Protein solubilising and stabilising ionic liquids†

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We report a family of biocompatible ionic liquids (ILs) which are able to dissolve significant amounts of proteins such as cytochrome c and in which ATR-FTIR spectroscopy results show retention of secondary structure to extreme temperatures.

Ionic liquids (compounds that consist only of ions and have a melting point <100 °C) have gained increasing attention as green, multi-use reaction media and solvents for a variety of applications.[1–3] ILs typically possess no vapor pressure, have fascinating features such as high thermal, chemical and electrochemical stability and widely tunable properties with regard to polarity, hydrophobicity and solvent miscibility.[3] ILs are also currently being researched for a variety of bio-applications including as media for biocatalytic reactions,[3,4] biosensors,[5] protein stabilization[6] and biopreservation.[7] They have been proposed as unique solvents for biomolecules such as proteins because of their unusual solvation characteristics. Work by one of the authors[5] and others[4,6] has shown that some proteins are, in fact, soluble, stable and remain active in some ILs. This is an important observation since proteins are sometimes unstable when handled *in vitro*, and stabilizing agents are a necessary component to ensure their long-term stability. This is especially true of proteins that have pharmaceutical potential since lack of stability is a limitation to widespread use of some protein therapeutics.

Thus, if it is possible to introduce a biomolecule into an appropriately biocompatible IL, it is hypothesized that stability and the technological utility of the biomolecule may be improved for reasons discussed further below. However, attention obviously must be paid to the effect of solubilization on the higher order structure of the protein, since most protein function is related to that higher order structure. Thus, this work aims to understand the effect of IL chemistry and structure on protein–IL interactions, and thereby develop ILs of utility in dissolving and handling proteins.

A number of enzymes of widely diverging types have been shown to remain catalytically active in an ionic liquid medium.[8,9] Lipases, in particular, maintain their activity in anhydrous ionic liquid media; their selectivity and operational stability are often better than in traditional media.[8,9] In most reports involving IL media, however, the enzyme is present not in a dissolved state but, rather, in a finely divided dispersed state and is therefore acting as a heterogeneous catalyst. In fact proteins are usually insoluble in organic solvents and ILs.[8,10–12] Thus, there are only a few reports concerning proteins dissolved in ILs. In one report monellin was found to be soluble in butylmethyl pyrrolidinium bis(trifluoromethanesulfonyl)amide, but only at low levels ($\sim 10^{-5}$ M); the structure in solution was detected *via* the intrinsic tryptophan fluorescence of the protein.[6] In two other reports, ether structures were used to induce solubility of the protein in a relatively traditional hydrophobic IL.[4,5]

Cytochrome c (cyt. c)[13] is chosen as a model protein for study here since it is one of the most thoroughly physicochemically characterized metalloproteins. From a biophysics point of view, it has played an indispensable role as a laboratory model in which to study the equilibrium and kinetics of protein folding.[14] From a medical standpoint, evidence that sickle cell anemia was caused by heme protein dysfunction demonstrated the molecular basis of this human disease.[15]

Thus in order to create ILs providing a high solubility for proteins such as cyt. c, a number of new ionic liquids have been prepared in this work based on potentially more biocompatible anions such as dicyanamide, saccharinate and dihydrogen phosphate. The cations chosen include the well known butylmethyl pyrrolidinium cation as well as an important biochemical cation, choline.

Cyt. c from horse heart was purchased from Aldrich and used without further purification. Choline saccharinate and *N*-butyl-*N*-methylpyrrolidinium ($p_{1,4}$) dicyanamide (dca) were synthesized according to literature methods.[16] 1-Butyl-3-methylimidazolium (bmim) dihydrogen phosphate (dhp), *N*-butyl-*N*-methyl pyrrolidinium dihydrogen phosphate ($p_{1,4}$ dhp) and choline dihydrogen phosphate were also synthesized according to literature methods as follows.[17] A slight excess of freshly prepared silver hydroxide was added to $p_{1,4}$ Br or choline chloride and the suspension stirred for 2 h. The precipitated solid (AgCl, AgBr and excess AgOH) was filtered off. A slight excess of phosphoric acid was added to the filtrate and the water removed by rotary evaporation. The subsequent residue was taken up in acetone to remove excess acid and any residual water. The mixture was then filtered, the solvent evaporated and the product was dried *in vacuo*. Salts were identified using ^{1}H NMR, DSC and electrospray mass spectrometry. Positive and negative ion electrospray mass spectra were recorded with a Micromass Platform electrospray mass spectrometer for samples dissolved in methanol. $P_{1,4}$ dhp: Ts-s_1 5 °C, Ts-s_2 91 °C, Tm 112 °C. ^{1}H NMR (D_2O, 300 MHz): δ 0.88 (t, 3H), 1.32 (m, 2H), 1.70 (m, 2H), 2.13 (s, 4H), 2.95 (s, 3H), 3.25 (t, 2H), 3.42 (s, 4H). ES-MS: ES^+ *m*/*z* 142 $p_{1,4}^+$, ES^- *m*/*z* 97 dhp^-. Choline dhp: Ts-s 23 °C, Tm 119 °C, ^{1}H NMR (D_2O, 300 MHz): δ 3.14 (s, 9H), 3.44 (t, 2H), 3.96–4.01 (m, 2H). ES-MS: ES^+ *m*/*z* 104 $choline^+$, ES^- *m*/*z* 97 dhp^-.†

