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Fluorescence and Circular Dichroism Spectroscopy of Cytochrome c in Alkylammonium Formate Ionic Liquids

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Abstract

The structural stability of cytochrome c has been studied in alkylammonium formate (AAF) ionic liquids such as methylammonium formate (MAF) and ethylammonium formate (EAF) by fluorescence and circular dichroism (CD) spectroscopy. At room temperature, the native structure of cytochrome c is maintained in relatively high ionic liquid concentrations (50%–70% AAF/water or AAF/phosphate buffer pH 7.0) in contrast to denaturation of cytochrome c in similar solutions of methanol or acetonitrile, with water or buffer co-solvents. Fluorescence and CD spectra indicate the conformation of cytochrome c is maintained in 20% AAF-80% water from 30 – 50 °C. No such temperature stability is found in 80% AAF-20% water. About one third of the enzyme activity of cytochrome c in 80% AAF-20% water can be maintained as compared to phosphate buffer and this is greater than the activities measured in corresponding methanol and acetonitrile aqueous solutions. This biophysical study shows that AAFs have potential application as organic solvent replacements at moderate temperature in the mobile phase for the separation of proteins in their native form by reversed phase liquid chromatography.

Introduction

Particularly in the last decade, ionic liquids have been used widely in various aspects of chemistry and biochemistry, such as electrolyte phases for biosensors (1), mobile or stationary phases for separation science (2), and as precipitating agents or additives for protein crystallization (3). Ionic liquids have also been used as solvents or co-solvents, rather than traditional organic solvents, to provide a non-aqueous environment for biocatalysts (4) and the effect of ionic liquids on the structure and activity of enzymes has been reviewed (5). Enzymatic activity in organic solvents requires the presence of trace water to be associated with the protein to maintain the many hydrogen bonds within the native structure. This is more likely with hydrophobic solvents such as hydrocarbons (6) but not more polar hydrophilic solvents such as ethers or ketones which can remove water from the enzyme leading to denaturation.

Ionic liquids with an N-alkylpyridinium cation or an 1-alkyl-3-methylimidazolium cation with certain anions have been shown effective for stabilizing proteins. In 2004, Baker and co-workers (7) provided an early detailed report of protein spectroscopy within an ionic liquid (1-butyl-1-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide, [bmpp][NTf₂]) by monitoring the fluorescence of the single tryptophan residue in the sweet protein monellin. The onset of thermal unfolding of monellin in [bmpp][NTf₂] occurred at about 100 °C as compared to 40 °C in water. In the same year, J. L. Iborra and co-workers (8) reported the use of both fluorescence and circular dichroism (CD) spectroscopy to analyze the α -chymotrypsin stabilization (at 30°C and 50°C) by the ionic liquid, 1-ethyl-3-

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methylimidazolium (emim)[NTf₂], as compared to water, 3M sorbitol, and 1-propanol. Kinetic analysis of the enzyme showed a strong stabilization power of [emim][NTf₂]; fluorescence spectra showed [emim][NTf₂] can maintain the native structure of α -chymotrypsin and CD spectra showed [emim][NTf₂] can enhance β -strands (a stretch of amino acids typically 5–10 amino acids long with fully extended peptide backbone) (8). Infrared spectrophotometry has been used to show that the secondary structures of protease (α -helix between 1650 and 1658 cm⁻¹, β -sheets between 1620 and 1640 cm⁻¹) in [emim][CH₃COO] were maintained the same as in water but were disrupted in acetonitrile (MeCN) (9). More recently, the vibrational reorientation dynamics associated with loop 1 of domain I in human serum albumin has been studied as a function of water addition to 1-butyl-3-methylimidazolium ionic liquids (10).

The solubility and stability of cytochrome c, one of the most thoroughly studied metalloproteins in various aqueous solutions (11) including those with alkylammonium surfactants (12) and solvents of different dielectric constants (13), has also been investigated in ionic liquids. The influence of the fluorescence emission spectrum upon addition of 5 mM ionic liquid 1-butyl-3-methylimidazolium chloride ([bmim]Cl) to a 10 μ M cytochrome c solution (pH 7.4) was significant (14). The native cytochrome c gives a single peak at 348 nm. In ionic liquid [bmim]Cl, the single tryptophan peak is red shifted to 369 nm and a new peak at 432 nm occurs. This change suggests that cytochrome c interacts with the [bmim]⁺ and forms a complex with a unique emission spectrum. A change of the ionic liquid to NTf₂ caused some loss of structure in the heme pocket of cytochrome c. The electron transfer of electrode immobilized cytochrome c during redox in the presence of butylimidazolium NTf₂ exhibited a loss of the characteristic Fe(II)/Fe(III) redox signal (14). In contrast, the peroxidase activity of cytochrome c in alkylimidazolium ionic liquids is retained (15) as well as that for cytochrome c immobilized without denaturation within a single-walled carbon nanotube – ionic liquid composite based on either 1-butyl-3-methylimidazolium tetrafluoroborate or 1-butyl-3-methylimidazolium hexafluorophosphate (16). Cytochrome c modified by poly(ethylene oxide) chains has enhanced solubility in the ionic liquid 1-ethyl-3-methylimidazolium NTf₂ without denaturation (17). In a 25 volume % 1-butyl-3-methylimidazolium chloride-75% Tris buffer (pH 7.4), the higher order structures of cytochrome c are largely retained; in a 50 % composition of the same solution, cytochrome c reached a state similar to a urea-denatured state (18).

