Specificity of Urea Binding to Proteins

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Abstract: The binding of urea to the small globular proteins bovine pancreatic trypsin inhibitor (BPTI) and PEC-60 was investigated by nuclear magnetic resonance (NMR) spectroscopy. The conformation of the proteins was unaffected in the temperature range 4–36 °C and urea concentrations up to 8 M. Intermolecular nuclear Overhauser effects (NOE) observed between the proteins and urea at 4 °C showed preferential binding of urea to pockets and grooves on the protein surfaces. These NOEs disappear at higher temperatures indicating kinetic lability of the urea binding on a nanosecond time scale. The binding constants were measured by following the amide proton chemical shifts as a function of urea concentration. The average values of the binding constants in the reaction protein + urea ⇌ protein·urea were between about 0.1 and 0.3 with a trend to lower values at higher temperatures. Some of the binding sites identified by intermolecular NOEs with urea showed binding constants above the average but never above 1. The data indicate that aqueous urea solutions present a rather uniform environment to proteins, where preferential binding of urea to defined conformational sites occurs, but the interactions are weak and short lived.

Although urea is widely used to denature proteins in studies of protein folding–unfolding equilibria,[1] the mechanism of urea-driven denaturation is not clear. According to a common picture, urea stabilizes both the native and the denatured protein conformation, but the larger number of binding sites exposed in the unfolded state favors denaturation.[2] While negative binding enthalpies observed for urea–protein interactions suggest hydrogen bonding of urea to exposed polar groups of the protein,[3] very little structural information is available on the urea–polypeptide interaction.[4,5] Clearly, the value of urea as a reagent to simulate the nascent, unfolded state of a protein critically depends on the absence of specific urea–protein interactions which could bias the unfolded state toward conformations particularly favorable for urea binding. Similarly, the pathway of urea-induced protein denaturation might be influenced by specific binding of urea to the native protein conformation.

Structural data on complexes of urea with proteins are scarce. A recent crystallographic study performed in the presence of increasing concentrations of urea characterized in detail nine different urea binding sites on the surface of native hen egg white lysozyme.[5] There, urea is bound via hydrogen bonds to polar groups of the polypeptide chain, while the structure of the protein itself was virtually unchanged. The structural integrity of the protein observed in the single crystal is corroborated in aqueous solution by nuclear magnetic resonance (NMR)[6] data which revealed only small chemical shift changes upon addition of urea.[7] However, most of the urea molecules observed in the single crystal do not seem to be present in aqueous solution as judged by the absence of any significant chemical shift changes of the protein protons at these sites. Clearly, the crystal lattice provides more specific urea binding sites than there are in solution, which is also suggested by the fact that most of the urea molecules observed in the crystal bridge between different lysozyme molecules.[5]

The present work aimed at the detection of specific urea binding sites on two small globular proteins, the basic protein bovine pancreatic trypsin inhibitor (BPTI) and the acidic protein PEC-60, in aqueous solution by NMR. The urea binding sites were detected by the observation of nuclear Overhauser effects (NOE) between protons of the proteins and urea. Binding constants were measured for individual urea binding sites and the results interpreted in terms of the surface structure of the proteins.

Materials and Methods

The line shape of the urea signal is most strongly affected by the acid–base catalyzed chemical exchange between the protons of urea and the water protons. A series of two-dimensional chemical exchange experiments recorded at 36 °C with different pH showed the slowest urea–water proton exchange rates at about pH 7.5 (pH measured at room temperature) with an exchange rate constant of about 2 s^{-1} at pH 7.0. The minimum exchange rate at 25 °C has been reported to be near pH 7.3.[8]

