Solubility and Stability of Cytochrome *c* in Hydrated Ionic Liquids: Effect of Oxo Acid Residues and Kosmotropicity

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Hydrated ionic liquids (ILs) were prepared by adding appropriate amounts of water to hydrophilic ILs. Some hydrated ILs show excellent solubilizing ability for proteins, keeping the basic properties of ILs. The solubility of cytochrome *c* (cyt *c*) depended on the structure of the component ions. When component anions have oxo acid residues, the resulting hydrated ILs solubilize cyt *c* quite well. In such hydrated ILs, the structure and activity of cyt *c* is influenced by the kosmotropicity of the component ions. We synthesized ILs from various ions having different kosmotropicity, including dihydrogen phosphate (dhp), dibutylphosphate, acetate, lactate, and methanesulfonate as anions. The activity of the dissolved cyt *c* depends on the permutations of kosmotropicity of the component ions. cyt *c* shows no structural change and retains its activity when dissolved in the hydrated choline dhp, which is an excellent combination of chaotropic cation and kosmotropic anion. Furthermore, cyt *c* dissolved in the hydrated choline dhp remained in a native state and was active after 18 months of storage at room temperature.

Introduction

Proteins generally exist in cellular compartments and the extracellular matrix under conditions of stable pH and osmotic pressure; they are stable in vitro only for short periods of time.[1] Carefully controlled conditions are needed to preserve these proteins, or they decompose and undergo agglutination. Freeze-drying is a general method for long-term preservation but may cause structural damage to protein molecules by freezing.[1] To prevent damage during preservation, chemicals such as freezing protective agents and preservatives need to be added to the protein solution. Since some proteins demand very precise conditions in the absence of heating or freezing, they can be handled only for a few days. Protein-based medicines have shown that they can control and cure diseases, and a market for protein-based pharmaceuticals has recently sprung up.[2] However, protein instability still limits protein-based pharmaceuticals and therapeutics; to this end, it is important to develop methods to preserve active proteins.[3]

Ionic liquids (ILs) are remarkable materials that have totally different properties from those of molecular liquids.[4−6] In many cases ILs have negligible vapor pressure and high thermal, chemical, and electrochemical stability, with widely tunable properties such as polarity, hydrophobicity, and solvent miscibility.[6] Ionic liquids are potential candidates as substituents of reaction media in electrochemistry[7] and enzymatic catalysis[6,8,9] as well as green chemistry[4,6] because of their excellent stability and unusual solvent properties. A number of enzymes have been reported to retain catalytic activity in an ionic liquid medium.[8,9] Lipases, in particular, maintain their activity in anhydrous hydrophobic ionic liquid media, and their selectivity and operational stability are often better than in traditional volatile solvents.[8,9] In most reports involving hydrophobic ILs, however, the enzyme is present in a dispersed (rather than a dissolved) state and is therefore acting as a heterogeneous catalyst. Except for membrane proteins, in general proteins are mostly insoluble in organic solvents and ILs.[8,10] Some hydrophilic ILs reportedly accelerate the dissociation of proteins, but in these cases the ILs lead to loss of the secondary and higher structure after dissolution.[11−14] Such structural changes generally cause loss of activity.[12,14] To induce solubility and maintain activity in ILs, poly(ethylene oxide) (PEO) chains have been modified onto the protein surface and/or on the component ions of ILs.[10,15−17] This PEO modification is quite effective, but it is rather troublesome to prepare and separate the PEO-modified proteins from unreacted PEO chains. Ionic liquids, having suitable affinity for (and no further influence to induce denaturation of) general proteins, have long been desired.

For handling of protein in ILs, we need detailed knowledge of the relationship between IL chemistry and protein solubility and stability. There is almost nothing in the literature. We propose a new IL system that maintains the basic properties of ILs but has a small amount of water added to considerably improve the protein solubility. To study the activity and stability of proteins in aqueous solutions, there are many reports on the effect of kosmotropicity of salts dissolved in the protein solutions.[18−20] Ions classified as kosmotropes strongly hydrate ions that accelerate the water structuring, whereas chaotropes weakly hydrate ions that suppress it. The Hofmeister series position correlates well with the effects of salt kosmotropicity.[21−23] It is well established in the aqueous phase that strongly kosmotropic anions stabilize proteins whereas strongly kosmotropic cations destabilize them; proteins are stabilized by kosmotropic anions and chaotropic cations.[20] The same effect