It is very likely that the solubility and stability of proteins in ionic liquids is affected by salt kosmotropicity, the ability to contribute to the stability and structure of water-water interactions therefore stabilizing proteins (19, 20). Different ionic liquid cation/anion pairings based on the Hofmeister series (a classification of ions in order of their ability to change water structure) can predict somewhat the stabilities of proteins (21). Strongly kosmotropic anions such as dihydrogen phosphate (dhp) and a chaotropic cation such as choline are expected to stabilize proteins (20). However, choline dhp is actually a white solid and not a room temperature ionic liquid (22). Patterns of protein unfolding and protein aggregation have been compared for ribonuclease A in choline dihydrogen phosphate and two imidazolium ionic liquid solutions from 0 to 4 M. The choline dihydrogen phosphate enhanced thermostability while the 1-ethyl-3-methylimidazolium dicyanide was a strong denaturant (23). A 20% choline dhp aqueous solution is considered to be a biocompatible solvent (19) as indicated by cytochrome c having no significant structure changes (20). Even after 6 months of storage at room temperature, the activity and structure of cytochrome c are maintained, compared with the usual inactivation after one week in buffered aqueous solution (20). At 80 wt%, the cytotoxicity of choline dhp and choline saccharinate is less than that for 1-butyl-3-methylimidazolium lactate (24).

One advantage of protic ionic liquids with nonquaternary-alkylammonium cations over aprotic ionic liquids is the proton activity is tunable by the choice of the organic weak base and this can be important for protein stabilization. Ethylammonium nitrate has been shown to prevent aggregation of denatured lysozyme and can bring back 75% of its activity at the 1.6 mg/mL level (25). More recently, ethylammonium nitrate at about 30 wt% in a sucrose-water solution has been shown to permit reversible thermal unfolding/refolding of lysozyme at a high concentration (200 mg/mL) as well as long term stabilization without aggregation (26). The availability of the N-H proton for alkylammonium nitrates has been shown to be a critical factor for maximal refolding of lysozyme as a function of temperature (27).

Short chain alkylammonium formate (AAF) compounds with a cation such as methylammonium or ethylammonium in conjunction with the formate anion are room temperature ionic liquids with freezing points well below 0 °C and can be considered to represent an amphiprotic buffer near a pH of 7.4. AAF ionic liquids are easy to synthesize inexpensively in 100–200 mL quantities (28), a definite advantage for analytical or commercial applications.

Solvatochromic studies of AAFs have shown some polarity factors similar to methanol (MeOH) but polarizability is more similar to water. As the alkyl chain of the alkylammonium Bronsted base decreases from n-butylammonium to methylammonium, the polarity parameter E_T^N of AAF (28) approaches the polarity of MeOH ($E_T^N = 0.8$), as measured by the Reichardt's betaine dye (29). The Kamlet-Taft (30) hydrogen-bond acidity or proton donating value (α) for methylammonium formate (MAF) of 0.91 or that for ethylammonium formate (EAF) of 0.85 was closer to the value of 0.93 for methanol (28, 31). The Kamlet-Taft hydrogen-bond basicity or proton acceptor β value of about 0.7 for MAF and EAF was much more similar to 0.62 of methanol as compared to 0.18 for water (28, 31). However, the Kamlet-Taft dipolarity/polarizability π^* values of 0.8 for EAF and 1.0 for MAF are quite similar to 1.1 for water as compared to 0.60 for MeOH (28, 31).

The polarity and dielectric constant similarities (32) between alkylammonium ionic liquids and methanol (MeOH) can explain the replacement of MeOH as the mobile phase modifier by an AAF to permit totally aqueous reversed-phase liquid chromatography (31, 33). Certain pharmaceutical mixtures such as nitrofurantoin-furazolidone, difficult to separate using a MeOH modifier, have been separated with better resolution in a shorter time using MAF (28). Ion-pair liquid chromatography of organic acids using ethylammonium formate (EAF) instead of MeOH in the mobile phase still requires the use of a conventional surfactant such as tetrabutylammonium ion (34). In contrast to alkylammonium nitrates, the formation of self-assembled aggregates in AAF ionic liquids seems to be limited (35), possibly due to the amphiphilic (both hydrophilic and hydrophobic) nature of AAFs as well as their likely neutral acid and base character (36). We have some liquid chromatographic evidence that proteins may be separated without extensive denaturation using EAF (37) but there is denaturation with MeOH as the mobile phase modifier.

