Enzymatic Activity and Thermal Stability of Metallo Proteins in Hydrated Ionic Liquids

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ABSTRACT:

Hydrated choline dihydrogen phosphate (Hy[ch][dhp]) containing 30 wt% water was investigated as a novel protein solvent. The Hy[ch][dhp] dissolved some metallo proteins (cytochrome c, peroxidase, ascorbate oxidase, azurin, pseudoazurin and fructose dehydrogenase) without any modification. These proteins retained the surroundings of the active site after dissolution in Hy[ch][dhp]. Some metallo proteins were found to retain their activity in the Hy[ch][dhp]. © 2010 Wiley Periodicals, Inc. Biopolymers 93: 1093–1099, 2010.

Keywords: ionic liquids; enzymatic activity; metallo proteins

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INTRODUCTION

Ionic liquids (ILs) are remarkable materials with very different properties from molecular liquids.[1,2] ILs are capable of having negligible vapor pressure and high thermal, chemical, and electrochemical stability.[3] They also have tunable properties including polarity, hydrophobicity, and solvent miscibility.[4] ILs have been applied in electrochemistry, enzymatic catalysis,[5] and green chemistry, exploiting their excellent stability and unusual solvent properties. A number of enzymes retain their catalytic activity in certain IL.[6,7] Lipases, in particular, maintain their activity in hydrophobic and anhydrous ILs. Previously published papers mentioned that their selectivity and operational stability are often better than these in traditional volatile solvents.[8] In most reports of hydrophobic ILs, the enzymes are not dissolved but are merely in a dispersed state and are therefore regarded as a heterogeneous catalyst. Some hydrophilic ILs accelerate the dissolution of protein, but in these cases the protein secondary structure has been lost after dissolution.[9,10] Such structural changes generally cause loss of enzymatic activity.[11] To induce solubility while maintaining the activity of enzymes in ILs, polyether structure and related additives have been employed.[12,13] These structures were incorporated into the protein surface as polyethylene oxide (PEO) modification and on the component ions of ILs. This PEO-modification is effective, but is difficult to prepare; it is also difficult to separate the PEO-modified proteins from unreacted PEO chains. Thus, there is a strong interest in preparing ILs having suitable affinity with proteins.

We have studied hydrated ILs as novel solvents for proteins.[8,14] The hydrated ILs maintain basic properties of pure ILs, but a small amount of water considerably improved protein solubility. Solubility varies with the component ions. Our previous work showed that hydrated ILs, comprising appropriate anions which have oxo acid residues, dissolved cyt c quite well. Furthermore, the structure and activity of cyt c differed according to the component ions. We investigated the effect of these ions on the structure and the activity of dissolved cyt c.[14] We expected kosmotropicity to be a factor influencing the properties of dissolved cyt c. Kosmotropicity (closely related to the Hofmeister series) is well established in the aqueous phase. In general in the aqueous phase, a pair of strong kosmotropic anions, and a chaotrope cation, stabilize proteins, whereas strong kosmotropic cations destabilize proteins.[15–17] Even in ILs, there have recently been many reports about the effect of kosmotro-

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picity on physical properties such as two phase behavior,[18] and on the activity of biomolecules.[19] In our previous report we found that the effect of component ions on cyt c was correlated with kosmotropicity.[14] In particular, hydrated choline dihydrogen phosphate (Hy[ch][dhp]), which is one of the best combinations of chaotropic cation and kosmotropic anion, is an excellent solvent for cyt c. We believe that kosmotropicity affects protein structure in hydrated ILs. Cyt c underwent no structural change and retained its activity in Hy[ch][dhp], even after 18 months stored at room temperature.

