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Fluorescence studies of protein thermostability in ionic liquids†

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Using the single tryptophan residue in the sweet protein monellin as a spectroscopic handle, we show the extreme thermodynamic stabilization offered by an ionic liquid; T_{un} ~ 105 °C in [C_4mpy][Tf_2N] compared to 40 °C in bulk water.

Although efficient, versatile and selective biotransformation is the hallmark of an enzyme, practical application is often limited by both fragility and expense. For this reason, stabilization and recovery strategies are often employed including immobilization (at designed solid-state surfaces, for example), chemical modification, and/or the use of osmolytes, macromolecular crowding agents or non-conventional/non-aqueous media. Within this latter category, the use of solvent engineering in general, and ionic liquids (as pure solvents, cosolvents, or discrete phases in bi- or triphasic systems)[1] in particular, has recently emerged as an interesting possibility. This interest has been driven in large part by the attitude that ionic liquids (ILs) hold potential as advanced solvents for cleaner, more eco-friendly chemistry, an attribute resulting largely from the very low to negligible vapor pressure common amongst ILs.

We now know that enzymes of widely diverging types are able to maintain their activities within ILs or aqueous biphasic IL systems, often with improved activity, enantioselectivity, reusability and/or operational stability.[2,3] Readers are referred to ref. 4 and the references cited therein for further details on recent developments in this field. While it is generally accepted that a certain amount of water is necessary to maintain enzyme activity, it is sometimes mistakenly perceived that the only suitable medium for biocatalysis is the aqueous environment. In fact, most enzymes can perform perfectly well under nearly anhydrous conditions. In addition, biocatalysis in reduced or restricted water systems may display a number of useful properties including the suppression of side reactions, increased solubility of nonpolar substrates, different selectivities, "reversal" of thermodynamic equilibria in favor of synthesis over hydrolysis (esterification using relatively inexpensive hydrolytic enzymes is possible, for example) and the elimination of microbial contamination.

Rational design of an effective biocatalytic process requires that one understand the influence of the solvent system on enzyme structure. Despite growing interest in the field of enzymology in ILs, almost nothing has been done to characterize the state of the protein in the solvent beyond the fact that it is capable of producing the desired reaction. This gap in knowledge is what motivates our current efforts in this area. Due to the very limited solubility of proteins in common ILs (coupled with other technical issues), however, these studies are often intractable by standard optical approaches. Because of its inherent sensitivity, fluorescence spectroscopy emerges as an attractive alternative and, in fact, as a research community we have already made significant inroads in the use of fluorescence spectroscopy to study IL physicochemical properties including solvent polarity and dynamics.[5] Now and for the first time, we extend this technique to the *in situ* study of protein conformation and thermostability within ILs.

† Electronic supplementary information (ESI) available: synthesis of [C_4mpy][Tf_2N]. See http://www.rsc.org/suppdata/cc/b4/b401304m/

We have selected monellin in the ionic liquid 1-butyl-1-methylpyrrolidinium bis(trifluoromethane sulfonyl)imide [C_4mpy][Tf_2N] for initial study; full experimental details for IL preparation have been deposited as ESI.† Monellin (Fig. 1) was chosen because while it is small (chains A and B contain 45 and 50 residues, respectively) and has no disulfide bridges it is still highly structured.[6] Most importantly, monellin contains a single tryptophan residue (W3), a remedy for the difficulties inherent in resolving the contributions of individual emissive centers in multi-tryptophan proteins.[7] Because of the sensitivity of the indole chromophore to the local environment, we were able to use intrinsic tryptophan fluorescence to monitor the unfolding transition for monellin at soluble levels (tens of micromolar) in IL. Results of steady-state fluorescence experiments performed in water and IL + water are compared in Fig. 2.‡ In each case, monellin exhibits a