All spectra were recorded on a Bruker AMX-2 600 NMR spectrometer. ROESY[9] and NOESY[10] spectra were recorded with a 20 mM solution of BPTI in 90% H_2O/10% ^2H_2O at pH 6.9 in the presence of 0, 1.0, 2.7, 4.9, and 7.8 M urea at 4, 10, 20, and 36 °C. The intense signals from water and urea were suppressed by the combination of a τ–SL sequence after the mixing time, where SL denotes a spin lock purge pulse of 2 ms duration,[11] and presaturation. For the detection of urea–protein NOEs, the carrier frequency was set at the frequency of the urea signal and the water resonance was suppressed by presaturation. Experiments for the

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(6) Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; PEC-60, peptide with amino-terminal glutamic acid, carboxy-terminal cysteine, and a total of 60 residues; NMR, nuclear magnetic resonance; 2D, two-dimensional; 3D, three-dimensional; DQF-COSY, double-quantum-filtered two-dimensional correlated spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy in the laboratory frame; ROESY, two-dimensional NOE spectroscopy in the rotating frame; TOCSY, two-dimensional total correlation spectroscopy.

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observation of the water–protein NOEs in the presence of urea were conducted by using the null of the excitation profile produced by the spin–lock purge pulse for water suppression while the urea signal was suppressed by presaturation.

A three-dimensional NOESY-TOCSY experiment was recorded at 4 °C and 7.8 M urea concentration to support the assignments of intermolecular NOEs between urea and BPTI. The pulse sequence used was $90°(\phi_1)$–t_1–$90°(\phi_2)$–τ_m(NOESY)–$90°(\phi_3)$–t_2–τ_m(TOCSY)–SL–(ϕ_4)–τ–SL(ϕ_5)–acquisition (t_3) with the phase cycle $\phi_1 = 2(x,-x)$, $\phi_2 = 4(x)$, $\phi_3 = 2(x)$, $2(-x)$, $\phi_4 = 4(y)$, $\phi_5 = 4(x)$ and receiver = x, $-x$, $-x$, x. In addition, all phases were incremented by 90° in the way of 2-step CYCLOPS,[12] yielding an 8-step phase cycle. Two homospoil pulses were applied in 18 ms intervals during the NOESY mixing period. The MLEV-17 sequence was used during the TOCSY mixing time τ_m(TOCSY).[13] Further experimental parameters were as follows: t_{1max} = 7 ms, t_{2max} = 11 ms, t_{3max} = 72 ms, τ_m(NOESY) = 40 ms, τ_m(TOCSY) = 24 ms, SL(ϕ_4) = 500 μs, τ = 100 μs, SL(ϕ_5) = 2 ms, total recording time about 48 h. In contrast to previous 3D NOESY-TOCSY experiments for the detection of water–protein NOEs,[11] the SL–τ–SL element was inserted after the TOCSY rather than after the NOESY mixing period with the carrier frequency set to the urea frequency, and the water resonance was suppressed by presaturation. The excitation profile of the resulting spectrum is uniform in the F_1 and F_2 dimensions and described by $\sin[0.38(\delta_3 - 5.95)]$ in the F_3 dimension, where δ_3 is the chemical shift in ppm along the F_3 frequency axis. This excitation profile is advantageous for the observation of urea–protein NOEs with resonances close to the water and urea resonances.

PEC-60 was studied in an approximately 10 mM solution in 90% H_2O/10% ^2H_2O at pH 7.0 in the presence of 0, 1, 2.8, 5.3, and 8 M urea at 4, 10, 20, and 36 °C. NOEs between urea and PEC-60 were measured from two-dimensional NOESY and ROESY spectra recorded in the same way as with BPTI.

The sequence-specific resonance assignments for the different conditions and the precise chemical shift values were established with use of the program EASY.[14] The error in the chemical shifts was minimized by referencing to internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$. The estimated uncertainty of the chemical shift values was ±0.003 ppm.

