A polar liquid zwitterion does not critically destruct cytochrome c at high concentration: an initial comparative study with a polar ionic liquid

A polar liquid zwitterion does not critically destruct cytochrome *c* at high concentration: an initial comparative study with a polar ionic liquid

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Abstract

A polar carboxylate-type zwitterion with a small volume of water can dissolve cytochrome *c* without significant disruption, compared to the case of a popular polar carboxylate-type ionic liquid, 1-ethyl-3-methylimidazolium acetate. The

change in Soret, Q, and 615 nm bands was not in the 80 wt% polar zwitterion solution, whereas the shift in Soret band, diminishing Q band, appearance 615 nm band was found in the 80 wt% polar ionic liquid solution. It suggests that the concentrated polar ionic liquid solutions critically disrupt the structure of cytochrome *c*, and the polar zwitterion solution used in this study was better than 1-ethyl-3-methylimidazolium acetate solution in high concentration range.

Introduction

Ionic liquids (ILs), defined as salts with melting point below 100°C, have attracted many interests as unique solvents. Polar ILs have extremely high hydrogen bond basicity[1-4] compared to polar organic solvents, and are known to be good solvents for poorly soluble materials such as proteins and polysaccharides.[1-3, 5-7] Carboxylate- and phosphonate-type ILs have been recently recognized as better polar ILs, compared to the chloride-type ILs, because they have higher polarity.[2, 3, 8]

Since polysaccharides, especially cellulose, are recalcitrant to many solvents due to the high crystallinity, there have been many studies regarding the enzymatic saccharification of cellulose after pretreatment with polar ILs.[9, 10] Here, these studies are categorized into two groups based on the reaction process: with or without washing the polar ILs before the enzymatic saccharification. The method without washing the polar ILs is quite preferable because only a dilution of the ILs by water is required in the process, suggesting a simple process. However, concentrated polar IL solutions are well known to denature enzymes.

Some studies have mentioned that the polar ILs strip water molecules around proteins, in addition to the disruption of the hydrogen bonds in the protein structure, due to the high hydrogen bond basicity of the polar ILs, resulting in the denaturation of proteins.[11, 12] Whereas chloride-type ILs dissolves cytochrome *c* (cyt. *c*) without denaturation,[13] carboxylate-type ILs denature it.

On the other hand, certain ILs with a small volume of water have

been reported to improve protein stabilization even at high temperatures.[14] The ILs are typically composed of choline dihydrogen phosphate. Similar to choline dihydrogen phosphate, some zwitterions (ZIs)[15] with a small volume of water have been reported as solutions for proteins. ZIs also have a unique hydrated state displaying a cold crystallization behavior, as well as choline dihydrogen phosphate.[16, 17] The hydrated state is often seen in biocompatible polymers and has been recognized as a key factor in the stabilization of biomolecules.[18] In the present study, we addressed whether polar carboxylate-type ZIs, which dissolve cellulose, can be used to dissolve proteins stably.

We used cyt. *c*, a robust and relatively low-molecular-weight protein, for this proof-of-concept study, which has been used as a model protein in many studies with ILs.[13, 14, 19-22] While cyt. *c* is not an enzyme, but a heme protein involved in electron transfer, its dissolved state can provide important insights to further address more complicated proteins such as hydrolytic enzymes. The robust protein structure of cyt. *c* is based on the H-bonding

network and the intramolecular linkage between His18 and Met80 mediated by the iron atom of heme.[23] In addition, it can reversibly be unfolded and refolded without critical denaturation and/or aggregation while severe condition, of course, causes irreversible denaturation and aggregation.[23] The state of cyt. *c* can be easily analyzed by using UV–vis spectroscopy, especially with focusing on the interaction between the ligands and the heme, whereas its secondary structures are difficult to distinguish. In detail, native state (III), unligated form between Met80 and heme (3.5), misligation between Lys and heme (IV), Lys-displaced form (V), and unfolded form (U) has been reported as the states of cyt. *c,* especially focusing the vicinity of heme.[24]

Results and Discussion

We used a ZI (OE_2imC_3C) shown in Fig. 1 in this study because it is the only ZI that is reported to dissolve cellulose so far.[25] It maintains liquid

state at room temperature due to the flexibility of its oligo-ether chain, while ZIs are typically solid. Therefore it can be used as a solvent to dissolve cellulose (solubility: 14 and 7 wt% at 120 °C with or without the co-solvent dimethylsulfoxide).[25, 26] OE_2imC_3C has high hydrogen bond basicity, and the β value of Kamlet-Taft parameters is 1.12, which is similar to or somewhat higher than that of a typical polar IL (0.95 for 1-ethyl-3-methylimidazolium acetate ($[C_2mim]OAc$, see Fig. 1)[27]). The high hydrogen bond basicity is the key factor for disrupting the hydrogen bond network of cellulose crystals to dissolve. We compared OE_2imC_3C with the typical cellulose-dissolving carboxylate-type IL, $[C_2mim]OAc$.