Quite recently (38), the thermal stability and activity of lysozyme has been reported in AAF ionic liquids such as EAF, propylammonium formate, 2-methoxyethylammonium formate, and ethanolammonium formate. Circular dichroism (CD) results of lysozyme in 25%, 50%, and 75% AAF indicated EAF and 2-methoxyethylammonium formate were effective refolding additives only at the 25% level as compared to the other AAFs. Propylammonium formate at about 60% and ethanolammonium formate, even at 75%, stabilized lysozyme from significant unfolding when the temperature was cycled from 25 °C to 90 °C and refolding occurred upon temperature reduction back to 25 °C. The activity enhancement of lysozyme followed the same trends as the protein denaturing-renaturing experiments.

In this work, cytochrome c, a simpler protein with only one tryptophan residue, has been chosen as the model protein to be studied with AAF protic ionic liquid solvents. To the best of our knowledge, a comparison of cytochrome c structure stability by both fluorescence and CD spectroscopy, particularly in MAF, either at ambient or elevated temperatures has not been done. This paper will show using fluorescence and CD that conformation stability of cytochrome c is maintained in different aqueous mixtures of MAF and EAF in contrast to aqueous MeOH and acetonitrile (MeCN) solutions. The thermal stability of cytochrome c from 20 °C to 80 °C in AAF, MeOH, and MeCN as well as its enzyme activity will be reported.

Materials and Methods

Instrumentation

All CD measurements were taken on a Jasco spectropolarimeter, Model J-810. The temperature was controlled at 20 °C $\pm$ 0.1 °C by circulating water through a jacket around the cell. The cell was 1.0 cm cylindrical quartz cuvet.

All fluorescence measurements were carried out on a Perkin-Elmer Model LS 55 fluorescence spectrometer. The excitation and emission wavelengths were set to 280 and 340 nm, respectively. A 4 mL quartz cell of 1 cm light-path was used for emission measurements. The cuvette temperature could be increased from room temperature (20 °C) to 80 °C, which was controlled by circulating water from an isothermopump (Fisher Isotemp Immersion Circulator Model 730).

Chemical Reagents

All water was distilled deionized water made in the laboratory using an E-pure Barnstead Millipore system (Dubuque, IA). Methylamine (33% wt. in absolute ethanol solution) and ethylamine (70% v/v in water) were supplied from Aldrich (Milwaukee, WI). The formic acid (98% assay) was obtained from Fluka (St. Gallen, Switzerland). Methanol (MeOH) and acetonitrile (MeCN) were purchased as HPLC grade from Pharmco Products (Brookfield, CT). ACS reagent, D-(+)-glucose, was obtained from Sigma-Aldrich (St. Louis, MO). Proteins, cytochrome c (99% purity) from horse heart prepared without using TCA, β -nicotinamide adenine dinucleotide hydrate from yeast, adenosine 5'-diphosphate sodium salt from bacterial source, adenosine 5'-triphosphate disodium salt hydrate, glucose-6-phosphate dehydrogenase Type VII (crystalline suspension in 3.2 M (NH₄)₂SO₄, pH 7.0) from bakers yeast, were also obtained from Sigma-Aldrich (St. Louis, MO).

Synthesis of Ionic Liquids

All AAF ionic liquids were synthesized in the lab by controlling the exothermic acid-to-base reaction between a solution of the alkylamine and one of formic acid (36). We improved the previous reported synthetic procedure by controlling temperature around 0 °C to reduce side reactions, protecting the reaction mixture using high purity nitrogen, and purifying AAF using vacuum freeze-drying (28). First the formic acid was diluted with 1:1 HPLC grade methanol to reduce synthesis temperature. Then the methylamine or ethylamine and the formic acid solutions were cooled for about 15 minutes in a mixture of ice and dry ice. After that, the chilled solution of formic acid and methanol was transferred to a three-neck round bottom flask, which was kept in the mixture of ice and dry ice. The methylamine or ethylamine solution was set to drip from an Aldrich addition funnel, cooled by pumping ice water through its jacket, at the rate of every 2~3 seconds per drop by adjusting the Teflon needle valve on the funnel. The addition funnel was capped with a high-vacuum valve adapter to minimize the evaporation of methylamine or ethylamine. The reaction solution in the flask was bubbled with high purity nitrogen and stirred gently for about 3 hours.

Ethanol or methanol was extracted from the product by a vacuum pump, and water and residual alcohol were removed from product by vacuum freeze-drying for 48 hours. After purifying, MAF or EAF remained a clear and colorless liquid, with a batch size of about 150 mL. Without reaction temperature control, the product MAF or EAF appeared yellow. For specific experiments, MAF or EAF was purified by solvent phase extraction (SPE) by adding activated carbon into 5 mL of ionic liquid, agitating, and filtering the ionic liquid. The AAFs were used shortly after synthesis and were always stored in the refrigerator to minimize the possibility of amide formation (36).

