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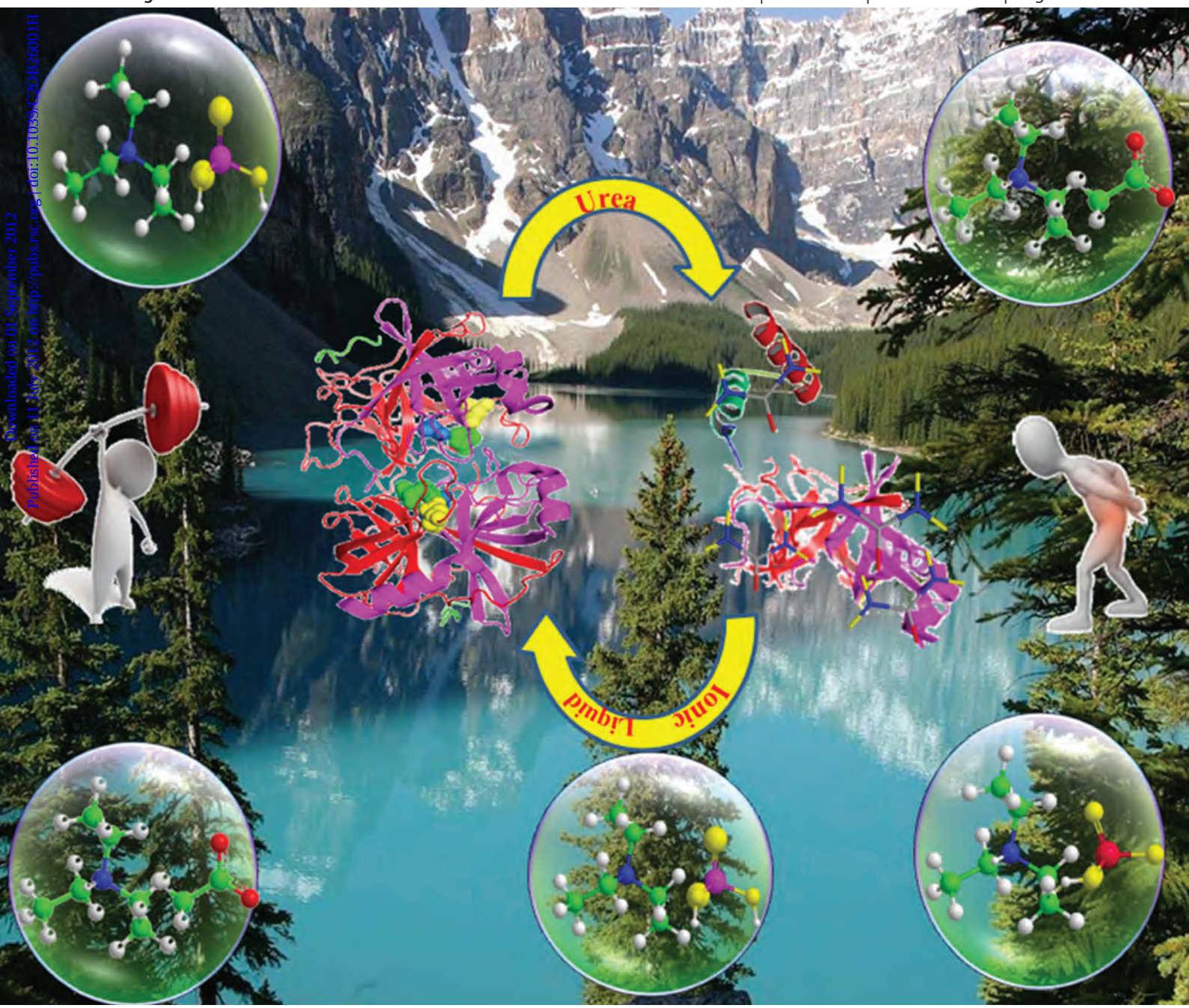

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COMMUNICATION

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COMMUNICATION

Water and a protic ionic liquid acted as refolding additives for chemically denatured enzymes†

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In this communication, we present the ability of water and a protic ionic liquid, triethyl ammonium phosphate (TEAP) to act as refolding additives for the urea-induced chemical denaturated state of the two enzymes, α-chymotrypsin and succinylated Con A. We show that the enzymatic activity is regained and in certain circumstances enhanced.