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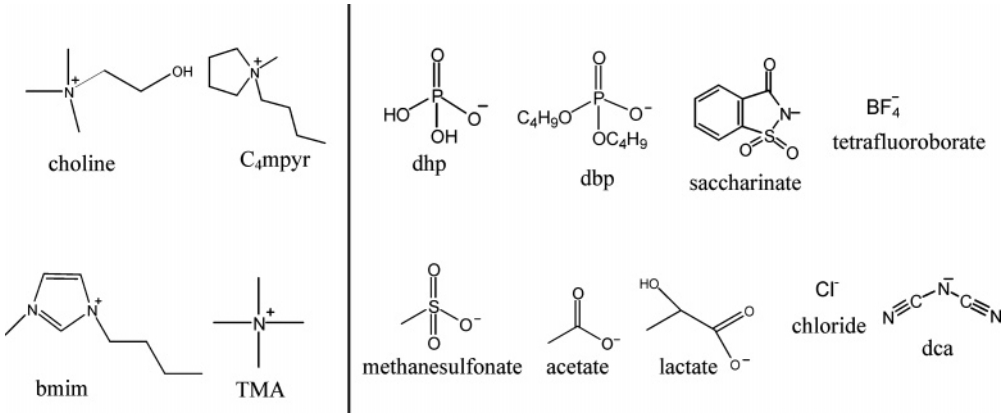

Figure 1. Structure of cations and anions for ILs examined in the present study.

has been reported in aqueous solutions with ILs as adding salt (~2.5 M), and biocatalysis was found to follow the kosmotropicity order.[24] We chose the combination of component ions according to kosmotropicity and synthesized the ILs to study the effect of the ILs as a solvent for proteins after addition of water. We have thus reported a new biocompatible ionic liquid based on choline cation and the dihydrogen phosphate anion[25,26] that offers dramatically improved protein stability with respect to both time and temperature. According to the kosmotropicity order, phosphate anion has strong kosmotropicity and tetramethylammonium (TMA) cation has strong chaotropicity. Of the phosphate anions, we adopt dihydrogen phosphate as a potentially biocompatible anion. In the present case we choose choline cation rather than TMA. The choline cation has very similar structure to TMA and occurs frequently in biological systems.

This work aims to elucidate the role of kosmotropicity of hydrophilic ILs for protein stability in the ILs. A number of ILs having different kosmotropicity were prepared, and the protein structure and activity in these ILs were investigated with respect to thermal and long-term stability. These are key features when proteins and biomolecules are handled in vitro. Cytochrome *c* (cyt *c*) is chosen as a model protein for study since it is one of the most thoroughly physicochemically characterized metalloproteins. Biophysically speaking, it has been a potential laboratory model to study the equilibrium and kinetics of protein folding.

Materials and Methods

Synthesis of Ionic Liquids. $AgNO_3$ and NaOH were purchased from Merck. Other chemicals were purchased from Aldrich. These reagents were used without further purification. Choline saccharinate and *N*-butyl-*N*-methylpyrrolidinium dicyanamide (C_4mpyr dca) were synthesized according to methods in the literature.[25,27] Choline dihydrogen phosphate (choline dhp), 1-butyl-3-methylimidazolium dihydrogen phosphate (bmim dhp), *N*-butyl-*N*-methyl pyrrolidinium dihydrogen phosphate (C_4mpyr dhp), choline dibutylphosphate (choline dbp), 1-butyl-3-methylimidazolium acetate, and 1-butyl-3-methylimidazolium methanesulfonate (bmim $MeSO_4$) were synthesized according to the published procedures.[27,28] 1-Butyl-3-methylimidazolium lactate was synthesized by the reaction of sodium L-lactate and bmim Cl in acetone.[27,29]

Salts were identified by ^{1}H NMR, differential scanning calorimetry (DSC) and electrospray ionization mass spectrometry (ESI-MS). Positive and negative ion electrospray mass spectra were recorded with a Micromass Platform electrospray mass spectrometer for samples dissolved in methanol. For choline dbp: T_m 79 °C; ^{1}H NMR (D_2O, 300 MHz) δ 0.84 (m, 6H), 1.32 (m, 4H), 1.53 (m, 4H), 3.12 (s, 9H), 3.44 (m, 2H), 3.80 (m, 4H), 3.99 (m, 2H); ESI-MS ESI$^+$ *m*/*z* 104 (choine$^+$), ESI$^-$ *m*/*z* 209 (dbp$^-$). For TMA dhp: T_m 127 °C; ^{1}H NMR (D_2O, 400 MHz) δ 3.18 (s, 12H); ESI-MS ESI$^+$ *m*/*z* 245 (2TMA$^+$ + dhp$^-$), ESI$^-$ *m*/*z* 268 (TMA$^+$ + 2dhp$^-$).

Structure and Conformational Stability of Cytochrome *c*. The cyt *c* solutions (from horse heart, Aldrich) were prepared to a final concentration of 3 mM in 50 mM sodium phosphate buffer (pH 7.4) or 10 mM Tris acetate buffer (pH 7.4) or ILs. It was found that the addition of 20 wt % water to the ILs used in this study was sufficient to render them liquid at room temperature and aided solubility. Therefore an IL/water mixture was used in all 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 a mercury−cadmium−telluride (MCT) detector. An aliquot (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.[30,31] The second derivative spectrum was obtained with Savitsky-Golay derivative function software for a nine data point window.