Binding constants of urea to BPTI and PEC-60 were measured by monitoring the chemical shifts of the protein resonances as a function of urea concentration. In the fast exchange regime, the chemical shift δ of a proton resonance is observed at an average value given by

$$\delta = ([p]\delta_f + [pu]\delta_c)/([p] + [pu]) \quad (1)$$

where [p] and [pu] denote the concentration of free and urea-complexed protein, respectively, expressed in moles per liter. δ_f and δ_c denote the chemical shifts of the free and complexed protein. Defining the binding constant K as

$$K = [pu]/([p][u]) \quad (2)$$

where [u] is the concentration of unbound urea in moles per liter, eq 1 can be rewritten as

$$\delta = (\delta_f + K[u]\delta_c)/(1 + K[u]) \quad (3)$$

For small binding constants, [u] is to a good approximation given by the total urea concentration, and the binding constant can be obtained from a fit of eq 3 to the chemical shift values observed at different urea concentrations.

Results

Structural Integrity in the Presence of Urea. BPTI and PEC-60, which structurally belongs to the Kazal-type family of trypsin inhibitors,[15] contain three disulfide bridges each and are not denatured by urea under the conditions used. The conservation of even conformational details is corroborated by unaltered NOE patterns observed in the NOESY spectra and by the fact that only small chemical shift changes were induced by urea. In particular, the chemical shifts of protons experiencing strong ring current effects are virtually unaltered. The possible influence of urea on side chain conformations was studied in special detail in BPTI: a DQF-COSY spectrum[16] recorded in the presence of 7.8 M urea resulted in qualitatively unaffected multiplet fine-structures of the αH-βH cross-peaks indicating conserved χ^1 angles. Furthermore, the line shapes of the aromatic protons observed in NOESY were virtually unchanged indicating preserved ring rotation rates, and intermolecular NOEs observed between water and BPTI[17] indicated the unaltered presence of the four hydration water molecules in the interior of the protein.

Another example for the structural integrity maintained in the presence of urea is represented by the 19 amino terminal residues of PEC-60, of which only the peptide segment with residues 7 to 11 is structurally stable in the native conformation via close contacts to the structurally well defined part of the protein, while relaxation measurements indicated increased flexibility on the nanosecond time scale for the other amino acid residues.[15] Although this segment might be expected to be most readily "denatured" by the presence of urea, there was no evidence for altered NOE patterns, which would indicate a change in conformation for residues 7 to 11 or a release of this segment from the structured part of the protein in the presence of 7.8 M urea.

Intermolecular NOEs between Urea and Protein Protons. The use of near neutral pH values was a prerequisite for the observation of the intermolecular urea–protein NOEs to avoid the urea signal being significantly broadened by fast proton exchange with the water. In our experiments, the urea line shape was virtually unaffected by the proton exchange in the temperature range between 4 and 36 °C, and the proton exchange rate between urea and the water was sufficiently slow to exclude the appearance of any exchange relayed NOEs that might otherwise arise from a pathway, where the transfer of magnetization from urea to the protein is mediated via rapidly exchanging protein protons or bound water molecules.

The intermolecular urea–protein NOEs were most intense at high urea concentration and low temperatures. Their assignment in BPTI was supported by a homonuclear 3D NOESY-TOCSY experiment recorded at 4 °C and 7.8 M urea concentration. Figure 1 shows part of the F_2–F_3 plane taken at the F_1 frequency of the urea signal. Diagonal peaks in this cross section are generated by the transfer of magnetization from urea to the resonances of the protein during the NOESY mixing time.[11] The off-diagonal peaks arise from the further transfer of magnetization during the TOCSY mixing time to scalar coupled protons. They enable the identification of urea–protein NOEs in those cases, where the assignment from the diagonal peaks alone would be ambiguous. In some examples, the TOCSY mixing step led to stronger off-diagonal than diagonal peaks.