In recent years, the stability of biomolecules in ionic liquids (ILs) has been a subject of investigation because of their biological and pharmaceutical applications.[1–9] Recently, several studies have highlighted the role of either the cation or anion of ILs and the specific biomolecular interactions.[10–13] As outlined in various recent studies, ILs have been successfully proven to increase stability of protein,[6,12,14] suppress aggregation,[1,15] as well as improve crystallization of proteins.[16] Very recently, we have showed the ability for the protic ionic liquid (pIL), triethylammonium acetate (TEAA) to attenuate the deleterious action of urea on α-chymotrypsin (CT).[5] We noticed that this TEAA reduces the urea-induced denaturation of enzyme, in a combination of urea and TEAA.[5] Flowers and Summers reported on the ability of the pIL, ethylammonium nitrate (EaN) to refold hen egg white lysozyme (25 mg ml^{-1} solution of denatured-reduced lysozyme was diluted with 143 mM Tris sulfate buffer ~pH 8.5 to give a final lysozyme concentration of 1.6 mg ml^{-1}) from an oxidatively reduced state.[14] In this communication, we present the refolding ability of water and TEAP against chemically unfolded structure. In this study initially we unfolded the 2 mg ml^{-1} enzyme structure in the presence of urea, later we observed the refolding ability of TEAP or water of 100 μl for the chemically unfolded enzyme structure. This communication presents the first investigation of the refolding ability of TEAP for urea-induced unfolded enzyme structures.

Obviously, water is playing an important role in the protein folding mechanism, however, the role of water as a refolding additive for chemically denatured enzymes has not been fully elucidated.[17] In this communication, we have exploited the possible versatility of water and TEAP as refolding additives for enzymes for the urea-induced denaturation of succinylated Con A (S Con A) and CT. We have used a combination of fluorescence, circular dichroism (CD), NMR and enzyme activity measurements.

Chemical denaturation of biomolecules by urea has been widely studied using *in vitro* experiments.[18] The chemical denaturation of S Con A was monitored using fluorescence spectroscopy. The denaturation process can be followed by changes in the maximal intensity of fluorescence (I_{max}), and the red-shift of the maximal emission wavelength (E_{max}). The E_{max} for S Con A in buffer pH 4 is ≈325 nm and is red shifted to E_{max} ≈ 330 nm in 1 M urea present in 2 mg ml^{-1} S Con A concentration (Fig. 1S†). When we add 100 μl of water to the 2 mg ml^{-1} enzyme denatured solution we observe a blue shift back to E_{max} ≈ 325 nm corresponding to native state of S Con A. To ensure that this is not just simply a dilution factor we tested the refolding of water at higher urea concentrations. Moreover, E_{max} shifted from 333 nm for 3 M urea to 325 nm for 100 μl of water in 3 M urea denatured solution leading to a blue shift, indicating that water is able to refold the denatured enzyme at 3 M urea (Fig. 1S†). However, water fails to refold the denatured S Con A structure at 4 or 5 M urea (334 nm or 337 nm, respectively) due to highly penetration of S Con A in 4 or 5 M urea (333 and 335 nm) (Fig. 2S†). As water refolded the 3 M urea denatured S Con A solution (2 mg ml^{-1}), we further carried out similar experiments for the industrially relevant enzyme CT. Fluorescence revealed that water failed to refold the 2 mg ml^{-1} CT solution in the presence of 1 M urea (Fig. 3S†). In the CT/buffer, urea and water solutions, the observed E_{max} is remarkably different for CT in buffer (Fig. 3S†). For a better understanding of the enzyme state during refolding, we also performed the kinetic experiment for both enzymes after 40 min incubation. Fig. 4S† reveals that the normalized I_{max} continuously falls with time in urea. Further, the addition of water to urea denatured enzyme solution (up to 3 M in 2 mg ml^{-1} enzyme solution) clearly showed that the water recovered the activity level, which was lost in the S Con A structure at 3 M urea. In an extensive study of CT, water fails to retain the activity at 1 M urea present in 2 mg ml^{-1} enzyme solution (Fig. 5S†), which resulted in a decrease of the I_{max} value to a greater extent after 40 min incubation. This may be due to the enzyme conformation that had

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probably been modified from the native state to the unfolded state with the addition of urea and fails to regain the native state after addition of water. To address the effect of water refolding ability, we further performed CD and NMR studies.