Choline formate was also synthesized in an analogous way but its yellow color caused a high background reading preventing any CD measurements and as expected was very alkaline in pH. Even diluted solutions remained highly basic in pH which would not be compatible for most proteins.

The concentrations of cytochrome c for the CD measurements were 10 μ M. This concentration can give at least 3 mdeg signal which is higher than three times of signal/noise. Since the tryptophan fluorescence is sensitive, the fluorescence of 10 μ M protein may exceed the maximum scale of the fluorescence instrument. The concentrations of cytochrome c for fluorescence measurements are 1 μ M.

The activity of cytochrome c was determined using a 735 μ L mixture containing 10 μ M cytochrome c, 100 μ M hypoxanthine, 5 milliunits/mL xanthine oxidase, and 50 mM phosphate buffer (pH 7.4). The reduction of cytochrome c can be monitored by the absorbance change at 550 nm. The measurement was carried out at 25 $^{\circ}$ C with 1 s intervals on Agilent 8453 UV-Vis spectrophotometer.

Results and Discussion

CD spectra of cytochrome c in AAF or organic solvent/water solutions

The CD background of both MAF and EAF in the far UV range (200 – 250 nm) was too noisy to reveal the changes of secondary structure of proteins. The high tension (HT) value (or dynode voltage) of both pure MAF and EAF solutions soared to the highest value, 1,000 V at 250 nm and below, due to the impermeability of ionic liquids at far UV range (31). The background of MAF and EAF in the far UV range could be improved by SPE. The noise range of MAF before SPE was from -100 mdeg to 150 mdeg, while the noise range of MAF after SPE was narrowed from 10 mdeg to 35 mdeg because of the likely absorption of some impurities by activated carbon. Although the noise range of MAF after SPE was reduced, it still had too big an influence on the signal to discern any secondary structure of proteins. Only the tertiary structure of proteins could be measured in AAF ionic liquid solvents from 250 – 500 nm.

Figure 1 shows a comparison of the Soret CD spectra of cytochrome c in 40% modifier solvent MeCN, MeOH, or MAF and 60% water as well as in 100% water. A Soret peak is a very strong absorption band in the blue region of the optical absorption spectrum of a heme protein (39). The bisignate band shape with a negative peak at 416 nm and a positive peak at 402 nm has been assigned to heme-polypeptide interactions close to the heme crevice (40) and indicates native cytochrome c. The bisignate spectra for cytochrome c in the MAF solution and 100% water are quite similar. Denaturation of cytochrome c in the MeOH and MeCN solutions is evident by the disappearance of the negative peak with only the positive peak present. CD studies of cytochrome c in which the phenylalanine (Phe) 82 residue has been replaced by an aliphatic amino acid have shown loss of the negative Soret peak (41). The increased positive peak and the disappeared negative peak suggested that the orientation of the heme with respect to aromatic residues such as Phe 82 in the polypeptide backbone of

the heme crevice became disordered through tilting of the heme plane in the pocket or through a loss of stable tertiary structure in the crevice (42). The denaturant-induced unfolding mechanism is shown as $F \rightleftharpoons MG \rightleftharpoons U$ where F represents natively folded state, U represents fully unfolded state and MG represents the molten globule-like intermediate (43). MG resembles a molten globule type of state in that the native tertiary structure is lost whereas the secondary structure is retained (42). With increasing MAF concentration, the positive peak increased and the negative peak disappeared (Figure 2, top). It appeared that cytochrome c started to denature at 50% AAF in water and is fully denatured at 70%. In a MeCN solution, cytochrome c started to denature at 30% and is fully denatured at 40%. In a MeOH solution, cytochrome c started to denature at 30% and is fully denatured at 70% (data not shown). However, as evidenced by the negative peak, cytochrome c denatured to a lesser extent in MAF than in MeOH and MeCN. The ranking of denaturation ability for cytochrome c was $\text{MeCN} > \text{MeOH} > \text{MAF}$. Only in 40% MeCN, cytochrome c had already fully denatured, but it still maintained its native structure in 40% MAF as shown in Figure 1.

The high tension (HT) signal is the dynode voltage of the CD detector, and can be detected in the CD measurement of the ellipticity, as shown in Figure 2 (bottom). The HT value increased with higher concentrations of MAF and the spectra showed about a 4 nm blue shift, indicating induced alterations in the electron configuration of the heme iron center from a typical low-spin species (Fe^{2+} , $S=0$, or Fe^{3+} , $S=1/2$) to one of mixed-spin state (40). The highest HT values in MeOH and MeCN were about 320 V (data not shown) while the highest HT value in MAF was about 300 V. This indicated that more cytochrome c in 90% MAF maintained its typical low-spin species than in 90% MeOH and MeCN.