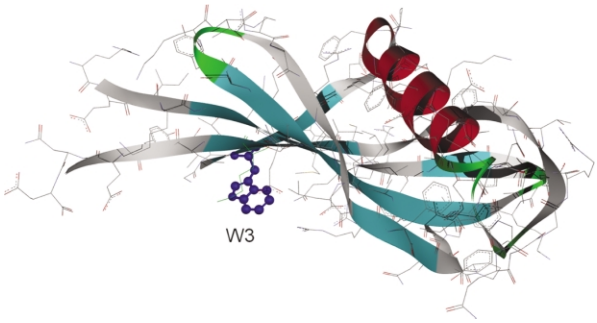

Fig. 1 X-Ray crystal structure of monellin (PDB code: 4mon). The crystal waters have been removed for clarity and only one of the identical monomers is shown, in accord with the known solution structure (see ref. 6). The single Trp residue pivotal to this study is located in chain B at position 3 from the N terminus.

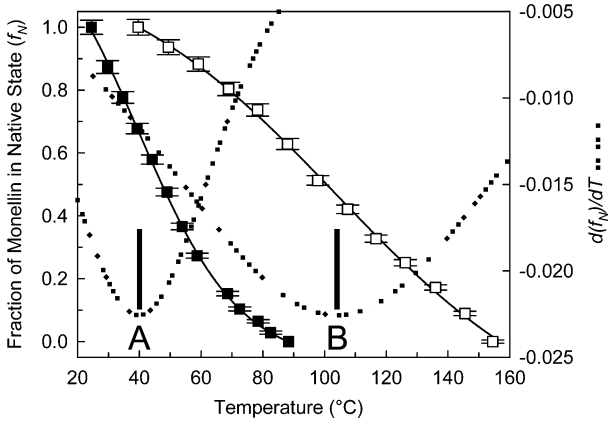

Fig. 2 Fluorescence-based thermal unfolding curves for monellin in water (■) and in [C_4mpy][Tf_2N] equilibrated with 2.0% (v/v) water (□). In each solvent system the unfolding temperature (T_{un}), denoted by solid vertical bars, was determined from the minimum of the first derivative ($d(f_N)/dT$, shown as dotted profiles A and B) of a sigmoid function fitted to the experimental data. In both cases, fluorescence was excited at 295 nm; a 2 nm spectral bandpass was used for excitation and emission.§

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monophasic unfolding curve signifying a simple two-state unfolding mechanism. Clearly, use of IL as solvent affords the protein with considerable thermodynamic stability; the onset of thermal unfolding shifts upward to over 100 °C in water-containing IL compared with *ca.* 40 °C in water. The first-derivative results also make clear that the unfolding transition is broadened within the IL possibly indicating a lower cooperativity for the transition relative to that in bulk water. Noting that the equilibrium constant for unfolding $K_{(F\rightarrow U)}$ is equal to $(1 - f_N)/f_N$, the relative free energy stabilization as a function of temperature, $\Delta\Delta G(T)_{(F\rightarrow U)}$, for monellin in IL can be estimated: $\Delta\Delta G(T)_{(F\rightarrow U)}/\mathrm{J\ mol^{-1}} \approx 115T + 1540$ for $30 \leq T/°\mathrm{C} \leq 80$. Results from a van't Hoff analysis reveal that this stabilization is entropically driven. That is, the entropies of unfolding, $\Delta S°$, are 250 and 136 $\mathrm{J\ K^{-1}\ mol^{-1}}$ for monellin in water and IL, respectively, consistent with more rigid solvation within an IL.

The spectral changes associated with unfolding also provide key information about the milieu surrounding W3. Upon unfolding, the emission contour in water is red shifted and approaches that of the 'naked' Trp analog *N*-acetyl-L-tryptophanamide (NATA) in bulk water (Fig. 3). The indication here is as expected, *i.e.*, W3 becomes more accessible to solvent as a result of unfolding. The highly blue-shifted emission observed for monellin in $[C_4mpy][Tf_2N]$, however, was completely unexpected and suggests that the state of the protein is significantly altered within the IL. While exceptionally short wavelength maxima are not unheard of (the known emission maxima for tryptophan in proteins span the range from 308 to 350 nm), they are relatively uncommon and generally implicate a complete shielding or isolation of the Trp by neighboring aromatic residues. As a benchmark, over the entire temperature range the emission maximum for NATA in IL lies within a narrow 336–339 nm window, irrespective of the state of hydration. This suggests a minimal exposure of W3 to the surrounding IL solvent as well as a tightening in protein structure which likely manifests in the higher thermal stability. Further increases in temperature result in additional blue shifting, possibly due to a progressive dissociation and stripping of biological water from the local protein surface.