The far-UV CD spectrum (190–240 nm wavelengths) shows that the wavelength of S Con A in buffer is located at ≈223 nm, which corresponds to a slightly more pronounced β-structure (Fig. 6S†). Urea-induced unfolding was monitored by CD analysis, up to 5 M urea in 2 mg ml^{-1} enzyme solution and results are displayed in the ESI (Fig. 6S†). The ellipticity of S Con A at ≈223 nm was decreased in 2 M urea or at a higher concentration of urea, indicating disruption of the secondary structure of the enzyme under this condition. The urea-induced unfolded (up to 3 M urea) S Con A was refolded by the addition 100 μl of water. The CD spectra of water in denatured S Con A up to 3 M urea are virtually identical to that of the S Con A in buffer solution (Fig. 6S†). On the contrary, the spectra for water in the highly denatured S Con A (in 4 or 5 M urea) is remarkably different to that of S Con A in buffer solution. Therefore, one can infer that water is able to act as a partially refolding additive for urea-induced states of S Con A. In addition, we also studied the near-UV CD spectra (240–300 nm) in order to obtain the water refolding ability on chemically denatured tertiary structure of the enzyme and the results are displayed in Fig. 7S.† The intensity of the 240–300 nm bands is affected by local conformational changes around these chromatophores. The near-UV CD spectrum of S Con A between 270 and 300 nm is mainly due to Tyr and Trp residues and Phe residues which strongly contribute to bands in the 258–270 nm regions. On addition of urea we found the induced unfolding of the α-subunit of Trp synthase, giving the observed changes in CD at 286 nm. While on the addition of water to this denatured solution, we observed the maxima for Trp residues at 254, 289, and 296 nm. Hence, we can conclude that water acts as a refolding additive for urea-induced unfolded of S Con A. Moreover, the experimental results revealed that there is no change in the α-subunit of Trp synthase in denatured CT at 286 nm with addition of water to the urea-denatured CT structure (Fig. 8S†). Therefore, we concluded that water can act as a refolding additive only for urea-induced unfolded S Con A while it fails for urea-induced denatured CT.

To ascertain the refolding ability of water on S Con A from a urea denatured state (3 M urea), we further performed ^{1}H NMR studies. Fig. 1 displays that the water is able to refold the S Con A structure even in the aliphatic region, where the refolding is generally irreversible from 0 ppm to 3 ppm,[19] this might be due to Asp-208 being hydrogen-bonded to water molecules[20] and helps in regaining the native structure of S Con A. The native structure of S Con A peaks is represented by peaks between 1.0 to 1.4 ppm and 2.2 to 2.8 ppm (Fig. 1a). These peaks disappear in the presence of urea (Fig. 1b). The addition of 100 μl of water results in the reappearance of the peaks between 1.0 and 1.4 ppm and 2.2 and 2.8 ppm (Fig. 1c). The refolding capabilities of water for S Con A were also supported using CD (Fig. 4S†).

ILs are frequently used to enhance the refolding ability for thermally denatured structure of biomolecules.[1,6,12–14] In order to assist the IL potential for a refolding additive, we hereby report the IL ability to act as a refolding additive for urea-induced unfolded structure of two different enzymes by the

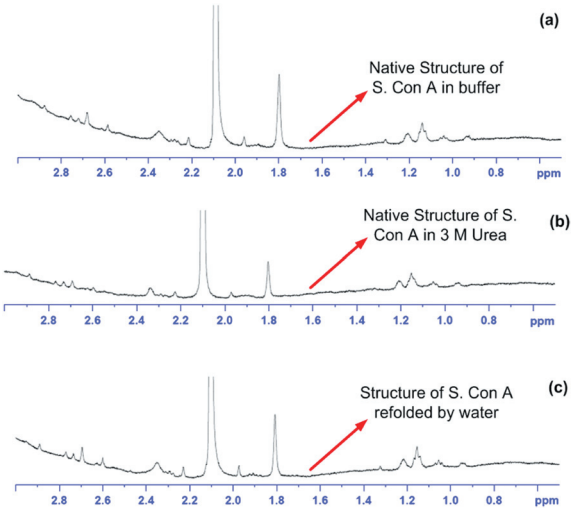

Fig. 1 ^{1}H NMR data for the refolding of urea (3 M)–unfolded S Con A in the presence of 100 μl of water at 25 °C for 2 mg ml^{-1} concentration of proteins.