Fluorescence of cytochrome c in AAF or organic solvent/water solutions

Fluorescence is another approach to monitor the denaturation process of cytochrome c that contains one tryptophan residue bonded to the heme. As cytochrome c denatures, the sixth coordination site binds to ligands, such as OH^- and CN^- , and the tryptophan moves away from the heme, inducing an increased fluorescence (44). As the change of emission wavelength maxima is less than 2 nm, we measured the emission signal at a typical maximum of 340 nm which was corrected for the solvent blank signal. A plot of tryptophan fluorescence of cytochrome c as a function of 10% volume percent increments from 10 to 90% of MAF, MeCN, MeOH, N-methylformamide (NMF), and dimethylsulfoxide (DMSO) in water is described in the following text but not shown. In MAF, cytochrome c showed consistently the lowest fluorescence, indicating that cytochrome c remained in a native conformation to about 80% solvent in water but even beyond that, the fluorescence was low. For MeCN, the fluorescence was next lowest increasing slightly from 30% solvent in water to a plateau at about 50%. The fluorescence profile for MeOH was higher than MeCN with an increase from 60% solvent in water to a plateau at 80%. A much more significant rise in fluorescence from 40% solvent in water to 70% for NMF and 50% solvent in water to 70% for DMSO was observed for these hydrophilic solvents confirming that highly polar organic solvents can cause significant denaturation of proteins such as cytochrome c.

CD spectra of cytochrome c in AAFs or organic solvent/phosphate buffer

Figure 3 shows the positive ellipticity peak height at 404 nm plotted as a function of concentrations of MeOH, MeCN, EAF, and MAF in phosphate buffer, pH 7.0. Both EAF and MAF had a wider stabilization range in phosphate buffer, from 10% to 70%, where cytochrome c denatured less in an AAF than in MeOH and MeCN. In 10% AAF and MeOH solutions, the ellipticity signals were about 3 mdeg, which was very close to the 2.7 mdeg in pure phosphate buffer 7.0 solution. The CD peak heights in 90% MeOH, EAF, and MAF in phosphate buffer solutions were all somewhat lower in the 5–6 range than that (8–10) for

90% MeOH- and 90% MAF- water solutions due to the stabilization effect of phosphate buffer to proteins. In addition, based on the magnitude of the CD signal profile in the intermediate solvent range from 30–70%, the order of the denaturation ability of solvent in both water and phosphate buffer solutions was consistent at MeCN > MeOH > EAF > MAF. This solvent order is the same if ordered from high to low in hydrophobicity based on Reichardt's polarity constant (28). Hydrophobic interaction of the organic solvent or AAF with the lone tryptophan and perhaps other aromatic amino acids is the likely cause of cytochrome c denaturation. The closer similarity of the Kamlet-Taft π^* polarizability value of MAF as compared to EAF to that of water could explain the measureable difference in cytochrome c stability between these two AAF ionic liquids (28). The reported lack of aggregation of formate based ionic liquids as studied by mass spectrometry (35) may also be an important factor in preventing protein aggregation.

Fluorescence of cytochrome c in AAFs or organic solvent/phosphate buffer

Figure 4 showed the tryptophan fluorescence of cytochrome c as a function of volume percent of EAF, MAF, MeCN, and MeOH in phosphate buffer 7.0 solutions. The plotted fluorescence signal has been corrected for the blank solvent. The tryptophan fluorescence of cytochrome c in MeOH/phosphate buffer solutions started to increase at 40% MeOH solvent composition and reached its peak at 70%, similar to the trend found in MeOH/water solutions. In MeCN/phosphate buffer solutions, the tryptophan fluorescence first increased to 28 units at 50% MeCN solvent composition and then it dropped to 12 units at 70%, before dramatically increasing to 38 units at 90% MeCN. In MAF/ phosphate buffer solutions, tryptophan fluorescence of cytochrome c was maintained as low as 20 units until 70% MAF solvent composition, and then it slightly jumped to 40 units at 90%. The similarity of the MAF and MeCN fluorescence profiles using phosphate buffer was also found using water as the co-solvent. Basically cytochrome c in MAF/phosphate buffer, pH 7.0 solutions showed weak tryptophan fluorescence, indicating protein conformation stability that was somewhat better than that using EAF. Fluorescence energy transfer kinetic studies (45, 46) of cytochrome c in AAF ionic liquids represent an interesting future endeavor.