The NOEs with Thr32 Hγ, Ser47 Hβ, Glu49 Hβ, and Asp50 Hβ arise from interactions with the hydroxyl protons of Ser47 and Thr32, which have similar chemical shifts as urea.[18] All other peaks identified in Figure 1 are with urea. In addition, NOEs with urea were identified for the amide protons of Glu7 and Ala25, the side chain amide protons of Asn43, and the Hδ of Tyr10. A comparison with the three-dimensional structure of BPTI[19] suggests four different binding sites of urea defined by the following groups of NOEs: (i) Leu6αH, Pro8αH and βH,

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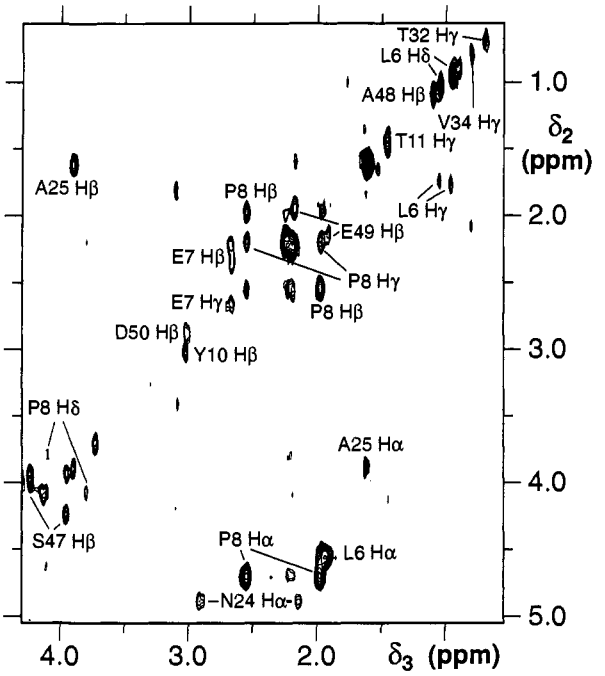

Figure 1. Spectral region of the F_2–F_3 plane taken at the urea chemical shift through a homonuclear 3D NOESY-TOCSY spectrum recorded with a 20 mM solution of BPTI at 4 °C, pH 6.9, in the presence of 7.8 M urea. Urea–protein NOEs appear on the diagonal. Diagonal peaks and off-diagonal peaks are identified by the assignment of the chemical shift in the F_2 dimension which corresponds to the protons involved in NOEs with urea or other resonances at the urea chemical shift.

Asn24αH, Ala25NH and βH, and Asn43δNH$_2$; (ii) Glu7βH, Pro8γH, and Tyr10βH and δH; (iii) Thr11γH and Val34γH of the γ_1CH$_3$ group; and (iv) Leu6γH and δH. The additional urea–protein cross-peaks with Ala25αH and Glu7γH (Figure 1) were absent or of vanishing intensity in two-dimensional ROESY spectra recorded with mixing times between 10 and 25 ms, suggesting significant contributions from spin-diffusion for the corresponding NOESY cross-peaks.

By far the strongest NOEs with urea were observed with the Ala25NH and βH resonances. Inspection of the surface of BPTI in a space-filling representation (see *e.g.* Figure 5 in ref 20) shows a deep cavity at that site lined by Leu6, Pro8, Asn24, and Ala25 with the δNH$_2$ group of Asn43 at the bottom. This site represents the deepest surface cavity in BPTI. The protons of the urea binding sites ii, iii, and iv are also lining grooves on the protein surface, albeit of increasing shallowness.

Only about ten urea–protein NOEs could be observed in the NOESY and ROESY spectra recorded with PEC-60 at low temperatures and high urea concentrations. Their assignment was impeded by the fact that the proton resonances of PEC-60 are broad at low temperatures and the NOEs with urea are weak, which abolished the possibility of using a 3D NOESY-TOCSY spectrum for their assignment. However, the second most intense NOE could be unambiguously assigned to the γCH$_3$ group of Ile23, and the strongest cross-peak has only two assignment possibilities, the δCH$_3$ group of Ile23 or the δCH$_3$ group of Ile46. In the structure of PEC-60 there is a deep pocket formed between the side-chain of Ile23 and residues around Cys39 which belong to the core of the protein.[15] In analogy to the observations made with BPTI, it seems plausible that urea would bind to that cavity.

