Communications

Crown Ether-Mediated Extraction and Functional Conversion of Cytochrome *c* in Ionic Liquids

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We report that a macrocyclic ligand enables transfer of a protein from an aqueous phase to ionic liquids. The extraction behavior of heme protein cytochrome *c* (Cyt-*c*) from an aqueous phase into ionic liquids was investigated with crown ethers. A hydroxyl-group-containing ionic liquid with dicyclohexano-18-crown-6 was found to be capable of quantitative partitioning of Cyt-*c*, whereas the protein transfer using conventional organic solvents was negligibly small. Furthermore, we clarified that Cyt-*c* solubilized in ionic liquids caused a structural transformation of Cyt-*c*, which triggers its functional conversion from an electron-transfer protein to peroxidase.

Introduction

Ionic liquids (ILs) are ambient temperature molten salts that possess many attractive properties such as negligible volatility, nonflammability, high thermal stability, and controllable hydrophobicity.[1] ILs are being extensively investigated as alternatives to volatile organic solvents.[2] In liquid−liquid extraction systems,[3] ILs show remarkably high extraction performance and separation ability for metal ions in comparison with conventional organic solvents when using certain neutral ligands.[4] Metal ions and simple organic compounds have been utilized as extraction targets,[5] and recently, several research groups have applied ILs to liquid−liquid extraction of amino acids. Armstrong et al.[6] reported the partition coefficients of a large variety of organic compounds with different functionalities and the moderate extraction of aromatic amino acids in the presence of dibenzo-18-crown-6. Subsequently, Pletnev et al.[7] reported that hydrophilic amino acids were quantitatively extracted into ILs containing dicyclohexano-18-crown-6 (DC18C6), even though amino acids in the presence of crown ethers are typically not extracted into conventional solvents. Wang et al.[8] investigated the effects of several parameters (the hydrophobicity of amino acids, the pH, the anionic nature of the ILs, and the alkyl chain length of the cation of the ILs) on the self-partitioning of five amino acids. Crespo et al.[9] carried out liquid−liquid extraction and supported liquid membranes transport experiments of amino acids and reported that the driving force for extraction of amino acids into ILs differed greatly from that for transport in the liquid membrane. ILs can provide an appropriate environment that contributes to the performance of extractants, and they offer considerable potential as diluents in liquid−liquid extraction. To the best of our knowledge, however, there are no studies showing effective extraction of biomacromolecules such as proteins from aqueous solution into IL phases, although several systems composed of conventional organic solvents have been reported.[10]

ILs have also been investigated as reaction media for biocatalysis.[11] ILs containing a strongly coordinating anion (alkyl sulfate, nitrate and lactate anions etc.) are known to dissolve proteins, but it was reported that dissolution could cause denaturation of proteins presumably due to their conformation change in ILs.[12−15] Thus, most of the biocatalytic reactions in ILs were conducted with dispersions of enzymes in weakly coordinating hydrophobic ILs. Several studies have been performed to maintain enzymatic activity in ILs, involving the addition of a small amount of water[14,16,17] or alcohol[18] to ILs, chemical modification or physical complexation of enzymes with poly(ethylene glycol),[12,15,16,19,20] and immobilization of enzymes to solid supports.[15,21]

In the present study, we employed cytochrome *c* from horse heart (Cyt-*c*) as a model protein. Cyt-*c* is a hemoprotein and possesses several lysine residues that can form a supramolecular complex with crown ethers.[22,23] We describe herein that Cyt-*c* was quantitatively extracted from an aqueous solution into IL phases with DC18C6. This is the first report showing protein extraction into ILs. It was found that simple but suitable combination of extractants and ILs can broaden the potential utility of ILs as homogeneous media for functionalization of proteins.