This work examines the value of Hy[ch][dhp] as a solvent for proteins. Some studies of proteins dissolved in ILs used mainly cyt c[8] and lysozyme.[20,21] Here, the effect on the structure and activity of various metallo proteins have been investigated in Hy[ch][dhp]. The effect of dissolution on the structure and activity are important when protein and other biomolecules are considered in vitro. Metallo proteins were chosen for this study because they are well understood physicochemically and they have many different functions in vivo, including enzymatic reaction, transport and storage of useful materials.[22] Furthermore, metallo proteins have been widely studied including electrochemically expecting bioapplications, such as biocatalytic reaction, biosensors, and biofuel cells. We have been analyzing the electron transfer reactions of metallo proteins in a nonaqueous phase.[12] In the present study, we evaluate the Hy[ch][dhp] as a reaction medium for metallo proteins.

RESULTS AND DISCUSSION

Hy[ch][dhp] was prepared with a small amount of water. It is plausible that these mixed water molecules hydrate the component ions so that they are bound water, not free water as in the buffer solution. When Hy[ch][dhp] prepared with different amounts of water was heated to 100°C, the water content became constant, at approximately two water molecules per ion pair (14 wt%). We suggest that all water molecules are strongly bound to the component ions of IL, and there are no free water molecules, when Hy[ch][dhp] is prepared with less than 14 wt% water. However, Hy[ch][dhp] with 14 wt% water has high viscosity, and it has difficult to use as a solvent. Addition of water considerably decreased the viscosity. The viscosity of Hy[ch][dhp] with 30 wt% water is much less than with 20 wt% water (further details of the relation between water content and viscosity will be stated below). A large excess of water might lead to a concentrated salt solution which is deleterious to proteins.[8,23,24] We therefore used Hy[ch][dhp] with 30 wt% water as a solvent in this work. In this solution, there are about five water molecules per ion pair. This Hy[ch][dhp] was examined as solvents for a series of metal proteins.

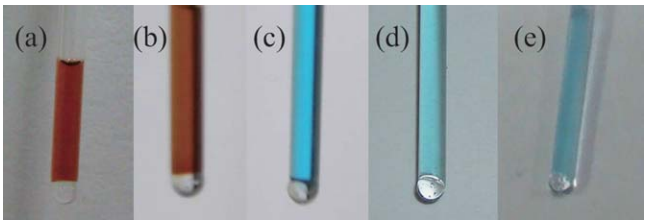

FIGURE 1 Photographs of dissolved metallo proteins in Hy[ch][dhp]. (a) cyt c, (b) HRP, (c) Az, (d) paz, and (e) ASOD.

Protein Solubility

Metallo proteins were mixed with [ch][dhp] containing 30 wt% water (about five water molecules per ion pair), and they were found to comprise two groups, soluble and insoluble. The proteins cyt c, HRP, Az, paz, and ASOD dissolved and gave clear homogeneous solutions; the original color is shown in Figure 1. These proteins were soluble in Hy[ch][dhp] with concentration over 1 m*M*. In contrast, hemoglobin and myoglobin scarcely dissolved at all in Hy[ch][dhp] and formed a dispersion in the vial. Their solubility was calculated to be below 0.5 μM in the clear layer with UV-vis spectroscopy after centrifugation. Solubility was still low when the water content was as high as 40 wt%. Furthermore, when myoglobin was dissolved in buffer first and then mixed with Hy[ch][dhp], the myoglobin still did not dissolve in the IL. The lyophilized condition of the sample did not affect the solubility. Cyt c and myoglobin therefore have very different solubilities despite their similar properties. Table I shows basic properties of the proteins studied here, molecular weight, number of amino acids, isoelectric point (PI), and secondary structure. However, these do not correlate with the solubility in Hy[ch][dhp], so that other factors must determine this; an investigation is in progress.