Thermal denaturation of cytochrome c in AAFs

Figure 5 shows the tryptophan fluorescence emission for cytochrome c as the temperature increases from 20 °C to 80 °C. In 40 mM, pH 7 phosphate buffer, the tryptophan fluorescence emission at 340 nm is stable throughout the temperature range, suggesting that the tryptophan does not move away from the heme. In 20% MAF- and 20% EAF-80% water, the fluorescence signals are about 1.5–2 times that in phosphate buffer, and are also stable although there is a small increase at 60 °C. In 80% MAF- and 80% EAF-20% water, the fluorescence intensities are about 5~6 times of that in phosphate buffer, and are also stable throughout the temperature range. But in 80% MeOH-20% water, the tryptophan fluorescence emission starts to drop at about 40 °C. At 80 °C there is only one fourth of the tryptophan fluorescence emission at 20 °C. When the experiment was stopped at 80 °C, there appeared to be aggregation of cytochrome c in the 80% MeOH solution near the inside cuvette wall. But in 80% MAF and EAF, there is no aggregation of cytochrome c in the solution.

Figure 6A shows the Soret CD spectra (350–450 nm) of cytochrome c as a function of increasing temperature from 20 °C (bottom curve at 416 nm) to 80 °C (top at 416 nm) in 40 mM phosphate buffer 7.0. As the temperature increases, the CD spectra still keep a bisignate band shape. The positive peak at 404 nm only increases about 0.5 mdeg, and the negative peak at 416 increases about 1 mdeg. A similar trend was found for cytochrome c in water. The stability of cytochrome c at elevated temperature in phosphate buffer or water is good.

As the temperature increases from 20 °C to 80 °C, the Soret CD spectra (350–450 nm) of cytochrome c in 20% MAF- and 20% EAF-80% water show that both the positive and negative peaks are disappearing (Figure 6B). Above 50 °C, the bisignate band shape totally disappears. This impact of increasing temperature on the structure changes of cytochrome c is different in CD spectral appearance (Figure 6B) from the CD profile (positive peak retained) of increasing concentration of the co-solvent (Figure 2). The 20% AAF ionic liquid composition in water may degrade the heme crevice at high temperature, while a high concentration of AAF ionic liquid only change the orientation of the heme with respect to aromatic residues in the polypeptide backbone of the heme crevice. Previously, stability of cytochrome c in a 20% choline dhp -80% water solution was evident up to 70 °C by infrared spectroscopy (47). Similarly, lysozyme also maintained stability, refolding between 25 and 50% EAF solvent composition, after exposure to 90 °C (38).

In 80% MAF- and 80% EAF-20% water at room temperature, positive peaks near 400 nm still exist in both AAF solutions. The CD traces as a function of temperature are similar for both MAF and EAF but are shown only for 80% EAF-20% water (Figure 6C). At a temperature of 30 °C, the positive peak disappears and the amplitude of the scan decrease as the temperature increases. Cytochrome c conformation stability apparently cannot be maintained at high temperature in high concentrations of AAF ionic liquids. Enhanced hydrophobic solvation of the aromatic amino acids by the AAF solvent which changes their orientation is a likely explanation.

Enzyme kinetics of cytochrome c in AAF

Cytochrome c activity can be measured by reduction assay with the superoxide anion which can be generated by the hypoxanthine/xanthine oxidase system (48).

When ferricytochrome c is present, it can react with the superoxide anion intermediate produced from the xanthine oxidase reaction as follows.

The reduction of cytochrome c can be monitored by the absorbance change at 550 nm. Figure 7 shows the cytochrome c activity assay in 50 mM phosphate buffer, MAF, EAF, MeCN, and MeOH (all 80% in water). The slopes (between 40 and 100 s) of phosphate buffer, MAF, EAF, MeOH, and MeCN are 6.2×10^{-4} , 2.1×10^{-4} , 2.1×10^{-4} , 1.8×10^{-4} , and 1.2×10^{-4} , respectively. The activity of cytochrome c in this high volume percent of MAF and EAF is about one third of that in 50 mM phosphate buffer (pH 7.4) but greater than that in either MeOH or MeCN. However, the possibility of xanthine oxidase activity loss due the presence of AAFs cannot be discounted. The activity of lysozyme in more dilute AAF solutions has been shown to be enhanced (38).

An interesting paper (49) has described the self-polymerization of 2-hydroxyethylmethacrylate in the ionic liquid choline formate without the need of light, heat, oxygen, or a free radical initiator. It has been postulated that formate may have a tendency to form radical products that initiate polymerization. A study of this potential radical activity in AAF ionic liquids and its biological implications is planned with the Miami University electron paramagnetic resonance laboratory.

Conclusion

The Soret CD region and tryptophan fluorescence spectra of cytochrome c have shown that AAF ionic liquids could maintain the native structure of cytochrome c in relatively high concentration (50–70 % AAF/water) compared to MeOH and MeCN. The CD and

fluorescence results of cytochrome c in phosphate buffer 7.0 have similar results to cytochrome c in water. The protic ionic liquids MAF and EAF can play a role as a compatible pH buffer. As temperature increases from 30 to 80 °C, cytochrome c does not seem to denature in phosphate buffer 7.0 but does so in 80% AAF- 20% water. The denaturation pattern of cytochrome by increasing temperature (losing both the positive peak at 404 nm and the negative peak at 416 nm in the CD spectra) is different from that by increasing the concentration of AAF ionic liquids (increasing positive peak at 404 nm and decreasing negative peak at 416 nm). At room temperature, cytochrome c activity in 80% MAF- or EAF-20% water is also diminished as compared to the activity in phosphate buffer. However, we anticipate AAF ionic liquids will be promising replacements at moderate percentages for organic solvent modifiers now commonly used for the reversed phase HPLC separation of proteins.