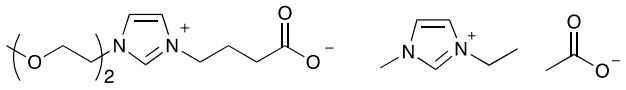

Fig. 1. Structures of OE_2imC_3C and $[C_2mim]OAc$.

We investigated the structure of ferric cyt. *c* in $OE_2imC_3C/[C_2mim]OAc$ solutions at various concentrations at room

temperature (Table S1 and Figs. S1–S4). A UV-vis spectrum of cyt. *c* in phosphate buffer (pH 7.0, 10 mM) was first measured as a reference: a sharp Soret band (408.9 nm), a broad Q band (529 nm), and a very small band derived from heme–Met80 bond (695 nm, absorbance: 0.005) were observed and it is confirmed to be native state, called III. In 30 wt% of OE_2imC_3C/water mixture, the Soret band shifted to 412.6 nm and the Q band was splitted at 520.7 and 549.5 nm. It is similar to that of ferrous cyt. *c*. Such ferrous cyt. *c*-like spectrum has been reported as state IV in alkali; the heme center undergoes at least partial autoreduction.[24] Carboxylate-type ZIs and ILs aqueous solutions are basically alkali (pH 7.7 for 30 wt% OE_2imC_3C solution), and there is no conflict to the previous report. The previous report[24] however mentioned that the transition to the state IV occurs at pH around 10, and thus the pH value is too low. It may involve ion concentration because 30 wt% OE_2imC_3C solution is very high concentration (~1.2 mol/L). In 50 wt% OE_2imC_3C, the same trend is observed. On the other hand, the Soret band of ferric cyt. *c* was observed at 408.4 nm, and a Q band was observed at 527.7

nm in the 70 wt% OE_2imC_3C solution at room temperature. They were also observed at 408.7 and 527.0 nm, respectively, in 80 wt% OE_2imC_3C solution. The results suggest that cyt. *c* is similar to native (III) structure in the 70 and 80 wt% solutions of OE_2imC_3C. Here we checked the band at 695 nm, which is attributed to the bond between Met80 and heme, and it is not observed in these solutions. It indicates the bond between Met80 and heme is not maintained in these solutions. The bond is very weak and it is known to be broken at pH 8.8. The OE_2imC_3C solutions show the pH of 10.1 and 10.9 at 70 and 80 wt%, respectively. From this fact, the state of cyt. *c* in 70 and 80 wt% solutions can be estimated as 3.5 (3.5 means a state between III and IV[24]), but it is not 3.5 because the cyt. *c* does not possess the characteristics of state 3.5: a flat Q band and the signal at 550 nm (the spectrum is similar to 30 wt% solution shown in Fig. S2). The cyt. *c* in 70 and 80 wt% is thus in state between III and 3.5 (defined as III–3.5 in this study) As a summary, cyt. *c* in 70 and 80 wt% solutions was not completely native but maintains major part of the structure at heme vicinity. It is noted that showing state IV and

3.5 at lower and higher OE_2imC_3C concentration, respectively, is unexpected and interesting. It also may involve with ion concentration because these solutions are extremely concentrated, 3–4 mol/L. In 90 wt% solution, Soret and Q bands shifted to 411.2 and 524.6 nm, respectively, and the absorbance of the bands significantly decreased. The state itself is still in III–3.5 based on the Q and 695 nm bands but clearly denatured, compared to the samples mentioned above.

To investigate the thermal stability of cyt. *c* in the OE_2imC_3C solutions, the samples were incubated at 80 °C. We first a cyt. *c* solution in buffer and the irreversible aggregation and visible precipitation of cyt. *c* were observed. On the other hand, the non-aggregated cyt. *c*, which was obtained through filtration, showed state III–3.5 because the band at 695 nm vanished (see Table S1). In all OE_2imC_3C solutions, there was no irreversible aggregation: OE_2imC_3C mitigated the heat shock. While the state in 70, 80, and 90 wt% solutions did not change after heat shock, the state changes from IV to 3.5 in 30 and 50 wt% solutions. It is interesting because of the opposite

shift which expected, i.e. cyt. *c* gets close to native state by heat shock.

