Renaturation of cytochrome C dissolved in polar phosphonate-type ionic liquids using highly polar zwitterions

Renaturation of cytochrome *c* dissolved in polar phosphonate-type ionic liquids using highly polar zwitterions

Kosuke Kuroda,* Yuki Kohno, Hiroyuki Ohno
Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo, 184-8588, Japan
Functional ionic liquid laboratories, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo, 184-8588, Japan

E-mail: kkuroda@staff.kanazawa-u.ac.jp

We present a method for renaturation of the denatured cytochrome *c* in a phosphonate-type ionic liquid with the addition of a suitable zwitterion and small amount of water. The polarity of zwitterions was found to be a critical property for renaturation of cytochrome *c*. The renatured cytochrome *c* was reduced by the addition of sodium dithionite, suggesting that the renatured cytochrome *c* retains its redoxability.

Keywords: ionic liquids | zwitterion | cytochrome *c*

Ionic liquids (ILs), which are salts with melting point below 100 °C, have gained interest as designer solvents. Polarity is one of the promising properties of ILs among their designable properties such as viscosity, hydrophobicity, and conductivity. Polar ILs exhibit extremely high hydrogen bond basicity[1-4] and are reported to be good solvents for some poorly soluble materials such as polysaccharides and proteins.[1-3,5-7] The second generation polar ILs, namely phosphonate- and carboxylate-type ILs, have recently gained special importance owing to their high polarity and low viscosity as compared to the chloride-type ILs.[2,3,8]

Since polysaccharides display high crystallinity and poor solubility, many studies have reported enzymatic hydrolysis of cellulose after pretreatment with polar ILs.[9,10] On the basis of the reaction process, these studies are categorized into two groups: with and without a washing step before the enzymatic hydrolysis. The method without washing ILs is preferable since it only requires dilution of ILs by water.

It is extremely difficult to reuse enzymes dissolved in polar ILs after their reactions. Since enzymatic reactions are carried out in dilute aqueous solutions of ILs, re-concentration of ILs is necessary for its reuse after the reaction and results in denaturation of the enzymes.[10,11] Separation of enzymes from IL media with liquid/liquid extraction technique is thought to be a potential method for reuse of proteins. However, no studies have reported the separation of enzymes from polar ILs owing to the high miscibility of polar ILs with other protein-soluble solvents. Therefore, renaturation methods of enzymes dissolved in concentrated polar ILs are strongly demanded.

Studies suggest that polar ILs strip water molecules around proteins, attributed to their extremely high hydrogen bond basicity, leading to protein denaturation.[11,12] While reports show that only chloride-type ILs can dissolve cytochrome *c* (cyt *c*) without denaturation,[13] phosphonate-type ILs cause its denaturation. On the other hand, Fujita et al. reported that certain ILs, typically composed of choline dihydrogen phosphate and a small volume of water, enhance protein stabilization without denaturation in presence of small volume of water.[14] These "hydrated ILs" were also reported to be useful for renaturation of aggregated proteins.[15] However, renaturation of proteins following their denaturation in polar ILs does not occur just by the addition of water.

In this study, we focused on zwitterions (ZIs)[16] for renaturation of proteins dissolved in phosphonate-type ILs. ZIs have been reported to have a unique hydrated state that displays a cold crystallization behavior, similar to that of choline dihydrogen phosphate.[17,18] The hydrated state is often a characteristic possessed by biocompatible polymers and is reported to play an important role in the stabilization of biomolecules.[19] In the present study, we have envisioned ZIs for renaturation of proteins dissolved in the phosphonate-type ILs through appropriate design of component ions of ZIs.

With respect to the design of ZIs, we focused on the polarity of ZIs to maintain the bound water in the polar phosphonate-type ILs. Less-polar ZIs may not be hydrated in the phosphonate-type ILs. To maintain their hydrated state, ZIs with higher polarity than that of phosphonate-type ILs are required. However, the polarity of ZIs has not been well studied, since ZIs are solids; we speculated the polarity of ZIs from that of analogous ILs. For instance, the β values for the Kamlet-Taft parameter (hydrogen bond basicity) are high for *N*-alkylimidazolium carboxylate-type ILs and 1.18 for 1-butyl-3-methylimidazolium acetate.[20] The resulting *N*-alkylimidazolium carboxylate-type ZI, 3-(1-methylimidazol-3-io)-butane-1-carboxylate (C_1im3C, shown in Figure 1), is expected to display similar β value. On the contrary, the β value for 1-ethyl-3-methylimidazolium methylphosphonate ([C_2mim][(MeO)(H)PO_2], Figure 1) is 1.00[3]; thus, C_1im3C has a β value enough to keep C_1im3C itself in hydrated state in [C_2mim][(MeO)(H)PO_2].