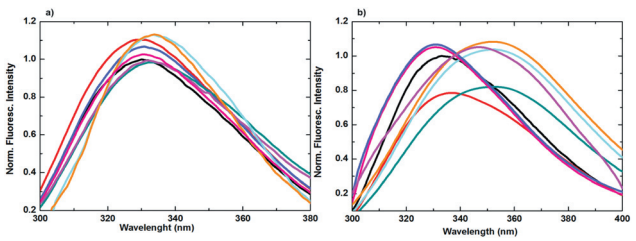

Fig. 2 The urea-unfolded enzyme was refolded by 100 μl of TEAP at 25 °C for 2 mg ml^{-1} concentration of protein. Fluorescence spectra for (a) S Con A and (b) CT in buffer (black), TEAP (red), 2 M urea (blue), 3 M urea (magenta), 4 M urea (orange), 5 M urea (purple) and TEAP in 2 M urea (dark cyan), in 3 M urea (light cyan), in 4 M urea (green) and in 5 M urea (pink).

aforesaid biophysical techniques. As shown in Fig. 2a, natural S Con A in TEAP has a characteristic fluorescence emission peak at ≈327 nm. After structural denaturation in urea (3 or 4 M urea in 2 mg ml^{-1} enzyme solution), there occurs a red shift, because of exposure of Trp residues to the solvent environment (Fig. 2a). It was observed that the E_{max} peak is blue shifted at ≈326 nm in the presence of 100 μl of TEAP, which is obviously quite consistent with E_{max} of the native structure of S Con A. However, we observed a slightly red shift during the refolding process in highly denatured S Con A in 5 M urea. These results clearly elucidate that TEAP is a refolding additive for urea-induced S Con A. To search for a convenient and efficient approach for the refolding ability TEAP, we have examined the similar experimental conditions on CT. Fig. 2b displays CT in TEAP has E_{max} at 331 nm. The change in the fluorescence of CT in the presence of urea (from 2–3 M urea) appeared at 352–360 nm confirming that CT is substantially unfolded under the urea. On the other hand, the denatured-unfolded (from 2–3 M urea) CT was refolded by 100 μl of TEAP (Fig. 2b). The fluorescence peak showed that TEAP fails the substantial urea-unfolded CT (at 4 M urea). A fluorescence kinetic experiment is

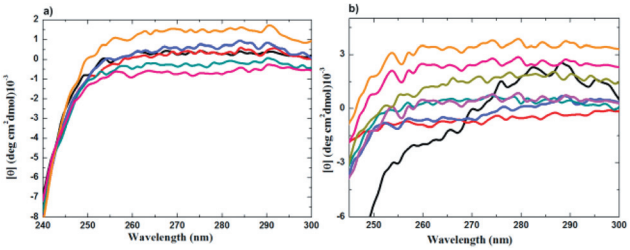

Fig. 3 The conformation of urea-unfolded enzyme was refolded by 100 µl of TEAP at 25 °C. Near-UV CD spectra for (a) S Con A and (b) CT in buffer (black), TEAP (red), 3 M urea (blue), 4 M urea (magenta), 5 M urea (orange) and TEAP in 3 M urea (dark cyan), in 4 M urea (dark yellow) and in 5 M urea (pink).

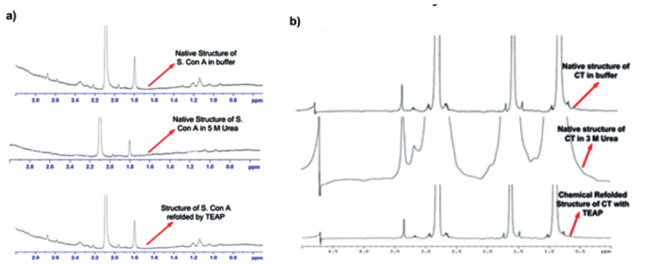

Fig. 4 ^{1}H NMR data for the refolding of (a) urea-unfolded S Con A and (b) urea-induced denatured CT in the presence of 100 µl of TEAP at 25 °C for 2 mg ml^{-1} concentration of enzymes.

also performed with TEAP in chemical denatured S Con A (Fig. 9S†). Discrete activity levels are observed as the I_{max} value of the TEAP added solution is higher than the control after incubation for 40 min. In the case of CT, TEAP is able to recover the enzyme activity of denatured solution up to 3 M urea and fails at a higher concentration (Fig. 10S†).