Protein Features After Dissolving in Hy[ch][dhp]

The conditions of the active site were important in determining the original characteristics of proteins. The environment of the active site of the dissolved proteins, cyt c, HRP, Az, and ASOD in the IL was analyzed by resonance Raman (RR) spectroscopy. The RR spectra of metallo proteins have been extensively studied. RR spectra are sensitive to the coordination state and spin state of the active site. All spectra show the original features based on the native structure of the active site (see Figure 2). These spectra were compared with those in buffer solution. For ASOD, the spectrum in Hy[ch][dhp] was compared with the difference spectrum in buffer solution, because it was difficult to observe a clear spectrum of ASOD in buffer (see Figure 3). We have previously reported the similarity of the RR spectra for cyt c in Hy[ch][dhp] and in buffer.[14] HRP is a heme containing

Table I Basic Properties of Dissolved Proteins in Hy[ch][dhp]

Protein	Molecular Weight	Hydrophobic Residue	pI	Helical (%)	Beta Sheet (%)
Cytochrome c from *horse heart*	12,400	44/104	10	40	1
Hemoglobin from *human*	64,500	316/574	6.8	76	0
Myoglobin from *bovin heart*	16,700	77/153	7.3	74	0
Peroxidase from *horseradish*	44,000	152/306	7.5	52	1
Azurin II from *Alcaligenes Xylosoxidans*	14,000	65/129	–	12	46
Pseudoazurin from *Achrom bactor cycloclastes*	13,100	71/123	8.4	16	35
Ascorbate oxidase from *Cucurbita*	140,000	283/552	5–6	13	36

enzyme which catalyses the oxidation of various organic compounds with hydrogen peroxide.[25,26] Figure 2a shows the ferric state of HRP in both buffer solution and Hy[ch][dhp]. These spectra imply that the iron axial ligand does not undergo change in Hy[ch][dhp]. Az is a type I copper containing protein; this family is called blue-copper proteins.[27,28] As shown in Figure 2b, the RR modes near 400 cm^{-1} in blue copper proteins have typically been assigned to Cu ligand stretching motions. In Hy[ch][dhp], the spectra suggest that the RR mode near 260 cm^{-1} comprising the Cu-N(His imidazole) stretching, and the modes near 400 cm^{-1} comprising the Cu-S(Cys) remain the same as in the native state. ASOD is one of the multi copper proteins which catalyse the oxidation of ascorbic acid (with oxygen).[29] Its spectrum in Hy[ch][dhp] showed that bands assigned to the Cu-N (peptide or amide side chain) and Cu-S (cystein) vibrations are maintained in Hy[ch][dhp] as shown in Figure 3. These findings show that these dissolved proteins maintained the native surroundings of the active site even in Hy[ch][dhp].

Next, the secondary and tertiary structures of proteins were investigated by circular dichroism (CD) measurements. Cyt c was used as the target protein for this analysis, because the relation between the structure and CD spectrum has been studied in detail before.[30,31] For comparison, cyt c was dissolved in buffer, Hy[ch][dhp], and similarly hydrated *N*-butyl-*N*-methyl pyrrolidinium dihydrogen phosphate ([C_4mpyr][dhp]). In the far UV region (250–250 nm), the CD spectrum is an effective probe for protein secondary structure such as α-helix and β-sheet. In the case of cyt c, this