Experimental Section

Material. Cytochrome *c* from horse heart (Cyt-*c*) was purchased from Sigma Co. (St. Louis, MO). Dicyclohexano-18-crown-6 (DC18C6)

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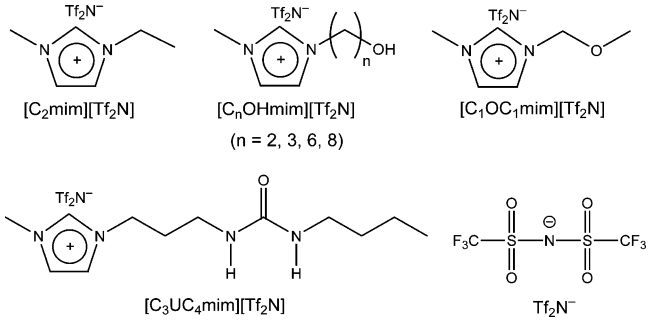
Chart 1. Molecular Structures and Abbreviations of Ionic Liquids



and 18-crown-6 (18C6) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). 2,6-Dimethoxyphenol (2,6-DMP) was obtained from Tokyo Kasei Kogyo Co., Inc. (Tokyo, Japan). All other reagents were of commercially available analytical grade and used as received.

IL Syntheses. A typical alkyl methylimidazolium bis(trifluoromethanesulfonyl)imide IL and ILs incorporating hydroxyl, ether, or urea groups were prepared as described elsewhere.[24] Details of Synthetic procedures are summarized in the Supporting Information. Molecular structures and abbreviations of ILs used in the present study were shown in Chart 1.

Liquid−Liquid Extraction of Cyt-*c* into ILs. An extracting phase was prepared by dissolving DC18C6 in ILs. An aqueous phase was prepared by dissolving Cyt-*c* in deionized water without buffer, because buffers containing alkali metal ions or amines may form complexes with DC18C6. Equal volumes of the IL solutions and aqueous solution were mixed and gently shaken at 30 °C for 6 h to attain equilibrium. After phase separation, the concentrations of Cyt-*c* in both phases were determined by the absorbance of the Soret band peak using a UV−vis spectrophotometer (JASCO U-best 570), which then allowed calculation of the degree of extraction (E = [Cyt-*c*]$_{IL.eq.}$/[Cyt-*c*]$_{aq.ini.}$). Extraction conditions were as follows: [Cyt-*c*] = 50 μM and [DC18C6] = 0−50 mM in the extraction behavior of Cyt-*c* with increasing DC18C6 concentration. [Cyt-*c*] = 10 μM and [DC18C6] = 20 mM in the extractability of Cyt-*c* with DC18C6 in various ILs.

Catalytic Oxidation of 2,6-Dimethoxyphenol by Cyt-*c*. Equal volumes of a [C_2OHmim][Tf_2N] solution ([DC18C6] = 20 mM) and an aqueous solution ([Cyt-*c*] = 10 μM) were mixed and gently shaken at 30 °C for 6 h to attain equilibrium. The IL solution containing Cyt-*c*−DC18C6 complex and a dry IL solution containing 2,6-dimethoxyphenol were added to a quartz cell. The mixture was diluted with water-saturated IL. The oxidative reaction was initiated by the addition of hydrogen peroxide methanol solution. The reaction was followed spectroscopically by monitoring the absorbance of product dimer at 469 nm.[25] Final conditions were as follows: volume 2.1 mL, [Cyt-*c*] = 1 μM, [2,6-DMP] = 10 mM, [H_2O_2] = 1 mM, and [DC18C6] = 2 mM. Catalytic oxidation of 2,6-dimethoxyphenol by Cyt-*c* in aqueous solutions without 18C6 or with 18C6 (2 mM) was also compared with that in IL.

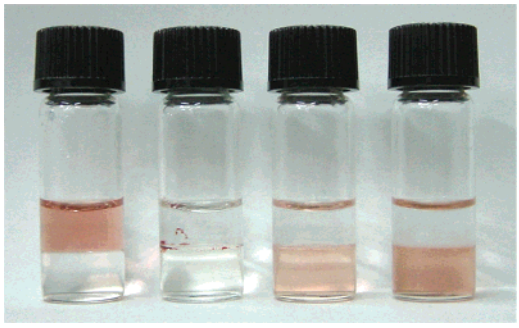


Figure 1. Extraction behavior of Cyt-*c* with increasing DC18C6 concentration in [C_2OHmim][Tf_2N]. [Cyt-*c*] = 50 μM, the ratio of [DC18C6]/[Cyt-*c*] is 0, 100, 200, and 1000 (left to right).