In summary, by monitoring the intrinsic emission from a single Trp protein, we have been able to follow its unfolding behavior within an IL. We believe that this is the first detailed report of protein spectroscopy of any kind within an IL. While the improved thermostability conferred upon proteins likely results from alteration in the protein hydration level and structural compaction, the underlying reasons are still largely speculative and may include additional factors such as free volume contributions, ionic interactions (salt bridges) and confinement effects. Nevertheless, this remarkable stabilization against thermal inactivation suggests a general and notable alternative to engineered or isolated thermophiles in high-temperature biocatalytic and biosensory applications.

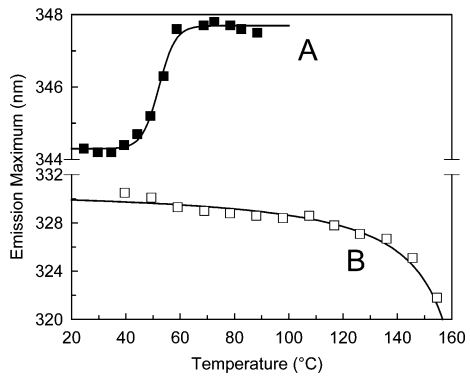

Fig. 3 Temperature-dependent emission maxima for monellin in water (■) and in $[C_4mpy][Tf_2N]$ equilibrated with 2.0% (v/v) water (□).¶

Notes and references

‡ IL was equilibrated overnight with aqueous monellin *via* gentle reciprocal shaking to result in a protein-containing IL with a water content of 2.0 vol%. Both aqueous and IL samples were centrifuged at 10 000*g* for 10 min prior to experimentation.

§ The fraction of native monellin remaining at each temperature, $f_N(T)$, was determined from the expression $f_N(T) = [F(T) - F_U]/[F_N - F_U]$ where $F(T)$ is the temperature-dependent fluorescence intensity integrated over a 4 nm slice centered about the emission maximum for the folded state, and F_N and F_U are the fluorescence intensities for the folded and unfolded conformations, respectively. Fluorescence intensities were background subtracted and corrected for solvent thermal expansion. Temperature was controlled using a home-built thermal stage.[8]

¶ Emission maxima were estimated from global minima of second-derivative spectra calculated from background subtracted experimental emission spectra or by fitting a central 50 nm window to a Weibull distribution; in either case, the uncertainty in estimation was ≤0.4 nm.

1 Several monographs and edited volumes dedicated to ionic liquids have appeared recently: *Ionic Liquids as Green Solvents: Progress and Prospects*, ed. R. D. Rogers and K. R. Seddon, ACS Symp. Ser. 856, American Chemical Society, Washington, DC, 2003; *Ionic Liquids in Synthesis*, ed. P. Wasserscheid and T. Welton, Wiley-VCH, Weinheim, 2003.

2 J. A. Garlitz, C. A. Summers, R. A. Flowers II and G. E. O. Borgstahl, *Acta Crystallogr., Sect. D*, 1999, **55**, 2037–2038; C. A. Summers and R. A. Flowers II, *Protein Sci.*, 2000, **9**, 2001–2008.