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References

1. Wei D, Ivaska A. *Anal. Chim. Acta.* 2008; 607:126–135. [PubMed: 18190800]
2. Shamsi SA, Danielson ND. *J. Sep. Sci.* 2007; 30:1729–1750. [PubMed: 17623452]
3. Judge RA, Takahashi S, Longnecker KL, Fry EH, Abad-Zapatero C, Chiu ML. *Crystal Growth and Design.* 2009; 9:3463–3469.
4. Weuster-Botz D. *Chem. Rec.* 2007; 7:334–340. [PubMed: 18050266]
5. van Rantwijk F, Sheldon RA. *Chem. Rev.* 2007; 107:2757–2785. [PubMed: 17564484]
6. Zaks A, Klibanov AM. *J. Biol. Chem.* 1988; 263:3194–3201. [PubMed: 3277967]
7. Baker SN, McCleskey TM, Pandey S, Baker GA. *Chem. Commun.* 2004:940–941.
8. Diego TD, Lozano P, Gmouh S, Vaultier M, Iborra JL. *Biotechnol. Bioeng.* 2004; 88:916–924. [PubMed: 15515169]
9. Zhao H, Jackson L, Song Z, Olubajo O. *Tetrahedron:Asymmetry.* 2006; 17:2491–2498.
10. Page TA, Kraut ND, Page PM, Baker GA, Bright FV. *J. Phys. Chem. B.* 2009; 113:12825–12830. [PubMed: 19711930]
11. Myer YP. *Biochemistry.* 1968; 7:765–776. [PubMed: 5689422]
12. Chamani J, Moosavi-Movahedi AA. *J. Colloid and Interface Science.* 2006; 297:561–569.
13. Sivakolundu SG, Mabrouk PA. *J. Am. Chem. Soc.* 2000; 122:1513–1521.
14. DiCarlo CM, Compton DL, Evans KO, Laszlo JA. *Bioelectrochemistry.* 2006; 68:134–143. [PubMed: 16009598]
15. Laszlo JA, Compton DL. *J. Mol. Catal. B Enzyme.* 2002; 18:109–120.
16. Du P, Liu S, Wu P, Cai C. *Electrochim. Acta.* 2007; 52:6534–6547.
17. Ohno H, Suzuki C, Fukumoto K, Yoshizawa M, Fujita K. *Chem. Lett.* 2003; 32:450–451.
18. Baker GA, Heller WT. *Chem. Eng. J.* 2009; 147:6–12.
19. Fujita K, Forsyth M, MacFarlane DR, Reid RW, Elliott GD. *Biotechnol. Bioeng.* 2006; 94:1209–1213. [PubMed: 16615145]
20. Fujita K, MacFarlane DR, Forsyth M, Yoshizawa-Fujita M. *Biomacromolecules.* 2007; 8:2080–2086. [PubMed: 17580947]
21. Constatinescu D, Weingärtner H, Herrmann C. *Angew. Chem. Int. Ed.* 2007; 46:8887–8889.
22. Cahill LS, Rana UA, Forsyth M, Smith ME. *Phys. Chem. Chem. Phys.* 2010; 12:5431–5438. [PubMed: 20376404]
23. Constatinescu D, Herrmann C, Weingärtner H. *Phys. Chem. Chem. Phys.* 2010; 12:1756–1763. [PubMed: 20145840]
24. Vrikkis RM, Fraser KJ, Fujita K, MacFarlane DR, Elliott GD. *J. Biomechanical Engineering - Transactions of the ASME.* 2009; 131 Art. No. 074514.