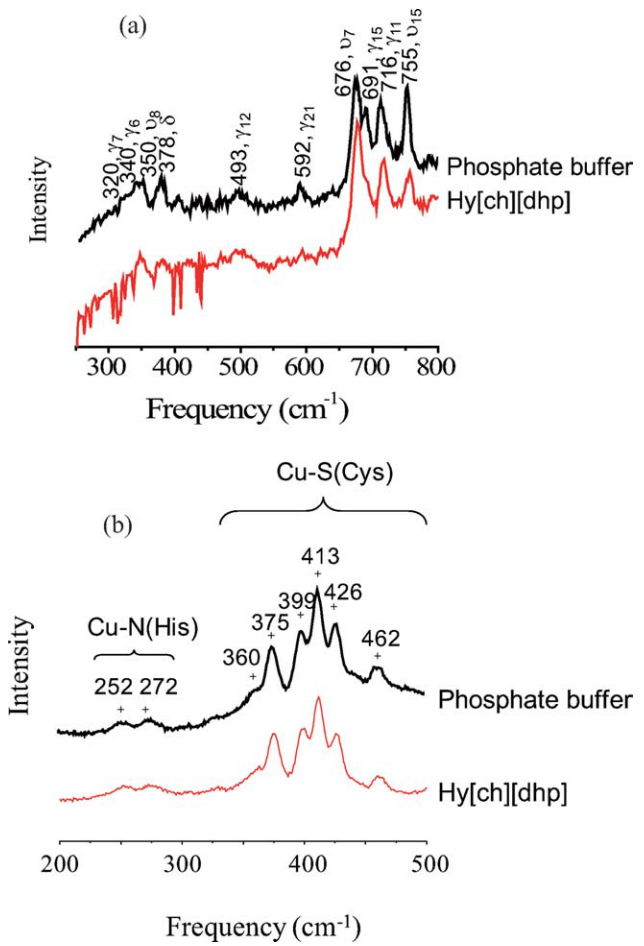

FIGURE 2 RR spectra of dissolved (a) HRP and (b) Az in Hy[ch][dhp] and buffer.

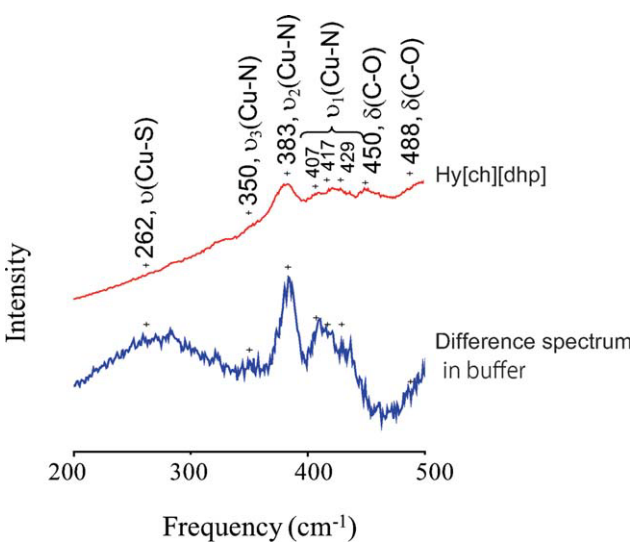

FIGURE 3 RR spectrum of dissolved ASOD in Hy[ch][dhp] and difference spectrum in buffer.

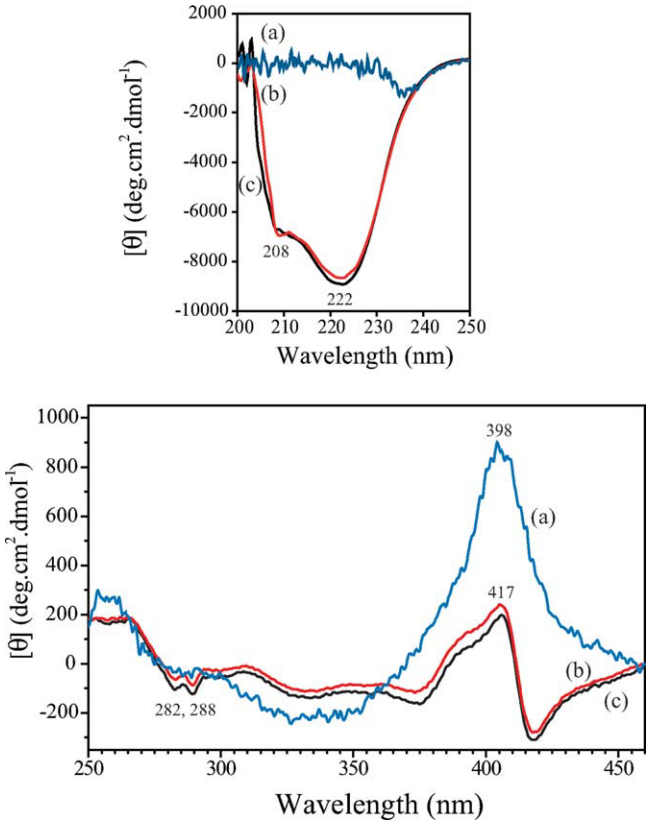

FIGURE 4 CD spectrum of dissolved cyt c in (a) [C_4mpyr][dhp], (b) Hy[ch][dhp], and (c) buffer.