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Differential scanning calorimetric (DSC) measurements for cyt. c were made with a Perkin-Elmer DSC7 at a scanning rate of 10 °C min^{-1}. The cyt. c solutions were prepared to a final concentration of 3 mM in either the 50 mM sodium phosphate buffer (pH 7.0) or the IL. It was found that the addition of 10–20 wt% water to the dhp salts was sufficient to render them liquid at room temperature and aid solubility and therefore an IL–water mixture was used in these cases. Single pass attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were measured with a Specac Golden Gate diamond ATR sampler fitted to a Bruker Equinox 55 with an MCT detector. Approximately 4 μl of cyt. c solution was pipetted onto a diamond window and a 100 scan interferogram was collected in single beam mode, with 2 cm^{-1} resolution, from 4000 to 600 cm^{-1}. Reference spectra were recorded under identical conditions with only the solvent media. The subtraction of the reference spectrum was carried out in accord with the criteria described by Dong *et al.*[18] The second derivative spectrum was obtained with Savitsky Golay derivative function software for a nine data point window.

A key feature of some of these new ILs is their ability to dissolve significant amounts of cyt. c, in contrast to previous reports where only very limited quantities of proteins including lipase,[19] thermolysin,[20] chymotripsin[21] and monellin[6] were found to be soluble in the ILs used. Fig. 1 illustrates these observations; the dca IL was surprisingly not a good solvent for cyt. c despite its slight basicity and hydrogen bonding potential, however $p_{1,4}$ dhp will dissolve up to 3 mM cyt. c (37 mg mL^{-1}) when a small amount (10–20 wt%) of water is added. The saccharinate ion was not as effective as the dhp anion. The cations studied did not appear to have a strong effect, $p_{1,4}$ dhp, choline dhp and bmim dhp all providing similar levels of solubility.

Very significantly, the secondary structure is retained in the IL solubilized protein, as shown in Fig. 2. Here the second derivative ATR-FTIR spectrum in the amide I region of cyt. c in the ILs is compared with that in the buffered aqueous control. The second derivative spectra are used as an extremely sensitive probe of structure. The key peaks at 1632, 1650, 1656 and 1680 cm^{-1} arising from the β-sheet, random coil, α-helix and turn structure[18] are retained. Previous work by one of the authors[5] has shown that cyt. c retained its redox acitvity in IL solution (the cyt. c was modified in those experiments to aid solubility in the IL used). This is in contrast to previous work where ILs lead to the loss of the secondary structure after dissolution.[6,19–21] Furthermore, the structural changes can lead to loss of the activity in some cases.[19,20]

Fig. 3 presents differential scanning calorimetry thermograms of the cyt. c solutions. The well known denaturing event[22] is seen in

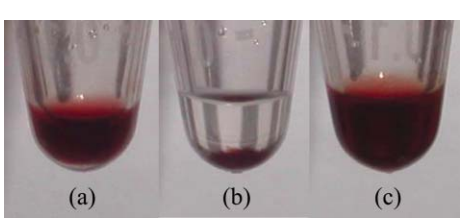

Fig. 1 Photographs of cyt. c in (a) phosphate buffer, (b) $p_{1,4}$ dca with 20 wt% water and (c) choline dhp with 20 wt% water.

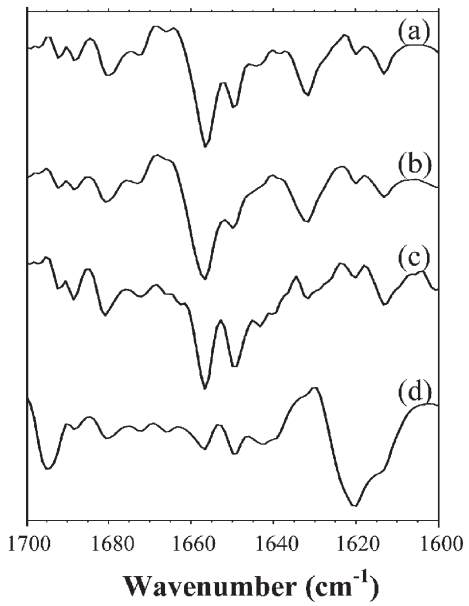

Fig. 2 Comparison of the second derivative spectra in the amide I region of cyt. c in (a) phosphate buffer, (b) choline dhp, (c) $p_{1,4}$ dhp at room temperature and (d) in phosphate buffer after incubation at 80 °C. The large new band at 1620 cm^{-1} in (d) indicates the denatured protein.