The Raman spectra were obtained on a Jasco NRS-1000 spectrometer with a Kaiser Optical holographic notch-plus filter and a liquid N_2-cooled charge-coupled device (CCD) detector. Data were accumulated for 200 s with spectral resolution of 4.0 cm^{-1}. The excitation source was a Coherent Innova 90C Kr laser with a 20 mW beam at a 413.1 nm excitation wavelength. Spectra were collected on samples in bulk condition at room temperature by use of a backscattering geometry. The peak frequencies were calibrated relative to an indene standard and are accurate to ±1 cm^{-1}.

Cytochrome *c* Reduction Assay for Superoxide Anion. Cytochrome *c* activity was determined via a reduction assay with the superoxide anion. Superoxide anion was generated by the hypoxanthine/xanthine oxidase system. The activity was monitored by the rate of reduction of cyt *c* indicated by the absorbance change at 550 nm.[32] Xanthine oxidase from bovine milk (specific activity = 27.3 mg mL^{-1}, Aldrich) and cyt *c* dissolved in ILs (3 mM) were diluted with 50 mM phosphate buffer (pH 7.4) to achieve 1.5 units mL^{-1} and 195 μM. Hypoxanthine (Aldrich) was dissolved in 30 mM NaOH to achieve a final concentration of 5 mM. A typical reaction mixture contained 10 μM cyt *c*, 100 μM hypoxanthine, 5 milliunits mL^{-1} xanthine oxidase, and 50 mM phosphate buffer (pH 7.4) in a final volume of 735 μL. Reactions were carried out at 25 °C. Changes in absorbance were monitored at 1 s intervals on a Cary 100 Bio spectrophotometer (Varian).

Results and Discussion

Selection of Component Ions and Solubility of Cytochrome *c* in ILs. Figure 1 shows the cation and anion structures of the ILs used in this study. We made a number of ILs having

different kosmotropicity. High protein stabilities have been achieved in aqueous solutions with salts composed of chaotropic cations and kosmotropic anions.[21,33] We concentrate the efforts to analyze the effect of the anion rather than the cation, because the kosmotropicity of the anions has a stronger effect on protein stability than that of the cations.

To prepare ILs, various cations were used such as the well-known 1-butyl-3-methylimidazoloim (bmim) cation, *N*-methyl-*N*-butylpyrrolidinium cation (C_4mpy), and strong chaotropic cations tetramethylammonium (TMA) and choline, which are both common in biological systems. The anions used in this study were hydrogen phosphate (dhp), methanesulfonate ($MeSO_4$), acetate, lactate, chloride, tetrafluorobrate, dicyanamide (dca), and saccharinate. Dibutyl phosphate (dbp) was also chosen because it has similar structure to phosphate but lacks a hydroxide group, so as to evaluate the contribution of hydrogen bonding. ILs that have dca and lactate anions are liquid at room temperature. C_4mpyr dhp, choline dhp, and TMA dhp had T_m values of above 100 °C.[25] On the basis of the definition of ILs, these salts may not be classified in the category of ILs but that of organic salts. All of these salts except for TMA dhp form colorless fluids on addition of 20 wt % water. Since TMA dhp did not give a homogeneous solution even with more than 30 wt % water, it was not used for further experiments. All other ILs were prepared by adding 20 wt % water, and the solubility of cyt *c* was examined. ILs containing anions such as saccharinate, chloride, or tetrafluoroborate are not good solvents for cyt *c* even after addition of 20 wt % water. Surprisingly, the ILs containing dca were also not good for cyt *c* even with more than 20 wt % water, despite the slight basicity and hydrogen-bonding potential. This result is consistent with the fact that cyt *c* is difficult to solubilize in most ILs even in the presence of some water.[10,25,34] UV−vis spectra showed that small amounts of cyt *c* (about 0.05 mM) dissolved in ILs containing anions such as saccharinate, chloride, or tetrafluoroborate, but this can be shown to be due to the water contained. However, other ILs solubilized significant amounts of cyt *c* (about 3 mM or 37 mg/mL), forming a dark red homogeneous solution. The solubility of cyt *c* was almost the same as that for ILs prepared with C_4-mpyr, bmim, or choline cations.

We next examined a neat bmim lactate to evaluate the effect of adding water to the IL on the solubility of cyt *c*. However, it proved difficult to dissolve cyt *c* in a neat bmim lactate, although bmim lactate with 20 wt % water dissolved cyt *c* well. After cyt *c* was kept in bmim lactate for several weeks, the solution remained homogeneous, but the solution color had changed from red to yellow, indicating significant structural change of the cyt *c*. This agrees with previous work in which the lactate anion leads to loss of the protein secondary structure after dissolution.[12,35] Thus, even in hydrophilic ILs containing small amounts of water, the solubility of cyt *c* varies widely depending on the structure of the component ions. The important IL structure involved in the solubility of cyt *c* appears to be the oxo acid residue. Addition of the proper amount of water is also important for dissolving protein in hydrophilic ILs without losing the IL properties. The determination of the appropriate amount of water for protein stability is important, and further analyses are under way.