region gives information about α-helix content. Features of the CD spectra of cyt c were almost exactly the same in buffer and in Hy[ch][dhp]. The band intensities at 222 and 208 nm, used for calculation of the α-helix content, were also similar. Consequently the α-helix content did not change after dissolution in Hy[ch][dhp]. In the case of [C_4mpyr][dhp], the cation has an aromatic ring structure, so the spectrum cannot be observed although cyt c dissolved well in it. In the near UV (250–350 nm) and Soret (350–450 nm) regions, the CD spectrum reflects changes in the tertiary structure of cyt c related to the environment of aromatic side chains and the heme pocket. The spectra show significant changes in [C_4mpyr][dhp] (see Figure 4). Similar spectra were observed in the unfolded state and when dissolved in organic solvents and ILs. This spectral change indicates that the distance and orientation of try-59 and phenylalanine are different in Hy[C_4mpyr][dhp]. In this state, one axial ligand Met-80 must be significantly weakened. This result agrees well with our previous finding that CT band absorption in UV-vis spectroscopy disappeared after dissolution in most hydrated ILs.[32] These spectral results together indicate that the secondary structure remains unchanged but the tertiary structure is loosened in most hydrated ILs. On the other hand, the CD spectrum of cyt c dissolved in Hy[ch][dhp] was almost identical to that in buffer, so that cyt c is expected to maintain the tertiary structure. Hy[ch][dhp] showed excellent affinity with some metallo proteins in which proteins dissolved well, and the active site and tertiary structure maintain the native state. It should be noted here that [ch][dhp] has a special ionic combination which has been chosen by natural selection as the head groups of lipid bilayers in living organisms.[33] This similarity may have certain reason for the stabilisation of proteins.

Enzymatic Activity

We have reported that cyt c retains its activity after dissolving in Hy[ch][dhp].[14] In this case the activity was investigated after dissolving in buffer, so that the cyt c was diluted. Conversely, some proteins, such as cellobiose dehydrogenase, exhibited activity in the dissolved state in Hy[ch][dhp].[34] In this work we made enzymatic assays of metallo proteins dissolved in Hy[ch][dhp], HRP, and ASOD.

HRP dissolved in Hy[ch][dhp] was added to the mixed solution of pyrogallol and hydrogen peroxide. Figure 5 shows the absorption spectra before and after mixing of HRP in Hy[ch][dhp]. After mixing of HRP, the absorbance at 420 nm was increased by generation of purpurogallin, indicating the progress of the enzymatic reaction. No spectral change was obtained in the absence of peroxidase addition. This means that the HRP was active and capable of performing the enzymatic reaction after dissolving in Hy[ch][dhp].

For the investigation of ASOD activity, ASOD and ascorbic acid (AA) were dissolved in Hy[ch][dhp], respectively and the spectral change was detected upon mixing of the two

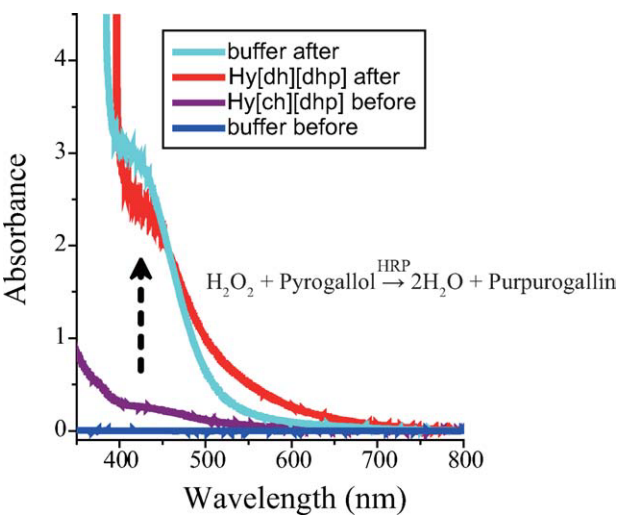

FIGURE 5 Spectral change of mixed solutions of pyrogallol and hydrogen peroxide in buffer and Hy[ch][dhp], before and after addition of HRP.