UV-vis spectra of cyt. *c* in [C_2mim]OAc solutions was measured at room temperature to compare. In the 30 wt% solutions, Soret and Q band were observed at 409.0 and 528.7 nm, and the signal at 695 nm was slightly observed: it is state III rather than 3.5. It suggests [C_2mim]OAc does not much perturb cyt. *c* at 30 wt%. The Soret band shifts to downside and the absorbance of Q band decreases with increasing the concentration of [C_2mim]OAc: Soret band at 406.8, 407.6, 406.3, and 401.9 nm (native: 408.9 nm) and Q band for absorbance of 0.043, 0.027, 0.027, and 0.017 (native: 0.037) for 50, 70, 80, 90 wt%, respectively. They, especially in 70–90 wt% solution, are thus far from native state at least at the vicinity of heme, compared to the cases of OE_2imC_3C, although they are still in state III–3.5 based on the Q and 695 nm bands. As a further proof of partial denaturation, new signal appeared at around 615 nm. The new signal is assigned to partial denaturation of cyt. *c* itself or ligation by [C_2mim]OAc because imidazolium cation and acetate anion can play a role as a ligand for heme[28]. Here we

assigned it to the former one because the same signal was found in the spectrum of cyt. *c* in pure water after 80 °C heat shock (Fig. S5). The signal at around 615 nm has been reported as that of free heme,[29] thus heme might be displaced from polypeptide whereas a signal at 493 nm, which is one of the signal of free heme in dimethyl sulfoxide aqueous solution[29], was not observed.

Stability against heat shock in the $[C_2mim]OAc$ solutions was investigated. There are almost no changes in the spectra after 80 °C heating and thus cyt. *c* in 70–90 wt% solutions are far from native state. From these results, cyt. *c* is relatively stable and become thermally stable in the concentrated OE_2imC_3C solutions (70 and 80 wt%), compared to that in $[C_2mim]OAc$ solutions, while the state is not completely native.

The difference in the cyt. *c* structures in the OE_2imC_3C and $[C_2mim]OAc$ solutions should be attributed to the specific hydrated state of ZIs. ZIs exhibit a specific hydrated state[30] that improves the stable dissolution of proteins as mentioned above;[17] it has been reported that

carboxylate-type ZIs also have the specific hydrated state[31] as well as biocompatible ZIs (sulfonate-type),[16, 17] ILs,[16] and polymers[18]. On the other hand, it is known that polar ILs, including carboxylate-type ILs, strip water molecules around proteins, which has been attributed to their extremely high hydrogen bond basicity; resulting in protein denaturation.[11, 12] The difference in cyt. *c* in the solutions are not attributed to the hydrogen bond basicity because OE_2imC_3C has higher β values in Kamlet-Taft parameters (1.12) than $[C_2mim]OAc$ (0.95).[25, 27] It is noted that the pH values of the solutions may not be a critical factor of the difference on cyt. *c* in the OE_2imC_3C and $[C_2mim]OAc$ solutions, either, because there was not significant difference on pH values of OE_2imC_3C and $[C_2mim]OAc$ solutions (see Table S1). Although there is difference only in the pH of the 30 wt% solutions (pH 7.7 and state IV for OE_2imC_3C solution, pH 7.7 and state III(–3.5) for $[C_2mim]OAc$ solution), it might not be the reason for the difference of the state of cyt. *c* because the state was also different in the 50 wt% solutions despite almost the same pH (pH 8.2 and state IV for OE_2imC_3C solution, pH 8.0 and state

III–3.5 for [C_2mim]OAc solution).

Thermal stability of cyt. *c* was investigated in detail in 70 and 80 wt% OE_2imC_3C/[C_2mim]OAc solutions. The wavelength of the Soret band at various temperatures in 70 wt% OE_2imC_3C/[C_2mim]OAc solutions is shown in Fig. 2 (the spectra are shown in Figs. S6 and 7, and detailed data set is also summarized in Table S2). In the 70 wt% OE_2imC_3C solution, the Soret band was observed at 408.4, 408.2, 408.2, 408.4, and 408.6 nm at room temperature, 50, 60, 70, and 80 °C, respectively; the Soret band barely shifted with the increasing temperatures. The Q band was also barely affected by the increasing temperature (527.7, 528.3, 527.9, 528.8, and 528.7 nm at room temperature, 50, 60, 70, and 80 °C, respectively). Here, there were no aggregates of cyt. *c*, and these observations demonstrate that cyt. *c* maintains state III–3.5, even at high temperatures while cyt. *c* aggregated in phosphate buffer at 80 °C. Here, the band at 615 nm was not observed even at high temperature; indicating cyt. *c* is not far from native state. In the 70 wt% [C_2mim]OAc solution, the Soret band was observed at 407.6, 405.9, 404.4,