Activity and Structure of Preserved Cytochrome *c* in ILs. Cyt *c* dissolved in some mixtures of hydrophilic ILs with water. The ILs (containing 20 wt % water) composed of dhp, dbp, lactate, acetate, and $MeSO_4$ anions were examined as solvents for cyt *c*, and the resulting activity was determined by superoxide reduction. Henceforth in this paper, IL will refer to a mixture of ionic liquid and 20 wt % water. This water content corresponds that about three water molecules are hydrated to a pair of ions, and accordingly no free water molecules exist. Native cyt *c* was dissolved in each IL at 3 mM concentration and was stored for 3 weeks under air at room temperature, and the activity was analyzed after dilution with buffer immediately prior to the measurement. Figure 2 shows the visible absorption change at 550 nm for superoxide reduction of cyt *c*; the increase in absorbance with time indicates the reduction activity of the cyt *c*. With less active cyt *c* in the solution, the superoxide reaction proceeded more slowly. As shown in Figure 2, the activity varies considerably depending on the ILs. Cyt *c* in choline dhp with 20 wt % water retained similar activity to that in fresh buffer solution. On the other hand, no activity was found in bmim lactate and bmim $MeSO_4$.

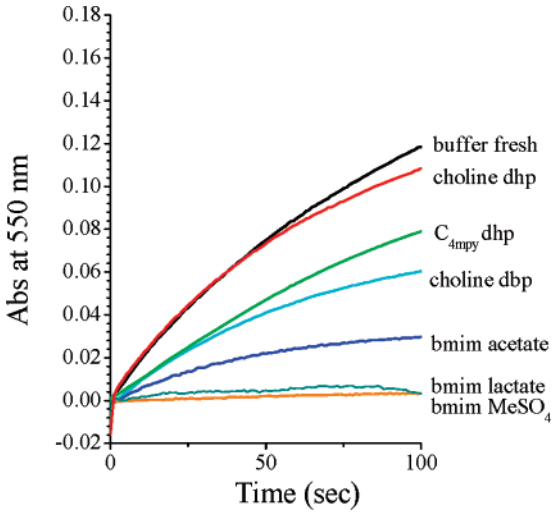

Figure 2. Cyt *c* activity assay in fresh buffer and after 3 weeks of storage: effect of ILs in which cyt *c* was dissolved. All ILs contain 20 wt % water.

The reason for these differences should be attributed to the kosmotropicity and chaotropicity of the ions used. There has, however, been controversy on the kosmotropicity order of several ions. The kosmotropicity cannot be discussed with only one parameter such as *B*-coefficient determined by the viscosity measurement. The relation between kosmotopicity and *B*-coefficient, Gibbs energy, ion radius, and so on should be analyzed for this.[19,20,36−38] For the chaotropic cations used in this study and tetra-*n*-butylammonium (TBA) cation, the chaotropicity should decrease in the following order: TBA ≫ C_4-mpyr ≫ bmim, according to the literature.[36] Larger cations are generally more kosmotropic. Proteins tended to be stabilized by the following chaotropicity order: $(CH_3)_4N^+$ (TMA) > $(C_2H_5)_4N^+$ > $(C_3H_7)_4N^+$ > $(C_4H_9)_4N^+$ (TBA).[39] Accordingly, choline is expected to be a strong chaotropic cation because it has similar structure to chaotropic TMA. However, the hydroxyl group makes the choline both more hydrophilic and more kosmotropic. There are no concrete data on the contribution of the hydroxyl group to the kosmotropicity of the ions. There are still no data on the strict kosmotropicity or chaotropicity of choline cation. Detailed analysis of the effect of the hydroxyl group on the kosmotropicity is underway.

Kosmotropicity cannot be determined simply by the *B*-coefficient; however, it may have a certain tendency and the *B*-coefficient of ions may help to roughly estimate the kosmotropicity order of ions used. The *B*-coefficient is 0.34, 0.25, and 0.13 for dhp, acetate, and $MeSO_4$, respectively.[40] When the kosmotropicity of dbp and lactate can be estimated from

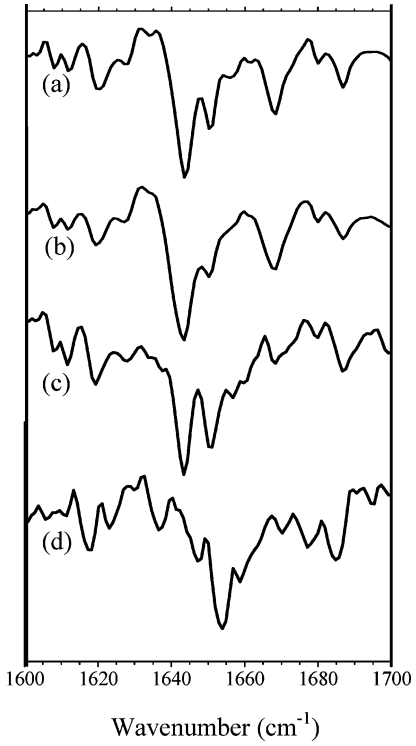

Figure 3. Comparison of the second-derivative spectra in the amide I region of cyt *c* incubated in (a) phosphate buffer, (b) choline dhp, (c) C_4mpyr dhp, and (d) bmim lactate. Spectrum a is fresh, whereas spectra b−d were incubated for 3 weeks.