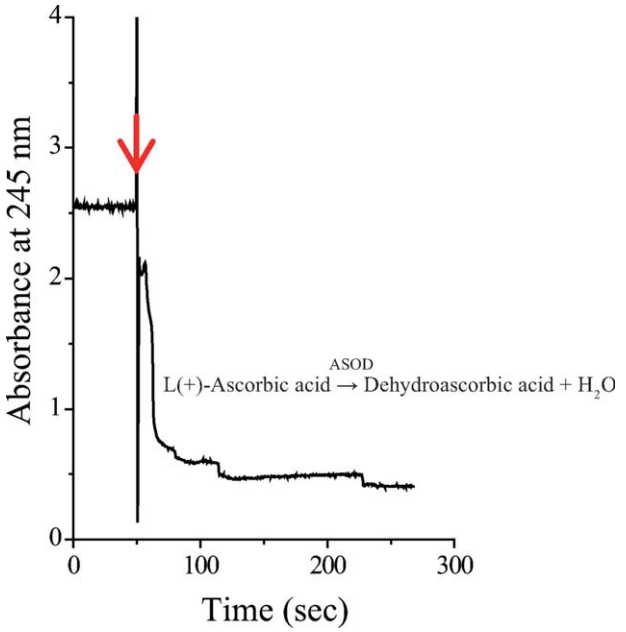

FIGURE 6 Absorbance change at 245 nm of AA before and after addition of ASOD.

liquids. Figure 6 shows the change in absorbance at 245 nm of AA in Hy[ch][dhp] from before to after addition of ASOD. When ASOD was mixed with AA at the arrow point, the absorbance at 245 nm based on the absorption of AA decreased with progress of the enzymatic reaction to produce the dehydroascorbate. This result shows that ASOD retains its activity after dissolving in Hy[ch][dhp], and the enzymatic reaction takes place in it. Although enzymatic assay of HRP and ASOD were detected in Hy[ch][dhp] that was used as a reaction solvent, the reaction rate was slow compared with that in buffer solution. These catalytic reactions progress under diffusion control. The viscosity is proposed to be one reason for this slowing. The viscosity of Hy[ch][dhp] with 30 wt% water was 30 cP at room temperature, whereas that of aqueous solution was 0.9 cP. The viscosity of Hy[ch][dhp] with 20 wt% water was 440 cp. In the Hy[ch][dhp] with 20 wt% water, both reactions for HRP and ASOD were much slower than those in Hy[ch][dhp] with 30 wt% water. However, the viscosity is considered to be one of factors for reaction rate, there are a few other possible factors to affect the enzymes such as pH, salt concentration, and hydration state. However, the change of apparent rate cannot fully be explained by the changes at active site of enzymes, because there are no changes in RR spectra.

Thermal Stability of FDH

We have reported the improvement of the thermal stability of cyt c dissolved in Hy[ch][dhp].[8] To investigate the thermal stability of other proteins in Hy[ch][dhp], FDH was chosen; it is commercially available and its thermal stability is widely known.[35] In this study, the FDH dissolved in buffer and in Hy[ch][dhp] were treated at 20–70°C for 15 min, and these underwent enzymatic assay after dilution with buffer. Figure 7 shows the residual activity in aqueous solution after incubation in buffer, in Hy[ch][dhp] with 30 wt% water, and in Hy[ch][dhp] with 80 wt% water. The activity at 20°C was defined as 100% residual activity. In both buffer solution and Hy[ch][dhp], the activity decreased gradually with increasing incubation temperature. The decreasing curve was totally different, however. In buffer solution, activity was started to decrease at 30°C and had almost vanished at 50°C. In Hy[ch][dhp], activity decreased from incubation at 40°C and no activity was observed at 70°C. In consequence, FDH that had been dissolved in Hy[ch][dhp] showed greater thermal stability at around 20°C than that in buffer solution. When FDH was incubated in Hy[ch][dhp] containing excess water (80 wt%), no improvement of thermal stability was observed (Figure 7c). This shows that when [ch][dhp] is present as solute in aqueous solutions, there is no significant difference from the buffer solution. This might be the same effect of thermal stability observed in cyt c in previous work.[8] The alleviation of degradation of proteins at higher temperatures in Hy[ch][dhp] suggests that the chemical