Urea Binding Constants. Urea might be expected to bind preferentially to polar groups, in particular to the backbone amide and carbonyl groups.[21,22] Measuring the ^{1}H chemical shifts in the presence of 0, 1.0, 2.7, 4.9, and 7.8 M urea in the case of BPTI and 0, 1.0, 2.9, 5.3, and 8.0 M urea in the case of PEC-60 showed indeed the largest titration shifts for the amide protons. However, the titration shifts observed between the lowest and highest urea concentration were quite small, with an average value of about 0.075 ppm at 4 °C and all individual values below 0.5 ppm. Both the average value and the maximum titration shifts decreased toward 36 °C. The titration shifts observed for the amide protons in BPTI were in qualitative agreement with the previously published titration shifts between 0 and 8 M urea at 30 °C and pH 3.5.[23] However, the titration shifts of the α-protons in BPTI were always below 0.2 ppm in the present study, whereas larger chemical shift differences have been reported at pH 3.5.[23] The cause for these discrepancies may be the different pH and protein concentration used.

The urea binding constants with BPTI and PEC-60 were determined by fitting eq 3 to the amide proton chemical shifts observed as a function of total urea concentration. The evaluation of the binding constant was attempted for all residues, where the titration shift between the lowest and the highest urea concentration was at least 0.04 ppm which applied to about half the amide protons. The binding constants were determined at 4, 10, 20, and 36 °C. Accepting only binding constants, for which the standard deviations derived from the fitting was smaller than the binding constant itself (with two exceptions, all these constants were smaller than 0.03), the average binding constants were 0.20 at 36 °C and 0.32 at 4 °C in BPTI and 0.08 at 36 °C and 0.11 at 4 °C in PEC-60. The trend to higher binding affinities at 4 °C is also observed if only those amide protons are considered for which the binding constants could be measured with reasonable accuracy at all four different temperatures. Because of the overall small chemical shift changes, the accuracy of the binding constants was insufficient to determine binding enthalpies and entropies for individual binding sites. However, the general trend of the temperature dependence both in the size of the titration shifts and the binding constants deduced from them indicates reduced binding constants at higher temperatures corresponding to negative binding entropies.

All measured binding constants were smaller than 1. The relatively small protein concentration and the small binding constants justify the use of the total rather than the free urea concentration in the fitting with eq 3.

Ala25 NH in BPTI shows one of the largest titration shifts at 4 °C and the largest binding constant (0.75 ± 0.06). Unfortunately, the titration shifts of this amide proton decreased with increasing temperature almost to zero, so that no binding enthalpies or entropies could be calculated from these data (Figure 2). The βCH$_3$ group of Ala25, which is also involved in a strong NOE with urea, did not show any significant titration shift at any temperature. However, two protons involved in NOEs with urea at the same site, Asn24αH and one of the side chain amide protons of Asn32, reflected binding constants of respectively 0.52 and 0.57 at 4 °C and 0.36 and 0.22 at 36 °C. Significantly smaller binding affinities were evidenced by the titration shifts of the amide protons near the other three sites characterized by NOEs with urea.

Because proton chemical shifts may reflect changes in chemical environment occurring relatively far from the proton site, it is difficult to estimate the number of urea binding sites from the binding constants alone. In particular, titration shifts were observed both to high-field and low-field values, which may lead to cancellation effects for protons with titration shifts from two different urea binding sites. However, at least two additional sites monitoring relatively large binding constants seem to be sufficiently far from the binding site near Ala25 NH to be independent binding sites for urea, although they are not documented by intermolecular NOEs with urea. These are