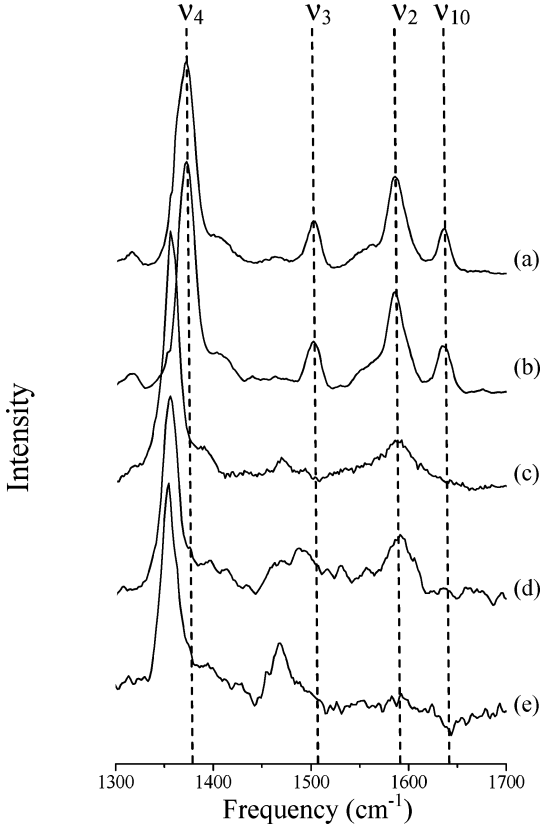

Figure 4. Comparison of the Raman spectra of cyt *c* in (a) phosphate buffer, (b) choline dhp, (c) bmim lactate, (d) bmim dhp, and (e) C_4-mpyr dhp.

the ion structure and size, the kosmotropicity order of anions might be dhp > dbp > acetate > lactate > $MeSO_4$. This order agrees well with Figure 2. We propose that the difference in activity of cyt *c* after time in ILs is based on the kosmotropicity and chaotropicity of the ions composing the ILs. This means that the kosmotropicity order determines protein stability not only in an aqueous solution with low salt concentration but also in ILs with a small amount of water. There are many theories explaining the effect of kosmotropicity on protein stability, such as "water structuring" and "water destructuring" or "salting-in" and "salting-out" interactions, preferential hydration, hydrophobic interactions, and removal of internally bound water molecules from the proteins.[18,19,36] Of course, in the case of bmim lactate, destabilizing proteins by the additional effect such as hydrogen bonding could be relevant.[11] Thus, selecting component ions on the basis of ion kosmotropicity of ILs is important. There are, however, many reports of biocatalysis in ILs containing PF_6^-, BF_4^-, and Cl^- anions.[8,9] Following the kosmotropicity order, these chaotropic anions render protein unstable. According to our results, these anions were not useful in spite of many papers studying enzymatic activities in ILs that include these anions.

Figures 3 and 4 show the effect of the IL structure on the structure of cyt *c*. Figure 3 shows the second-derivative ATR-FTIR spectra of the amide I region of cyt *c* incubated in buffer solution, choline dhp, C_4mpyr dhp, and bmim lactate for 3 weeks. These spectra were compared with that in the fresh buffer solution. The second-derivative spectra are highly sensitive to the secondary structure of proteins. The strong band at 1656 cm^{-1} originates from the α helix of the native structure,[30,31] and bands between 1642 and 1624 cm^{-1} are associated with the *β*-sheet structures in the native state. The spectra in choline dhp and C_4mpyr dhp show no significant difference from spectra in buffer solution, which strongly suggests that cyt *c* retains its secondary structure after dissolution in dhp ILs. On the other hand, the spectrum in the incubated bmim lactate shows a significant change in the main amide bands. We have already found above that cyt *c* undergoes structural change in bmim lactate. ATR-FTIR spectra show the structural changes even when the solution has the same red color.