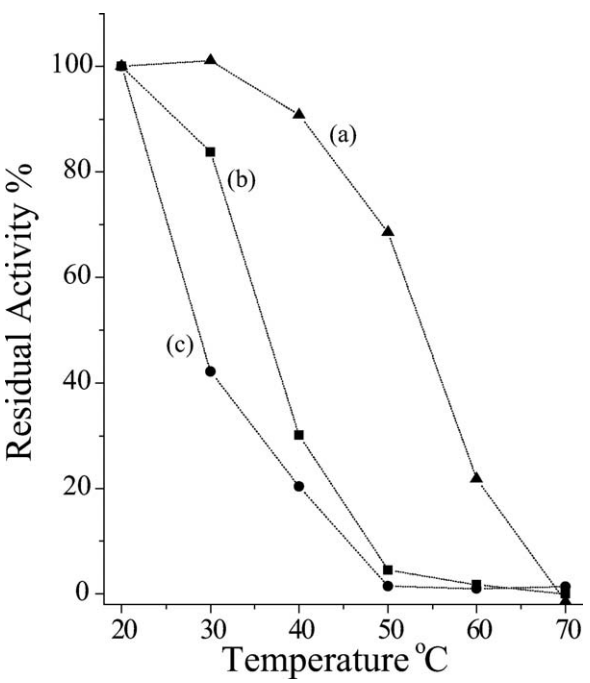

FIGURE 7 Residual activity of FDH dissolved and treated in (a) [ch][dhp] with 30 wt% water, (b) buffer and (c) [ch][dhp] with 80 wt% water.

and structural steps involved are greatly diminished perhaps due to the low water content. Viscosity is expected as other possible factor, that the increase in the thermal stability of some proteins has been induced by increasing solution viscosity though the lowering thermal motion of these proteins. In view of the thermal stability of FDH in Hy[ch][dhp], long-term stability is also likely to be increased. Investigation of the long term stability of FDH in Hy[ch][dhp] is under way.

These observations suggest that complex forces are exerted on proteins dissolved in Hy[ch][dhp]. For example, the affinity of proteins and component ions and/or water which is interacting with ions is one possible force. The amount of water, and the water state provides another. These factors might have a complex outcome on the solubility, activity and thermal stability. Recently the effect of the water state has been studied as part of the concept of molecular crowding.[36] Biomolecules occupy a significant fraction of the cellular volume, leading to a crowded intercellular environment. Thus, proteins and other polymers exist at high concentration. The water state, water activity and the behavior of biomolecules are then different from diluted conditions. Hydrated ILs might generate a pseudo environment for molecular crowding. Further experiments will study the water in relation to protein stability and whether the water condition is controllable by the water content and the IL involved.

CONCLUSIONS

We have studied the effectiveness of Hy[ch][dhp] as a novel solvent for metallo proteins. Hy[ch][dhp] dissolves some metallo proteins, although the mechanism is not yet clear. Proteins dissolved in Hy[ch][dhp] were shown to keep the surroundings of the active site and secondary structure the same as in buffer solution. The enzymatic reactions were generally influenced by the salt concentration, because concentrated salt water causes the deactivation of enzymes. The electrostatic interactions may also reduce even the activity of those enzymes remaining in the native state. It might cause the decrease of the interaction between enzymes and substrate, and the dissociation of subunit structure. However, various metallo proteins retained their original native form and activity in Hy[ch][dhp]. Significant thermal stability of FDH was also observed. The surprising results we found could be caused by the combination of restraint of hydrolysis, the water circumstances due to the component ions, the minimized mobility of molecules, and other environmental conditions. Hy[ch][dhp] is likely to be a significant matrix for proteins which realize the long shelf life in vivo, and further investigation is in order. This new medium should expand the condition for many functional proteins. Many scientific applications should be developed with the hydrated ILs.