Polar ILs are recognized as extremely strong denaturants and thus it is quite challenging to use complicated proteins at this primitive stage. In this study, we

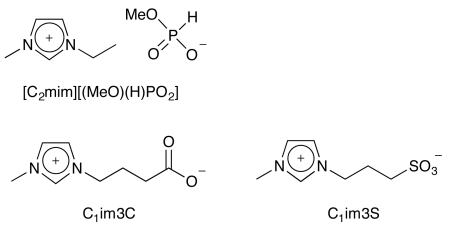

Figure 1. Structures of an IL and ZIs used in this study.

used cyt *c*, a relatively low-molecular-weight and robust protein instead. While cyt *c* is not an enzyme, gaining insights into its dissolved state is very important to further address more complicated proteins such as hydrolytic enzymes.

Cyt *c* is a heme protein where the protoporphyrin IX prosthetic group is fixed to the protein moiety through thioether linkages. The robust protein structure is due to the sophisticated H-bonding network and the intramolecular linkage between Met80 and His18 mediated by the iron atom.[21] In addition, it can be unfolded and refolded reversibly without degradation of the protein.[21] The state of cyt *c*, especially interaction between the heme and the ligands, can easily be analyzed with UV-*vis* spectroscopy while it is difficult to distinguish its second structures.

We preliminarily investigated the structure of cyt *c* (oxidized state; iron (III)) in $[C_2mim][(MeO)(H)PO_2]$ free from ZIs with or without water. In the dried $[C_2mim][(MeO)(H)PO_2]$, a shift in the Soret band of cyt *c* from 408 (in water) to 398 nm was observed, indicating denaturation of cyt *c*. It is noted that there is no precipitation, indicating that cyt *c* did not aggregate. Furthermore, the cyt *c* denaturation was observed even with 70 wt% water content (molar ratio of IL/water was 1/27). The spectrum of cyt *c* in the $[C_2mim][(MeO)(H)PO_2]$/water mixture eventually showed a good agreement of the spectrum of that in water when the molar ratio of IL/water was 1/46 (water content: 80 wt%).

Next, we investigated the effect of ZIs on the dissolved state of cyt *c*. We prepared cyt *c* solution (the molar ratio of $[C_2mim][(MeO)(H)PO_2]$/C_1im3C/water was 1/1/11, water content: 35 wt%). We observed the Soret and Q bands at 408 and 528 nm, respectively, which corresponded to the spectrum in water while the solution did not correspond when the water ratio was below 1/1/11. As expected, polar ZIs enhanced cyt *c* stabilization in polar ILs in the presence of small amount of water.

ZIs exhibit specific hydrated state that enhances the stable dissolution of proteins as mentioned above[18]; however, there are no reports on carboxylate-type ZIs. We analyzed the hydrated state of C_1im3C using differential scanning calorimetry. With C_1im3C/water mixture, an exothermic peak was observed from –70 to –50 °C (Figure S1, see Supporting Information). The peak derived from the cold crystallization is a characteristic of freezing bound water, frequently seen in biocompatible ZIs[17,18], ILs[17], and polymers[19].

We studied the effect of the polarity of ZIs by means of a potentially less polar ZI, 3-(1-methylimidazol-3-io)-propane-1-sulfonate (C_1im3S, shown in Figure 1) because analogous ILs have relatively low hydrogen bond basicity. For instance, the β value for *N*,*N*-dimethylimidazolium methylsulfate is 0.61[22], which is less than that of $[C_2mim][(MeO)(H)PO_2]$. When C_1im3S was added to $[C_2mim][(MeO)(H)PO_2]$ aqueous solution at a final molar ratio of 1/1/11 and 1/1/30 (IL/ZI/water), the Soret band of cyt *c* was observed at 400 and 401 nm, respectively, indicating its denaturation (Figure S2). When the final ratio of IL/ZI/water changed to 1/1/46, the spectrum of cyt *c* corresponded to that in water (Figure S2). Thus, C_1im3S seems to have no positive effect on the stabilization of cyt *c*.