Figure 4 shows resonance Raman (RR) spectra of cyt *c* in aqueous buffer and several ILs (choline dhp, bmim lactate, bmim dhp, and C_4mpyr dhp). RR spectra of heme proteins have been extensively studied. Particularly useful modes are those in the high-frequency region of the spectrum, which are sensitive to both axial coordination and spin state of the iron atom of heme.[41,42] The RR spectrum of cyt *c* in choline dhp showed intense bands at 1372 (ν_4), 1502 (ν_3), 1586 (ν_2), and 1634 (ν_{10}) cm^{-1}, which indicate that the central iron ion of the heme complex is in the oxidized form and in a six-coordinated low-spin state (Figure 4b).[41] This characteristic is similar to that for cyt *c* in buffer solution as shown in Figure 4a, suggesting that the dissolution of cyt *c* in choline dhp does not produce any structural change in the vicinity of the heme. Furthermore, the low-frequency region between 325 and 625 cm^{-1}, which is the fingerprint region for interaction of the heme with the surrounding protein matrix, also agrees well with observations on buffer solution (data not shown).

On the other hand, the RR spectra of cyt *c* in bmim lactate and bmim dhp showed different characteristics. These spectra showed intense bands at 1355, 1469, and 1592 cm^{-1}, which correspond to a five-coordinated high-spin state in the reduced form.[41] In this state, the bond with one axial ligand, Met-80, is significantly weakened. This result agrees with that inferred from UV−vis spectroscopy.[26] This change of the coordination shell is probably not the result of extensive rearrangement of the polypeptide chain. In the ATR-FTIR measurement, as shown in Figure 3, the secondary structure of cyt *c* in C_4mpyr dhp is

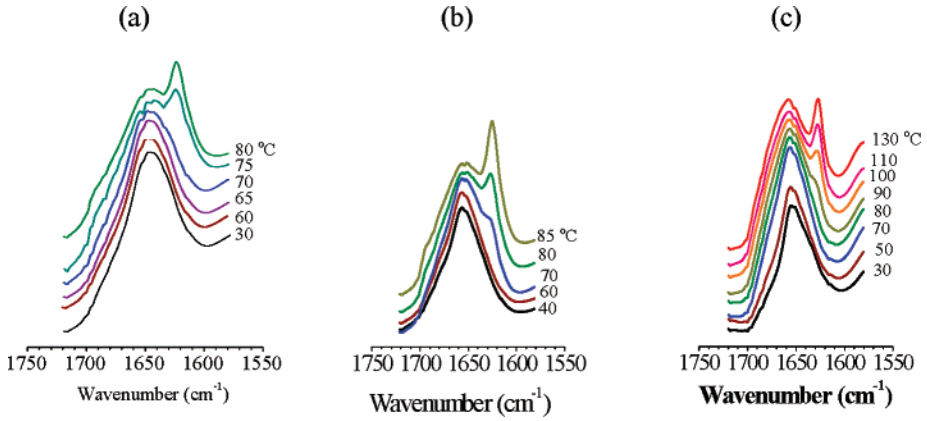

Figure 5. ATR-FTIR spectra in the amide I region of cyt *c* in (a) phosphate buffer, (b) choline dhp with 80 wt % water, and (c) choline dhp with 20 wt % water.

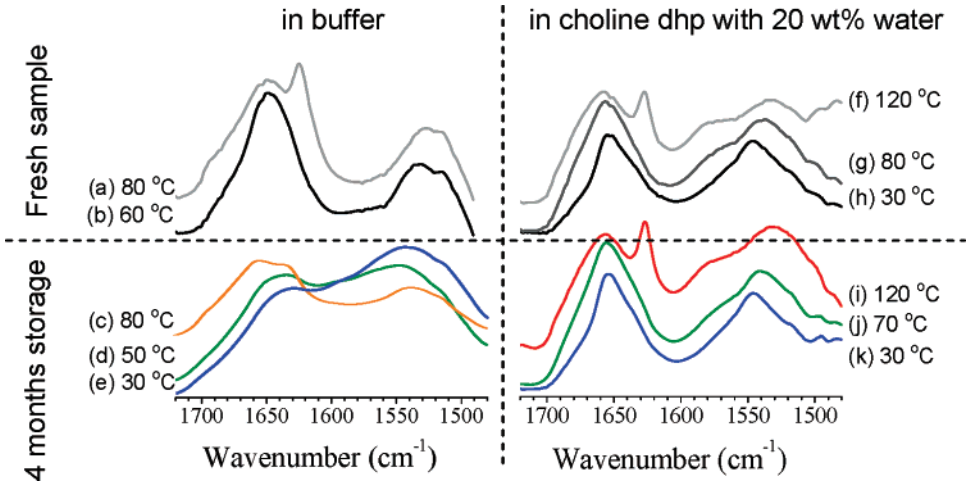

Figure 6. ATR-FTIR spectra in the broader amide I and II region of cyt *c* as a function of temperature. Curves a, b, and f−h show the thermal behavior of a freshly prepared solution of cyt *c* in buffer and choline dhp, respectively. Curves c−e and i−k are related to samples of cyt *c* stored for 4 months at room temperature in buffer or choline dhp and then treated at the indicated temperature.