Results and Discussion

In a preliminary experiment, we carefully examined the extractant for protein extraction and found that a macrocyclic ligand crown ether enables transfer of a lysine-rich protein from an aqueous solution to a hydrophobic ionic liquid. Therefore, we first investigated the extraction behavior of Cyt-*c* in [C_2OHmim][Tf_2N], which provides the highest hydrophilicity among the ILs tested in the present study (Figure 1). The red color of Cyt-*c* visually indicated its distribution between the phases. In the absence of DC18C6, the partitioning of Cyt-*c* into [C_2OHmim][Tf_2N] was negligible. Precipitation of Cyt-*c* at the IL−water interface was observed when the concentration of DC18C6 was relatively low. However, further increase of the DC18C6 concentration promoted the transfer of Cyt-*c* into [C_2OHmim][Tf_2N], and finally, quantitative extraction was achieved when the DC18C6 concentration was around 1000-fold larger than that of Cyt-*c*. If converted to the molar ratio to lysine residues, the DC18C6 concentration is around 50 equiv. We confirmed that native Cyt-*c* was not dissolved in [C_2OHmim][Tf_2N] although it had been expected to interact with proteins through hydrogen bonding. The Cyt-*c* transfer from the aqueous phase into the IL phase was thus solely due to mediation by DC18C6. This phenomenon can be explained as follows: Native Cyt-*c* from horse heart has 19 lysine residues. The isoelectric point (p*I*) value of horse heart Cyt-*c* is 10.6, indicating that Cyt-*c* exists as a cationic species under neutral conditions. DC18C6 has a strong affinity for the ammonium cation ($-NH_3^+$) due to the tripodal hydrogen bonding interaction, the ion−dipole interaction, and the inclusion effect. Thus, plural DC18C6 can coordinate with lysine residues and form a supramolecular complex with Cyt-*c*. The complex formation can enhance the solubility of Cyt-*c* in [C_2OHmim][Tf_2N], which allows the transfer into the IL phase. It was worth noting that Cyt-*c* remains solubilized in [C_2OHmim][Tf_2N] even after the

Table 1. Physical Properties of ILs and Extractability of 10 μM Cyt-*c* with 20 mM DC18C6 into ILs

IL	extractablity (%)[a]	density (g/mL)	water content in wet IL (w%)[b]	IL solubility in water (mM)[c]
[C_2OHmim][Tf_2N]	100.0 ± 0.3	1.57	17.2 ± 0.6	259.9 ± 2.3
[C_3OHmim][Tf_2N]	90.5 ± 4.2	1.54	17.1 ± 0.2	175.9 ± 1.6
[C_6OHmim][Tf_2N]	55.8 ± 1.2	1.43	10.8 ± 0.3	55.4 ± 1.8
[C_8OHmim][Tf_2N]	28.4 ± 8.9	1.38	7.5 ± 0.2	20.0 ± 1.2
[C_2mim][Tf_2N]	2.1 ± 0.2	1.52	3.9 ± 0.0	54.8 ± 1.9
[C_1OC_1mim][Tf_2N]	55.6 ± 6.5	1.57	5.2 ± 0.1	91.8 ± 0.3
[C_3UC_4mim][Tf_2N]	5.0 ± 0.2	1.38	11.3 ± 0.2	*d*

[a] The number of measurements is 3. [b] Water content in wet ILs was determined by Karl Fischer's method. The number of measurements is 3. [c] IL solubility in water was determined by measuring the absorbance of the aqueous phase at 211 nm. The number of measurements is 3. [d] No sharp peak at 211 nm was observed.

extracting phase was dried at 30 °C under vacuum (the water content was 0.11 ± 0.05 wt %). This suggests that most of the water was not required for the solubility of the Cyt-*c*−DC18C6 complex once it was solubilized in ILs.