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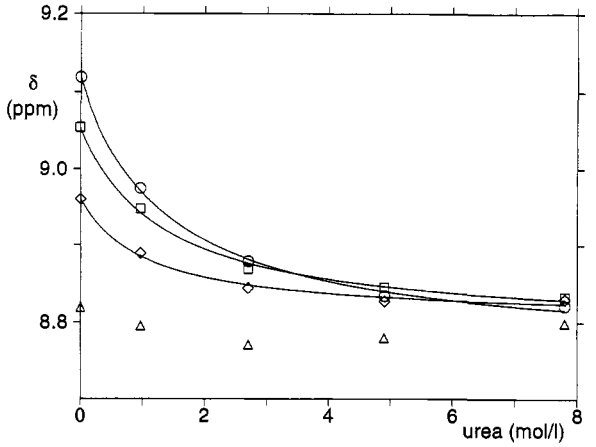

Figure 2. Chemical shifts of the amide proton of Ala25 in BPTI as a function of urea concentration. The data points correspond to 4 °C (circles), 10 °C (squares), 20 °C (diamonds), and 36 °C (triangles). The curves represent the best fits of eq 2 to the data which yielded the binding constants to urea. No fitting was attempted for the data at 36 °C.

reflected by the titration shifts of the amide protons of Arg42 and Gln31. The binding constant indicated by Arg42 NH is between 0.6 and 0.8 at all temperatures investigated, whereas the binding constant evidenced by Gln31 NH decays from about 0.5 at 4 °C to less than 0.1 at 36 °C.

The largest binding affinity measured in the structured part of PEC-60 was about 0.2 pertaining to Cys39 NH. This observation confirms the preferential binding of urea to the pocket formed between the side chain of Ile23 and the core of the protein. The titration shifts of the protons of Ile23 itself were too small to obtain a reliable value of the binding constant.

Binding constants between about 0.01 and 0.1 were observed for residues 3 to 5 in the unstructured, mobile amino-terminal pentapeptide segment of PEC-60, which can be thought of as being representative of a non-native, random coil conformation. The largest titration shifts in PEC-60 were observed for the flexible loop between residues 12 and 23. The largest binding constant at 4 °C was 0.5 measured for His13 NH, and the largest binding constant at 36 °C was about 0.3 which was measured for Thr15 NH. However, it is not possible to identify the binding sites reflected by these binding constants, because this loop is characterized by increased mobility on the nanosecond time scale and structural disorder already in the native protein structure.[15] Furthermore, the chemical shift of the side chain of His13 titrated by about 0.4 ppm between 1 and 8 M urea concentration which suggests a urea-dependent change of its pK value. A change in charge of this side chain would influence the small titration shifts of the nearby amide protons.

Discussion

The present study shows that urea binding sites at native protein structures can be detected by intermolecular urea–protein NOEs. At a first glance, the situation is similar to the observation of intermolecular water–protein NOEs:[17] only a single urea signal is observed because of rapid chemical exchange between the urea molecules in the different chemical environments, and there are only a few distinct binding sites giving rise to positive intermolecular NOESY cross-peaks. In the NOE experiments with water, the sign of the water–protein NOEs is inverted for increasing diffusion rates of the hydration water molecules.[20,24] The fact that no sign inversion was detected for the urea–protein NOEs in the present experiments may be attributed to the insufficient signal-to-noise ratio observed for the intermolecular NOE cross-peaks, which is also limiting in the protein hydration studies.[20]