not significantly different from that in buffer, though the RR spectra suggest a change in the vicinity of heme, indicating a five-coordinated high-spin state in the reduced form, in contrast to the six-coordinated low-spin state in the oxidized form. Consequently, the structural change associated with the transition to the five-coordinated high-spin state is restricted to the heme crevice. These results suggest that though the secondary structure remains unchanged, the tertiary structure is loosened in most ILs. Interestingly, the changes in the secondary structure and at the heme crevice were not observed in choline dhp. The extent of structural change agrees with the decrease of activity in Figure 2. Loss of defined tertiary structure is well-known in studies of the degradation of cyt *c*.[43] Furthermore, it is consistent with previous work reporting that structural changes involving ILs can lead to loss of activity.[12,14] From these results, we suggest that the secondary structure and intramolecular interactions of cyt *c* dissolved in ILs are affected by the kosmotropicity of the component ions, affecting the cyt *c* activity. Dissolved cyt *c* showed no significant structural change in hydrated choline dhp, which is one of the best combinations of chaotropic cations and kosmotropic anions examined in this study.

Thermal Stability and Long-Term Stability of Cytochrome *c* in Choline dhp. In the previous sections, we mentioned that the choline dhp with 20 wt % water is effective as a solvents for cyt *c* for 3 weeks. We now describe the thermal and long-term stability of cyt *c* in choline dhp. Cyt *c* was dissolved in hydrated choline dhp. Differential scanning calorimetry (DSC) thermograms show a dramatic increase in the thermal stability of cyt *c* in choline dhp with 20 wt % water, based on the disappearance of the denaturing peak at about 80 °C in aqueous solutions.[25] Increased thermal stability is further confirmed by the ATR-FTIR spectra at differing temperatures as shown in Figure 5. Thermal denaturation of the protein is indicated by the new band at 1626 cm^{-1}. In the control buffer solution, a new band appeared when the solution was heated to 80 °C. In choline dhp, the band corresponding to the denaturation appears above 100 °C. At 130 °C this band is still slightly less significant than in the aqueous solution at 75 °C.

To investigate the effect of choline dhp on protein stability, the water content was varied. When excess water (80 wt %) is added to the choline dhp, a decrease in thermal stability is suggested by both DSC and ATR-FTIR measurements. Denaturation of cyt *c* is suggested in the DSC trace at 77 °C and in the ATR-FTIR spectrum at 70 °C (Figure 5b). These results show that when choline dhp is used as a solute in aqueous solutions, it is not different from the buffer solution. Thus, it is necessary to keep a high choline dhp salt content, which maintains the IL-like properties of the mixture, and at most a solute quantity of water is tolerable to maintain the high protein thermostability as seen in Figure 5c. The critical amount of water is probably related to the water activity required to maintain the presence of the water molecules involved in the structure of the protein.

Figure 6 shows further evidence of the remarkable stabilization of the protein in the choline dhp. As stated above, significant thermal denaturing of the protein in this IL takes place at around

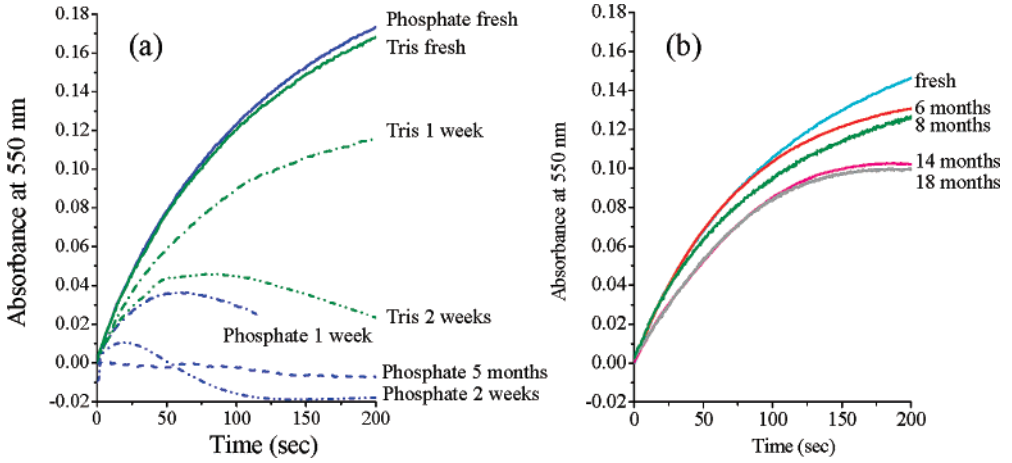

Figure 7. Cyt *c* activity assay: effect of storage time on the activity in phosphate and Tris buffer (a) and choline dhp with 20 wt % water (b) at room temperature.