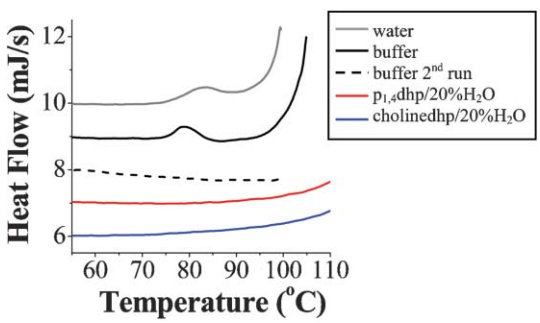

Fig. 3 DSC thermograms of 3 mM cyt. c in water, sodium phosphate buffer, $p_{1,4}$ dhp and choline dhp.

the DSC trace of the aqueous solutions. The thermal stability of cyt. c is dramatically increased in the dhp mixtures, as evidenced by the disappearance of the denaturing peak from their DSC traces. Increased thermal stability is further confirmed by the variable temperature ATR-FTIR spectra shown in Fig. 4. The unfolding of the protein chain is evidenced in the aqueous buffer control solution of Fig. 4a as the temperature approaches 80 °C by the appearance of a new band at 1626 cm^{-1} and the gradual loss of the α-helix band at 1657 cm^{-1}. The appearance of this band corresponds to the thermal event seen in the DSC traces (Fig. 3). Fig. 4b presents the same experiment carried out in choline dhp and in this case the band corresponding to the denatured protein only appears above 100 °C. At 130 °C this band is still slightly less significant than it is in the aqueous solution at 75 °C. Fig. 4c shows the complete absence of the 1626 cm^{-1} band during the temperature excursion to 130 °C in the $p_{1,4}$ dhp solution. This correlates with the absence of a thermal event in the DSC results. To clarify the effect of these dhp salts on protein stability, other water contents were also investigated. When

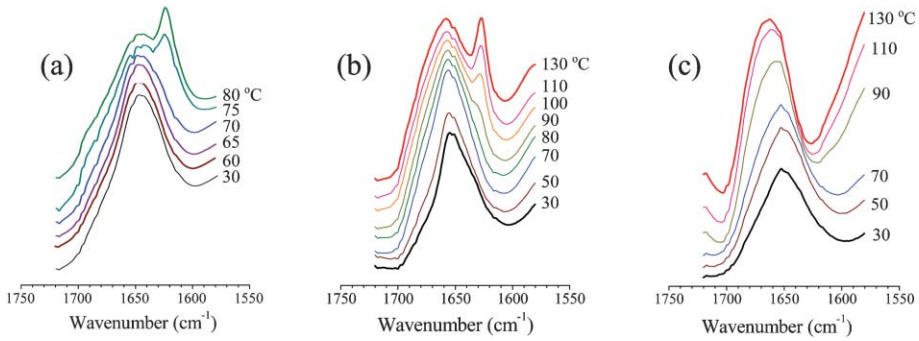

Fig. 4 ATR-FTIR spectra in the amide I region of cyt. c in (a) 50 mM sodium phosphate buffer, (b) choline dhp and (c) $p_{1,4}$ dhp *versus* temperature.

excess water (80 wt%) is added to choline and $p_{1,4}$ dhp, the denaturation event of cyt. c is seen in the DSC traces at 77 and 62 °C, respectively (data not shown). A decrease in thermal stability is therefore suggested from these DSC results. Furthermore, a clear decrease in thermal stability is confirmed by the ATR-FTIR spectra. A new band based on unfolding of the protein chain is obtained at 70 and 35 °C in choline and $p_{1,4}$ dhp with 80 wt% water, respectively. These results show that when the dhp salts are present as solutes in aqueous solution they produce a slight destabilisation or at the very least are not different to the buffer solution. Thus a high content of dhp salt and at most a solute quantity of water are necessary to produce the high protein thermostability seen in Fig. 3 and 4. To investigate the effect of dhp ILs in the complete absence of water, synthesis of new dhp ILs which are liquid at room temperature is currently in progress.

The retention of structure after an excursion to an extreme temperature is further confirmed by a comparison of the ATR-FTIR second derivative spectrum at room temperature before and after the temperature excursion. These data show that the buffered aqueous cytochrome c solution remains denatured post thermal excursion to 80 °C whilst the $p_{1,4}$ dhp solubilized protein returns to room temperature after the treatment at 110 °C practically unchanged.

In summary, enhanced solubility and stability of a model protein dissolved in novel biocompatible IL solutions based on the dihydrogen phosphate anion are reported. Retention of the secondary structure has been confirmed by vibrational spectroscopy. The nature of the anion appears to be the important factor in these effects, the dhp anion providing both a proton activity similar to that in neutral water as well as hydrogen bonding donor and acceptor sites. The role of the water component in assisting solubilization is also significant and future work will further probe the role of this water.

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