In contrast to NMR studies of protein hydration where positive NOESY cross-peaks are observed only for a few water molecules which play a structural role in the protein conformation,[17] the observation of intermolecular NOEs with urea appears to correlate mainly with the presence of pockets and crevices on the protein surface, since no intermolecular NOEs were observed with more exposed protons even when their titration shifts reflected urea binding affinities above average. Furthermore, the intermolecular NOEs with urea vanish almost completely at 36 °C in both NOESY and ROESY, while the average binding affinity for urea drops only a little. These observations are most readily explained by residence times of the urea molecules at their binding sites, which are of the order of the rotational correlation times of BPTI and PEC-60 or shorter. Residence times shorter than a few nanoseconds would significantly decrease the intermolecular urea–protein NOE intensities relative to the intra-protein NOEs.[20] Because of the apparent sensitivity of the urea–protein NOEs with respect to the lifetime of the urea–protein interaction, the NOEs observed at low temperature primarily indicate somewhat extended residence times of individual urea molecules in the grooves of the proteins. Consequently, not all the sites identified by urea–protein NOEs displayed an increased urea binding affinity as measured by the amide proton titration shifts at these sites. Only the urea binding sites with the most intense intermolecular NOEs in BPTI and PEC-60 were also characterized by increased binding constants.

The temperature sensitivity of the intermolecular urea–protein NOEs is in line with the observation of small urea binding constants, with all values below 1. The binding affinities determined for BPTI and PEC-60 are in good agreement with previous results on lysozyme, for which average urea binding constants of 1 and 0.17 were estimated from NMR[7] and circular dichroism studies,[25] respectively, and peptide models like acetyl-tetraglycyl-methyl-ester with binding constants in the range of 0.2 to 0.5,[21] and diketopiperazine with a binding constant of about 0.04.[26]

In the bimolecular binding equilibrium

$$\text{protein} + \text{urea} \rightleftharpoons \text{protein·urea} \qquad (4)$$

the occupancy of the binding site is above 50% for urea concentrations larger than the inverse of the binding constant K. For a site with a binding constant of 1, the occupancy would still be less than 90% at 8 M urea concentration. Incomplete complex formation even at the best binding sites is also experimentally documented by the continuous increase of the intensities of the intermolecular urea–protein NOEs between 1 and 8 M urea concentration. Considering that a single binding site is usually reflected by chemical shift changes of several amide protons, there seem to be only a few sites that are preferentially occupied by urea rather than water at 8 M urea concentration.

In accord with the previous studies on lysozyme,[5,7] urea hardly affects the native conformation of either BPTI or PEC-60, as long as their polypeptide backbone fold is intact. Most notably, urea does not even influence the side chain conformations in the native protein structures in a significant way, which is most clearly evidenced by the small chemical shift changes induced by urea. For comparison, a local conformational change involving the χ^1 angle of Cys38 in BPTI causes larger chemical shift changes than the presence of 8 M urea.[27] The conformational stability of the native state in the presence of urea is remarkable, since even a protein as stable as BPTI is not entirely inert with respect to denaturation. For example, the unfolding rates of BPTI are increased by urea as indicated by significantly enhanced exchange

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rates observed for the most slowly exchanging amide protons which are buried in the interior of the protein.[23] Furthermore, BPTI has been found to be in equilibrium with denatured forms in the absence of urea if some of its disulfide bridges are broken.[28] It seems likely that the resistance of PEC-60 with respect to denaturation also depends on the presence of the three disulfide bridges.

In conclusion, the binding interaction of urea with the native proteins BPTI and PEC-60 is overall weak and short lived on a nanosecond time scale. The short lifetime of the interaction suggests that the presence of urea presents no significant kinetic barrier in folding–unfolding reactions of proteins. The structures of the proteins appear to be completely preserved in the presence of urea, including conformational details of the amino acid side chains. Although there are preferential binding sites in grooves and pockets on the protein surfaces, these are never filled more than 90% of the time even in the presence of 8 M urea, which limits the specificity of the urea–protein interaction. No example was observed, where an intra-protein interaction was replaced by an interaction with urea in the folded structures. Although protein unfolding events are much rarer than the formation and breaking of the protein–urea complexes, it appears unlikely in light of the present results that urea would initiate unfolding by actively and specifically disrupting the native protein conformation. Rather, the main role of urea could be the stabilization of spontaneously formed, unfolded protein conformations.

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Supplementary Material Available: A listing of the titration shifts and binding constants with standard deviations (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.