3 M. Erbeldinger, A. J. Mesiano and A. J. Russell, *Biotechnol. Prog.*, 2000, **16**, 1129–1131; R. M. Lau, F. van Rantwijk, K. R. Seddon and R. A. Sheldon, *Org. Lett.*, 2000, **2**, 4189–4191; T. Itoh, E. Akasaki, K. Kudo and S. Shirakami, *Chem. Lett.*, 2001, 262–263; J. A. Laszlo and D. L. Compton, *Biotechnol. Bioeng.*, 2001, **75**, 181–186; M. Eckstein, M. Sesing, U. Kragl and P. Adlercreutz, *Biotechnol. Lett.*, 2002, **24**, 867–872; J. L. Kaar, A. M. Jesionowski, J. A. Berberich, R. Moulton and A. J. Russell, *J. Am. Chem. Soc.*, 2003, **125**, 4125–4131; D. L. Compton and J. A. Laszlo, *J. Electroanal. Chem.*, 2003, **553**, 187–190; P. Lozano, T. De Diego, D. Carrié, M. Vaultier and J. L. Iborra, *Biotechnol. Prog.*, 2003, **19**, 380–382; L. Gubicza, N. Nemestóthy, T. Fráter and K. Bélafi-Bakó, *Green Chem.*, 2003, **5**, 236–239; M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey and R. D. Rogers, *Green Chem.*, 2003, **5**, 443–447.

4 For two brief reviews on the use of ionic liquids as media for biotransformations see: U. Kragl, M. Eckstein and N. Kaftzik, *Curr. Opin. Biotechnol.*, 2002, **13**, 565–571; F. van Rantwijk, R. M. Lau and R. A. Sheldon, *Trends Biotechnol.*, 2003, **21**, 131–138.

5 K. A. Fletcher, I. A. Storey, A. E. Hendricks, S. Pandey and S. Pandey, *Green Chem.*, 2001, **3**, 210–215; S. N. Baker, G. A. Baker, M. A. Kane and F. V. Bright, *J. Phys. Chem. B*, 2001, **105**, 9663–9668; K. A. Fletcher, S. Pandey, I. K. Storey, A. E. Hendricks and S. Pandey, *Anal. Chim. Acta*, 2002, **453**, 89–96; R. Karmakar and A. Samanta, *J. Phys. Chem. A*, 2002, **106**, 4447–4452; K. A. Fletcher and S. Pandey, *Appl. Spectrosc.*, 2002, **56**, 266–271; K. A. Fletcher and S. Pandey, *Appl. Spectrosc.*, 2002, **56**, 1498–1503; J. H. Werner, S. N. Baker and G. A. Baker, *Analyst*, 2003, **128**, 786–789; J. Lu, C. L. Liotta and C. A. Eckert, *J. Phys. Chem. A*, 2003, **107**, 3995–4000; K. A. Fletcher, S. N. Baker, G. A. Baker and S. Pandey, *New J. Chem.*, 2003, **27**, 1706–1712; G. A. Baker, S. N. Baker and T. M. McCleskey, *Chem. Commun.*, 2003, 2932–2933; J. A. Ingram, R. S. Moog, N. Ito, R. Biswas and M. Maroncelli, *J. Phys. Chem. B*, 2003, **107**, 5926–5932; S. N. Baker, G. A. Baker, C. A. Munson, F. Chen, E. J. Bukowski, A. N. Cartwright and F. V. Bright, *Ind. Eng. Chem. Res.*, 2003, **42**, 6457–6463.

6 J. A. Morris, R. Martenson, G. Deibler and R. H. Cagan, *J. Biol. Chem.*, 1973, **248**, 534–539; L. Zheng, W. R. Reid and J. D. Brennan, *Anal. Chem.*, 1997, **69**, 3940–3949.

7 J. W. Longworth, in *Time-Resolved Fluorescence Spectroscopy in Biochemistry and Biology (NATO Series I, Vol. 69)*, ed. R. B. Cundall and R. E. Dale, Plenum Press, New York, 1983, pp. 651–778; M. R. Eftink, *Biophys. J.*, 1994, **66**, 482–501; K. Flora, J. D. Brennan, G. A. Baker, M. A. Doody and F. V. Bright, *Biophys. J.*, 1998, **75**, 1084–1096; Y. Engelborghs, *J. Fluoresc.*, 2003, **13**, 9–16.

8 S. N. Baker, G. A. Baker, C. A. Munson and F. V. Bright, *Appl. Spectrosc.*, 2001, **55**, 1273–1277; C. A. Munson, G. A. Baker, S. N. Baker and F. V. Bright, *Langmuir*, 2004, **20**, 1551–1557.