We briefly investigated whether ZIs are able to maintain their hydrated state in $[C_2mim][(MeO)(H)PO_2]$. The saturated aqueous solutions of ZIs were added to dry $[C_2mim][(MeO)(H)PO_2]$. C_1im3S immediately recrystallized in the IL after the addition, suggesting that the lower hydrogen bond basicity of C_1im3S resulted in "torn off" of the bound water of C_1im3S in $[C_2mim][(MeO)(H)PO_2]$. On the other hand, no precipitation was observed for C_1im3C, suggestive of the retention of its bound water molecules even in $[C_2mim][(MeO)(H)PO_2]$ due to sufficiently high hydrogen bond basicity for C_1im3C. Thus, the bound water of C_1im3C may enhance the stable dissolution of cyt *c*.

Renaturation of cyt *c* was carried out by adding C_1im3C and water to the cyt *c* solution of dry $[C_2mim][(MeO)(H)PO_2]$. As described above, cyt *c* was denatured in the $[C_2mim][(MeO)(H)PO_2]$ (the Soret band was at 398 nm), showing a green-colored solution. Addition of C_1im3C and water at final concentration of 1/1/11 ($[C_2mim][(MeO)(H)PO_2]$/C_1im3C/water) led to a drastic color change to red (Figure 2). The UV spectrum of the solution is shown in Figure 3. A strong Soret band and a broad Q band were observed at 407 and 530 nm, respectively, suggesting that cyt *c* was efficiently renatured to its original state.

The redox ability of the renatured cyt *c* in $[C_2mim][(MeO)(H)PO_2]$/C_1im3C/water mixture (1/1/11) was confirmed. As cyt *c* is known as a typical redox-active protein, the activity of the cyt *c* in the solution can be measured with UV-*vis* spectroscopy. In the renatured condition, we observed cyt *c* to be in an oxidized state. We measured the UV-*vis* absorption spectrum of the solution after adding a small excess amount of sodium dithionite as a reducing agent (Figure 3). Sharp α- and β bands in a Q-band

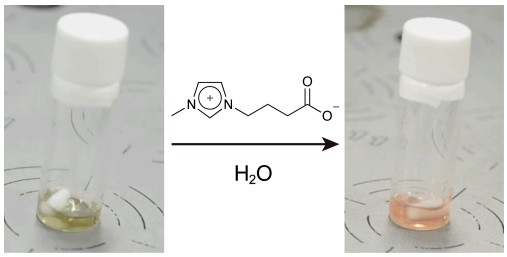

Figure 2. Images of cyt *c* dissolved in $[C_2mim][(MeO)(H)PO_2]$ before and after addition of C_1im3C and water (final ratio: 1/1/11).

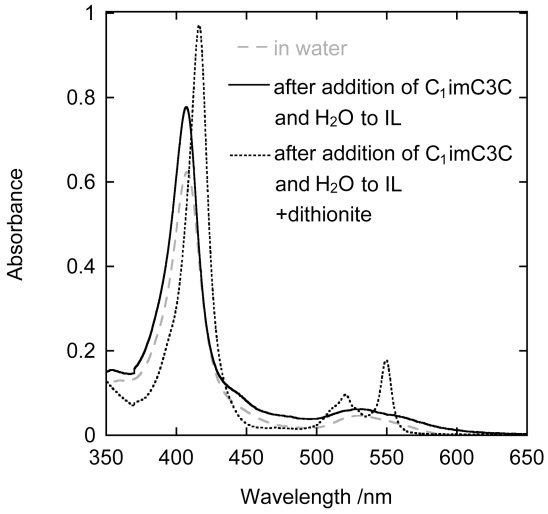

Figure 3. UV spectra of cyt *c* in water (grey dashed line), $[C_2mim][(MeO)(H)PO_2]$ after addition of C_1im3C and water (1/1/11, black continuous line), $[C_2mim][(MeO)(H)PO_2]$ after addition of C_1im3C and water (1/1/11) as well as sodium dithionite (black dotted line).

region were observed at 550 nm and 522 nm, respectively, and Soret band was observed at 417 nm, as seen for reduced cyt *c* in water. This clearly shows that cyt *c* retained the redox activity in the solution.

As we mentioned above, hydration state of ZIs is a key to stabilization and renaturation of proteins. Therefore cyt *c* may be surrounded by hydrated C_1im3C instead of $[C_2mim][(MeO)(H)PO_2]$ in the $[C_2mim][(MeO)(H)PO_2]$/ C_1im3C/water mixture. Stripping $[C_2mim][(MeO)(H)PO_2]$, a strong denaturant, from cyt *c* and interacting between cyt *c* and hydrated C_1im3C, a stabilizer, could be a possible reason for the renaturation. It is however noted that the exact mechanism of renaturation is unclear, and further investigation is needed for the clarification.