MATERIALS AND METHODS

Materials

Cytochrome c (cyt c) from *horse heart* and peroxidase from *horseradish* (HRP) were purchased from Sigma-Aldrich and used without further purification. Ascorbate oxidase from *Cucurbita* (ASOD) was purchased from Wako and used without further purification. Azurin II from *Alcaligenes Xylosoxidans* (Az) was expressed and purified as described previously.[37] Pseudoazurin was isolated from *Achromobacter cyclastes* IAM 1013 (Paz) as described previously.[38] *Gluconobacter sp.* D-Fructose dehydrogenase (FDH) was purchased from Toyobo enzymes and used without further purification. [ch][dhp] was synthesized according to the method[14] of choline bromide solution was treated on an ion exchange resin (Amberlite IRN77) and mixed with phosphoric acid solution. The solvent evaporated and the product was dried in vacuo. The resulting [ch][dhp] was identified using ^{1}H NMR, DSC, elemental analysis and electrospray mass spectrometry.

CD Measurement

CD spectra were recorded with a JASCO J-720 at 25°C. A square quartz cuvette (path length: 1 mm) was used for CD measurement. Solutions of 0.1 m*M* cyt c were prepared with phosphate buffer (pH 7.0) or Hy[ch][dhp] with 30 wt% water. These samples were recorded in the wavelength ranges 200–250 nm and 250–470 nm.

Raman Spectroscopy

Raman spectra were obtained using a JASCO NRS-1000 spectrometer with a Kaiser Optical holographic super-notch filter and a liquid N_2-cooled CCD detector. A detailed description has been given elsewhere.[14] Proteins were dissolved in phosphate buffer or Hy[ch][dhp]. The protein concentrations were typically 1.0–3.0 m*M*, and an excitation wavelength was 568 nm. Spectra were collected on samples in the bulk condition at room temperature, using a backscattering geometry. The peak frequencies were calibrated relative to an indene standard, and are accurate to ± 1 cm^{-1}.

Enzymatic Activity

Enzymatic assay of HRP,[39] ASOD,[40] and FDH[35] was performed according to the published test procedure. Absorption spectra and the change in absorbance were recorded using a Shimadzu UV-2450. For the enzymatic assay of HRP, 0.5% (w/w) hydrogen peroxide solution (H_2O_2) and 5% pyrogallol solution were prepared with deionized water. Peroxidase enzyme solution containing 0.4–0.7 unit/ml was prepared using 100 m*M* potassium phosphate buffer (pH 7.0). [ch][dhp] (675 mg) was mixed with phosphate buffer (80 μL), H_2O_2 solution (40 μL), and pyrogallol solution (80 μL). The absorption spectrum was monitored before and after addition of HRP solution (25 μL).

For the analysis of ASOD activity, [ch][dhp] with 30 wt% water was used as the solvent for ASOD (0.1 m*M*) and AA (20 μM). The absorbance at 245 nm based on the AA was monitored before and after addition of ASOD in Hy[ch][dhp].

The thermal stability of FDH was investigated by enzymatic assay in aqueous buffer solution, following incubation in McIlvaine buffer (pH 4.5) or Hy[ch][dhp] containing 30 wt% water at 20–70°C for 15 min. The incubated 4 unit/mL FDH samples (25 μL) in MacIlvain buffer or Hy[ch][dhp] were equilibrated at 37°C for 5 min after mixing with 1M D-Fructose solution (25 μL) and McIlvaine buffer (175 μL). Potassium ferricyanide solution (25 μL) was added to the incubated solution in order to start the enzymatic reaction. After 5 min of reaction at 37°C, ferric sulfate SDS solution (125 μL) was added to stop the reaction, and then incubation proceeded at 37°C for a further 20 min. The absorbance at 660 nm was observed after dilution by distilled water (850 μL). The viscosity was measured using a DV-I+ viscometer (Brookfield) with a CPE-40 spindle.

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