25. Summers CA, Flowers RA. Protein Science. 2000; 9:2001–2008. [PubMed: 11106174]
26. Byrne N, Wang L-M, Belieres J-P, Angell CA. Chem. Commun. 2007:2714–2716.
27. Byrne N, Angell CA. J. Molec. Biol. 2008; 378:707–714. [PubMed: 18384810]
28. Grossman S, Danielson ND. J. Chromatogr. A. 2009; 1216:3578–3586. [PubMed: 18849044]
29. Reichardt C. Green Chem. 2005; 7:339–351.
30. Kamlet MJ, Abboud JL, Taft RW. J. Am. Chem. Soc. 1977; 99:6027–6038.
31. Waichigo MM, Hunter BM, Riechel TL, Danielson ND. J. Liq. Chromatogr. Relat. Technol. 2007; 30:165–184.
32. Huang M-M, Weingartner H. Chem. Phys. Chem. 2008; 9:2172–2173. [PubMed: 18792901]
33. Waichigo MM, Danielson ND. J. Sep. Sci. 2006; 29:599–606. [PubMed: 16605076]
34. Waichigo MM, Danielson ND. J. Chrom. Sci. 2006; 44:607–614.
35. Kennedy DF, Drummond CJ. J. Phys. Chem. B. 2007; 113:5690–5693. [PubMed: 19385688]
36. Greaves TL, Weerawardena A, Fong C, Krodkiewska I, Drummond C. J. Phys. Chem. B. 2006; 110:22479–22487. [PubMed: 17091990]
37. Waichigo, MM. Ph.D Dissertation. Miami University; 2006.
38. Mann JP, McCluskey A, Atkin R. Green Chem. 2009; 11:785–792.
39. Myer YP. Current Topics in Bioenergetics. 1985; 14:149–188.
40. Myer YP. Biochemistry. 1968; 7:765–776. [PubMed: 5689422]
41. Rafferty SP, Pearce LL, Barker PD, Guillemette JG, Kay CM, Smith M, Mauk AG. Biochem. 1990; 29:9365–9369. [PubMed: 2174257]
42. Thomas YG, Goldbeck RA, Kliger DS. Biopolymers (Biospectroscopy). 2000; 57:29–36. [PubMed: 10679637]
43. Goto Y, Hagihara Y, Hamada D, Hoshino M, Nishii I. Biochemistry. 1993; 32:11878–11885. [PubMed: 8218260]
44. Rodriguez-Cruz SE, Khoury JT, Parks JH. J. Am. Soc. Mass Spec. 2001; 12:716–725.
45. Lyubovitsky JG, Gray HB, Winkler JR. J. Am. Chem. Soc. 2002; 124:14840–14841. [PubMed: 12475313]
46. Lee JC, Engman KC, Tezcan FA, Gray HB, Winkler JR. Proc. Nat. Acad. Sci. 2002; 99:14778–14782. [PubMed: 12407175]
47. Fujita K, MacFarlane DR, Forsyth M. Chem. Commun. 2005:4804–4806.
48. Fridovich I. J. Biol. Chem. 1970; 245:4053–4057. [PubMed: 5496991]
49. Winther-Jensen O, Vijayaraghavan R, Sun J, Winther-Jensen B, MacFarlane DR. Chem. Commun. 2009:3041–3043.

Figure 1.
Overlapping Soret CD spectra of cytochrome c in 40% MeCN-, 40% MeOH-, 40% MAF-60% water, and 100% water (from top to bottom).

A

B

Figure 2. The Soret CD spectra (A) and HT spectra (B) of cytochrome c as a function of MAF concentration (bottom scan 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and top scan 90%) in water.

Figure 3.

Ellipticity at 404 nm (positive peak) as a function of concentration of MeOH, MeCN, EAF, and MAF in phosphate buffer (top to bottom at 60%) as well as phosphate buffer itself. Each data point is the average of 3 replicates. The relative standard deviations (RSDs) of the points are less than 3%.

Figure 4. Tryptophan fluorescence of cytochrome c as a function of volume percent of MeOH, EAF, MAF, and MeCN, in phosphate buffer (top to bottom at 90%) and phosphate buffer itself. Each data point is the average of 3 replicates. The RSDs of the points are in the 3% range.

Figure 5.

Changes of fluorescence emission intensity at 340 nm for cytochrome c excited at 280 nm as temperature increases from 20 °C to 80 °C for various solvent percentages in water and phosphate buffer itself. Top to bottom at 20 °C: 80% MeOH, 80% EAF, 80% MAF, 20% EAF, 20% MAF, and phosphate buffer. Each data point is the average of 3 replicates. The RSDs of points are in the 3% range.

a

b

c **Figure 6.**

- a. The Soret CD spectra (350–450 nm) of cytochrome c as a function of increasing temperature [20 °C (bottom curve at 416 nm), 30°C, 40°C, 50°C, 60°C, 70°C, 80 °C (top curve at 416 nm)] in 40 mM phosphate buffer 7.0.
- b. The Soret CD spectra (350–450 nm) of cytochrome c as a function of increasing temperature [20 °C (bottom curve at 416 nm), 30°C, 40°C, 50°C, 60°C, 70°C, 80 °C (top trace at 416 nm)] in 20% EAF-80% water.
- c. The Soret CD spectra (350–450 nm) of cytochrome c as a function of increasing temperature [20 °C (peak at 400 nm), 30°C, 40°C, 50°C, 60°C, 70°C, 80 °C (bottom scan)] in 80% EAF-20% water.

Figure 7.
Cytochrome c activity assay at room temperature in phosphate buffer, 80% MAF-, 80% EAF-, 80% MeOH-, and 80% MeCN- 20% water (top to bottom trace at 100 s).