In conclusion, a highly polar ZI, C_1im3C, promoted renaturation of cyt *c* dissolved in a polar IL, $[C_2mim][(MeO)(H)PO_2]$, in the presence of a small amount of water. The renatured cyt *c* showed redox ability in the corresponding hydrated ZI/polar IL mixture.

Mr. Y. Ito should be acknowledged for the differential scanning calorimetry measurement. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 21225007, 15K17867). This study was also partly supported by the Japan Science Technology Agency, JST-CREST program.

Supporting Information is available on http://dx.doi.org/10.1246/cl.******.

References and Notes

Present address: Division of Natural System, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan (K. Kuroda), National Institute of Advanced Industrial Science and Technology (AIST), 4-2-1, Nigatake, Miyagino-ku, Sendai 983-8551, Japan (Y. Kohno).

1 R. P. Swatloski, S. K. Spear, J. D. Holbrey, R. D. Rogers, *J. Am. Chem. Soc.* **2002**, *124*, 4974.
2 Y. Fukaya, A. Sugimoto, H. Ohno, *Biomacromolecules* **2006**, *7*, 3295.
3 Y. Fukaya, K. Hayashi, M. Wada, H. Ohno, *Green Chem.* **2008**, *10*, 44.
4 K. Kuroda, H. Kunimura, Y. Fukaya, H. Ohno, *Cellulose* **2014**, *21*, 2199.
5 A. Brandt, J. Gräsvik, J. P. Hallett, T. Welton, *Green Chem.* **2013**, *15*, 550.
6 D. M. Phillips, L. F. Drummy, D. G. Conrady, D. M. Fox, R. R. Naik, M. O. Stone, P. C. Trulove, H. C. De Long, R. A. Mantz, *J. Am. Chem. Soc.* **2004**, *126*, 14350.
7 H. Xie, S. Li, S. Zhang, *Green Chem.* **2005**, *7*, 606.
8 Y. Qin, X. Lu, N. Sun, R. D. Rogers, *Green Chem.* **2010**, *12*, 968.
9 A. P. Dadi, S. Varanasi, C. A. Schall, *Biotechnol. Bioeng.* **2006**, *95*, 904.
10 N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto, H. Takahashi, *Biotechnol. Lett.* **2008**, *30*, 1037.
11 M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey, R. D. Rogers, *Green Chem.* **2003**, *5*, 443.
12 S. Park, R. J. Kazlauskas, *Curr. Opin. Biotechnol.* **2003**, *14*, 432.
13 K. Tamura, N. Nakamura, H. Ohno, *Biotechnol. Bioeng.* **2012**, *109*, 729.
14 K. Fujita, D. R. MacFarlane, M. Forsyth, M. Yoshizawa-Fujita, K. Murata, N. Nakamura, H. Ohno, *Biomacromolecules* **2007**, *8*, 2080.
15 K. Fujita, M. Kajiyama, Y. Liu, N. Nakamura, H. Ohno, *Chem. Commun.* **2016**, *52*, 13491.
16 M. Yoshizawa, M. Hirao, K. Ito-Akita, H. Ohno, *J. Mater. Chem.* **2001**, *11*, 1057.
17 K. Fujita, Y. Nikawa, H. Ohno, *Chem. Commun.* **2013**, *49*, 3257.
18 Y. Ito, Y. Kohno, N. Nakamura, H. Ohno, *Chem. Commun.* **2012**, *48*, 11220.
19 M. Tanaka, T. Motomura, N. Ishii, K. Shimura, M. Onishi, A. Mochizuki, T. Hatakeyama, *Polym. Int.* **2000**, *49*, 1709.
20 T. V. Doherty, M. Mora-Pale, S. E. Foley, R. J. Linhardt, J. S. Dordick, *Green Chem.* **2010**, *12*, 1967.
21 S.-R. Yeh, S. Han, D. L. Rousseau, *Acc. Chem. Res.* **1998**, *31*, 727.
22 K. Kuroda, H. Kunimura, Y. Fukaya, N. Nakamura, H. Ohno, *ACS Sustain. Chem. Eng.* **2014**, *2*, 2204.