To corroborate the fluorescence results, we have also investigated TEAP refolding ability through CD analysis. S Con A in TEAP has a similar profile to the spectra observed in buffer, and showed a minima at ≈223 nm (Fig. 11S†). In case of TEAP, the far-UV CD spectrum of S Con A was similar to that observed in buffer (Fig. 11S†). Experimental results revealed that TEAP is able to recover the S Con A structure from urea denatured structure up to 5 M. TEAP may be able to recover all the local polar interactions and disruption of non-local interactions from denatured state of S Con A. Similarly, the near-UV CD spectra (240–300 nm) spectra also illustrated that TEAP is able to refold the S Con A structure (Fig. 3a). Further, to demonstrate the TEAP potency on urea-induced denatured CT, we explored similar CD analysis and the results are presented in Fig. 3b. Adding 100 µl of TEAP leads to the perturbation of urea-induced in CT (up to 3 M) then TEAP significantly recovers both secondary and tertiary perturbation structure, indicating TEAP is behaving as refolding enhancer for chemically denatured CT structure (up to 3 M urea). In other words, TEAP fails to recover the chemically denatured CT structure at 4 M urea or higher concentration of urea in the same solution (Fig. 3b). The combined fluorescence and CD results clearly revealed that TEAP has more refolding ability in urea-induced unfolded S Con A than that of CT structure.

To verify the structural changes during the refolding process by TEAP from a quenched chemically unfolded enzyme structure, we further performed ^{1}H NMR studies. TEAP is able to chemically refold the S Con A structure up to 5 M, which might be due to Asp-208 hydrogen-bonded to water molecules[20] and water molecules remain in a compact state due to the highly viscous TEAP, which in turn help to recover the native structure of S Con A. Hence, refolding is observed in the TEAP for chemically denatured S Con A (up to 5 M urea) (Fig. 4a). While in the case of CT, the hydrogen-bonded proton located between the carboxylate group of aspartate 102 and $N^{\delta 1}$ of histidine 57 renatures the native conformation during the refolding process. Strong hydrogen bonds, those found in water and TEAP shift the proton resonance peaks downfield by about 2 ppm while weaker hydrogen bonds, such as between carbonyls and amino protons, only shift the resonance peaks downfield by approximately 1 ppm in the aliphatic region. Therefore, TEAP is able to refold the urea-unfolded CT (up to 3 M) (Fig. 4b). TEAP fails to refold the chemical denatured structure of CT at higher concentrations. On the basis of these results, the refolding ability of TEAP varies from enzyme to enzyme which in turn depends on the functional group arrangement in the protein.

Conclusions

In summary, we have demonstrated that water and protic IL TEAP to be efficient and effective refolding additives for urea-induced denatured enzymes on the basis of fluorescence, CD and NMR analysis. Clearly, water refolds only up to 3 M urea denatured S Con A structure present in 2 mg ml^{-1} S Con A solution whereas, it fails to refold the chemically-denatured CT structure.

In the case of TEAP, it refolds the urea-induced denatured S Con A and CT structure up to 5 M and 3 M present in 2 mg ml^{-1} enzyme solutions, respectively. These surprising results show that the refolding ability of water and TEAP varies from enzyme to enzyme. These results also raise the question about why the solvent (water or TEAP) refolding ability limits up to 5 M urea-unfolded enzyme present in 2 mg ml^{-1} solution of S Con A, whereas renaturation propensity prevails up to 3 M urea on CT. These refolding effects on chemical-induced unfolded structures are dependent not only on denaturant concentration but also on the number and type of functional groups, aliphatic molecules present in the native structure of the enzyme. Further, the ability of protic TEAP IL can strengthen its applicability to wider biological systems with more compatibility and biocompatibility.

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