120 °C, based on the new band at 1626 cm^{-1} in the ATR-FTIR spectra shown in Figure 6 (curves f−h). The conformational differences between native and thermal denatured states have previously been associated with a new band appearing at 80 °C due to partial aggregation (curve a).[44,45] After storage of the cyt *c* in hydrated choline dhp for 4 months under air at room temperature, the bands (curves i−k) changed a little. The thermal denaturatation shows the same course of events as fresh cyt *c*. While the choline dhp solution of cyt *c* remains unchanged during storage, the buffer solution becomes distinctly more viscous, implying that the protein has denatured. Furthermore, the second-derivative ATR- FTIR spectra of cyt *c* in buffer solution before and after storage for 4 months show significant change, indicating loss of the secondary structure.[26] The charge-transfer band at 695 nm in UV−vis spectroscopy also indicates that the peptide chain undergoes a transition from the native-state globular form to an extended random configuration, and loses an axial ligand on the heme after storage in a buffer solution. On the other hand, these spectra revealed no significant change in the cyt *c* structure when preserved in the hydrated choline dhp.[26]

After various periods of storage in these solutions, the activity of cyt *c* was then determined by superoxide reduction. Figure 7 shows a typical superoxide reduction, monitored by the visible absorption at 550 nm. The conditions were the same as those in Figure 2. The less active is the cyt *c* in the solution, the slower is the superoxide reduction. Figure 7a shows activity of cyt *c* in both phosphate and Tris buffers. After several weeks, the cyt *c* activity, in the buffered solutions at room temperature shown in Figure 7a, is effectively extinguished, in agreement with other reports (e.g., Sigma product information). On the other hand, as seen above, the choline dhp cyt *c* solution remains almost as active as the fresh samples after being stored for at least 6 months at room temperature.[26] The extended experiments reported here show that even after storage for *18 months at room temperature*, the activity remained. Clearly, cyt *c* can retain its activity for a remarkably long time in choline dhp. The absence of degradation processes in dhp-containing ILs suggests that the chemical and structural steps involved in the degradation processes are much diminished, perhaps as a result of the low water content. Unfolding, relaxation of the polypeptide chain, and breakdown of the associated hydrogen bonds are general reasons for chemical degradation in proteins, so that the stabilizing effects obtained in choline dhp should be applicable to a wide range of proteins. The dhp-containing ILs might also be effective for the refolding of polypeptide chains.

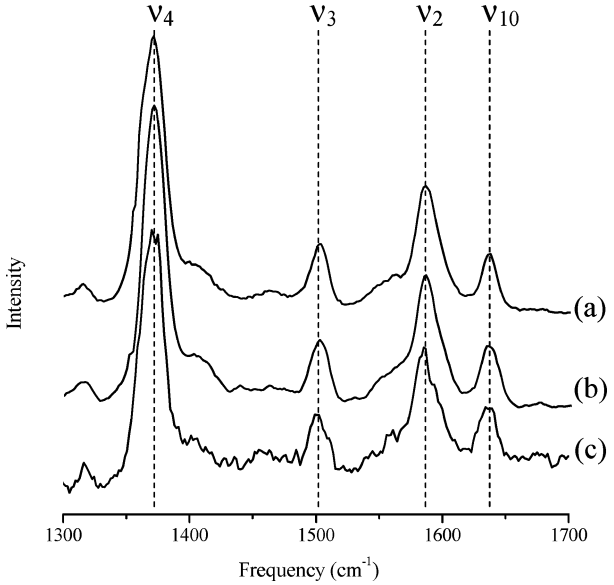

Figure 8. Comparison of the Raman spectra of cyt *c*: in (a) phosphate buffer, fresh; (b) choline dhp, fresh; and (c) choline dhp, storage for 1 year.

The superoxide assay indicates that activity is retained during long periods in choline dhp. To confirm the retention of structure after long-term storage, Figure 8 c shows RR spectra of cyt *c* in choline dhp dissolved and stored for 1 year at room temperature. The characteristics indicate that iron is in the oxidized form and in a six-coordinated low-spin state. This is similar to results with cyt *c* in fresh buffer solution, as shown in Figure 8a, suggesting that dissolution of cyt *c* in hydrated choline dhp does not cause structural change at the vicinity of the heme even after storage for over 1 year at room temperature. In a buffer, the RR spectrum based on dissolved cyt *c* could not be detected because of strong fluorescence after 1 year of storage. It is thus confirmed that a great improvement in conformational stability results from storage in choline dhp with 20 wt % water, giving rise to significant long-term activity.

Conclusion

High solubility of cyt *c* was achieved in hydrated ionic liquids (ILs) that had oxo acid residues. A hydrated choline dhp retains ionic liquid properties even with a small amount of water being present. The secondary structure and intramolecular interactions

(heme crevice) were strongly influenced by the kosmotropicity of the component ions. Kosmotropicity thus clearly plays an important direct regulatory role on ILs in biochemistry. Dissolved cyt *c* showed no significant structural change in the hydrated choline dhp, which is one of the best combinations of chaotropic cations and kosmotropic anions. Furthermore, dissolved cyt *c* showed excellent thermal stability and long-term stability in the hydrated choline dhp, exhibiting activity even after 18 months of storage.

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