolding rates of the non-isolated processes may be increased by urea. This is likely due to the decreased refolding rate of the isolated processes since their current state affects the stability of the non-isolated processes. It can be calculated from Table 1 that the mean time that the S1–N24 bond stays in the unfolded, open position after each unfolding event is 2 ns and 5 ns, for CI2 in water and in 10 M urea, respectively. The decreased refolding rate of the S1–N24 bond in the urea solvent gives more time for other local processes to occur, such as solvation of the newly exposed protein surface area. This would further destabilize the protein region and could trigger other unfolding events, for example the unfolding of the hydrogen bonds at the lower end of the α-helix. The primary mechanism of urea

is therefore to decrease the refolding rates rather than to increase the unfolding rates.

- Is the proportionality between ΔSASA and the *m*-value[19] also valid for local unfolding processes? If not, what other factors might influence the magnitude of the effect of urea on the free energy of a local protein process?

A consequence of the model proposed above for the affect of urea on the kinetics of local processes is that the proportionality between ΔSASA and the *m*-value is not valid for local processes. Both the unfolding rate and the refolding rate of an isolated process are decreased by urea and the decrease in ΔG^0 is therefore small. The unfolding rate is increased for a non-isolated process while the refolding rate is decreased. The free energy change ΔG^0 is therefore decreased more for a non-isolated process than for an isolated process, even though ΔSASA may be the same. Both the processes of rupture of the S1–N24 bond and the rupture of the hydrogen bonds in the backbone are connected with a change in SASA, but urea only affects ΔG^0 of the hydrogen bond process. The *m*-value can differ for local processes with the same magnitude of ΔSASA since other factors, such as the amount of solvent contact versus protein contact as proposed in the mechanism above, can also affect the *m*-value. A direct interpretation of the *m*-value for a local process in terms of ΔSASA, as sometimes seen in hydrogen exchange studies, is therefore not possible.

Studies concerning the affect of urea on the kinetics of local processes in proteins are very scarce. The MD simulation study by Stumpe and Grubmüller [40] has a similar objective as this paper, but with a focus directly on the global unfolding. Partially unfolded structures of the Cold Shock protein are simulated in water and in a urea solution and the evolution in time of the protein SASA is analyzed. The results show that urea impedes the reduction of SASA that occur for the same protein structure in water. The authors conclude that urea stabilize partially unfolded protein structures, which is in accord with our results. They argue that this occurs as a result of an ability of urea to impede the hydrophobic collapse of expanded proteins. However, in our opinion this is not thoroughly investigated in the article. It may be that urea impedes a collapse of partially unfolded proteins regardless of whether this collapse would consist of the formation of polar or apolar protein contacts. Since the paper focuses on the affect of urea on the global unfolding/refolding, the affect of urea on the kinetics of local processes in the protein is not thoroughly studied. However, it is noted that a local contact between a trypthophan and a serine residue ruptures several times in both water and in urea solution, but the refolding is impeded by urea. Similarly to this study, Stumpe and Grubmüller therefore concluded that urea stabilizes protein structures that have unfolded by thermal fluctuations, rather than actively unfolding the protein.

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