Since the properties of the extracting solvent affect the extraction efficiency, we carried out the protein extraction using seven different ILs. Table 1 shows the physical properties of various ILs, which includes ethyl, hydroxyl, ether, or urea groups and the extractability of Cyt-*c* with DC18C6 into ILs. In the typical IL [C_2mim][Tf_2N], Cyt-*c* was not extracted at all. In [C_nOHmim][Tf_2N] (n = 2, 4, 6, and 8), the degree of Cyt-*c* extraction decreased considerably as the alkyl spacer between the hydroxyl group and imidazolium was elongated. These results indicate that increasing the hydrophobicity of ILs reduces the degree of Cyt-*c* extraction. To investigate the effect of functional groups introduced onto ILs on the efficiency of DC18C6-mediated Cyt-*c* extraction, we employed ILs with ether or urea groups. The degree of Cyt-*c* extraction in [C_1OC_1mim]-[Tf_2N] was 55.6 ± 6.5%, which is comparable to that in [C_6OHmim][Tf_2N]. By contrast, little extraction was observed in [C_3UC_4mim][Tf_2N], although urea groups are capable of hydrogen bonding with proteins, implying that the urea group may inhibit interaction between Cyt-*c* and DC18C6. These results clearly demonstrated that the partitioning of a protein into ILs is controllable on the basis of hydrophobicity and the type of functional groups of ILs.

The extraction behavior of Cyt-*c* with DC18C6 in conventional organic solvents was also examined, to compare it with that in ILs. Chloroform, toluene, isooctane and 1-octanol were tested and no transfer of Cyt-*c* was observed. These results indicate the potential of designer ILs as extracting media for proteins.

Several research groups have focused on the functional conversion of Cyt-*c* to a catalytically active heme enzyme in a specific medium.[10b,10c,18,23,26] Peroxidase activity of Cyt-*c* in [C_2OHmim][Tf_2N] was thus explored by oxidative reaction of 2,6-dimethoxyphenol (2,6-DMP) with hydrogen peroxide (See the Supporting Information). Cyt-*c* in water exhibited very weak peroxidase activity regardless of the presence or absence of 18-crown-6 (18C6). On the other hand, Cyt-*c* extracted into [C_2-OHmim][Tf_2N] accelerated the initial reaction rate 5.5-fold relative to that of Cyt-*c* in water, indicating functional conversion of Cyt-*c* from an electron-transfer protein to peroxidase. Native Cyt-*c* has two coordinative residues (His 18 and Met 80) for forming a six-coordinate low-spin heme complex. In the extracted Cyt-*c*, however, the LMCT band due to Met 80 coordination disappeared at 695 nm (see the Supporting Information). This suggests the presence of five-coordinate heme with an open site by the supramolecular complex formation with DC18C6 in ILs.

Conclusions

In the present study, we found that hydroxyl-group-containing ionic liquids with DC18C6 are capable of quantitative partitioning of heme protein Cyt-*c*, although the partitioning of Cyt-*c* with DC18C6 into conventional organic solvents was negligibly small. It was clarified that Cyt-*c* extracted into ILs adds new functionality to an electron-transfer protein such as peroxidase activity. These results highlight the great potential of ILs not only as extracting phase but also as novel reaction media for biocatalysis. Furthermore, we confirmed that recovery of Cyt-*c* from ILs is readily achieved by the addition of potassium chloride, which can form an inclusion complex with DC18C6. Further studies on extractive solubilization of lysine-rich proteins are currently underway in our group.

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Supporting Information Available. Synthetic procedures for ILs, catalytic oxidation of 2,6-dimethoxyphenol by Cyt-*c*, and absorption spectra of Cyt-*c* at the LMCT band. This material is available free of charge via the Internet at http://pubs.acs.org.

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