403.6, and 403.0 nm at room temperature, 50, 60, 70, and 80 °C, respectively; the Soret band was significantly shifted with increasing temperatures (see Fig. 2). This shows that the structure of cyt. *c* was disrupted, although the Q band did not change with increasing temperature (528.5, 528.4, 528.4, 528.4, and 528.1 nm at room temperature, 50, 60, 70, and 80 °C, respectively). These results indicate the state of cyt. *c* itself maintained III–3.5. However, the absorbance of Q band became very small with increasing temperature: 0.027 and 0.018 at room temperature and 80 °C, respectively (cf. 0.037 in buffer at room temperature, 0.042 and 0.034 in OE_2imC_3C solution at room temperature and 80 °C, respectively). The absorbance of the band at 615 nm increased from 0.001 (room temperature) to 0.003 (80 °C); presumably indicating that heme displacement was enhanced by heating (see Table S2). In addition, it was noted that the solution turned from red to somewhat green at higher temperatures, although there were no irreversible aggregates of cyt. *c*. The green color is a typical color of disrupted cyt. *c*.

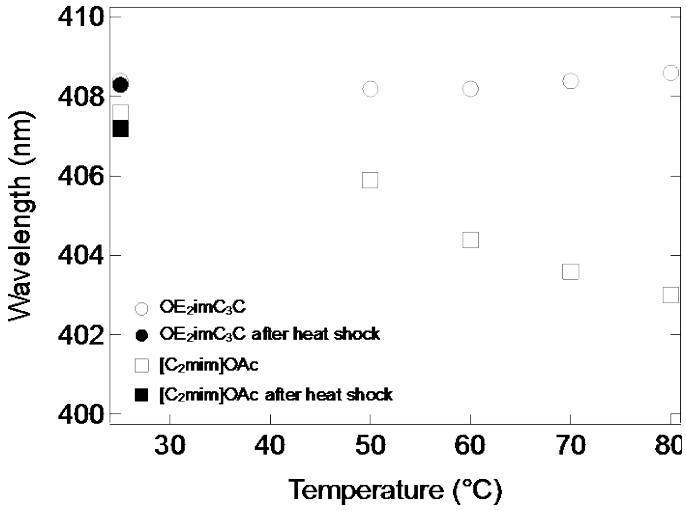

Fig. 2. Wavelength at the maximum absorbance of the Soret band of cyt. *c* in 70 wt% solutions of OE_2imC_3C or $[C_2mim]OAc$ at various temperatures. The filled markers indicate those measured at room temperature after heated at 80 °C. The Soret band in phosphate buffer was observed at 408.9 nm.

A similar trend was observed in 80 wt% solutions (Fig. 3). Regarding OE_2imC_3C, the Soret band was observed at 408.7, 408.9 409.1, 409.1, and 409.1 nm at room temperature, 50, 60, 70, and 80 °C, respectively. The Q band did not shift either; 527.0, 528.5, 528.5, 527.8, and 528.3 nm at room temperature, 50, 60, 70, and 80 °C, respectively. These results showed that

cyt. *c* maintained state III–3.5, in 80 wt% OE_2imC_3C solutions even at high temperatures. The band at 615 nm was not observed even at high temperature; indicating cyt. *c* is not far from native state. On the other hand, we investigated the structure of cyt. *c* in the 80 wt% $[C_2mim]OAc$ solution at higher temperatures, although the Soret band shifted even at room temperature. The Soret band was observed at 406.3, 403.2, 402.4, 401.8, and 402.2 nm at room temperature, 50, 60, 70, and 80 °C, respectively, showing a shift as the temperature increased, similar to that with the 70 wt% solution. The Q band was observed at 528.3, 528.6, 526.1, 526.5, and 526.4 nm at room temperature, 50, 60, 70, and 80 °C, respectively; even the Q band shifted at higher temperatures. Although the state of cyt. *c* in the 80 wt% $[C_2mim]OAc$ solution itself is also categorized into III–3.5 from the shape of Q band, the absorbance of Q band became very small with increasing temperature: 0.027 and 0.020 at room temperature and 80 °C, respectively (cf. 0.037 in buffer at room temperature, 0.039 and 0.035 in OE_2imC_3C solution at room temperature and 80 °C, respectively). The absorbance of the band at 615 nm

increased from 0.002 (room temperature) to 0.004 (80 °C); presumably indicating that heme displacement enhanced by heating (see Table S2). From these results, cyt. *c* is durable to heat in the OE_2imC_3C solutions while the disruption of cyt. *c*, at least at the vicinity of heme, was enhanced by heating in the $[C_2mim]OAc$ solutions.

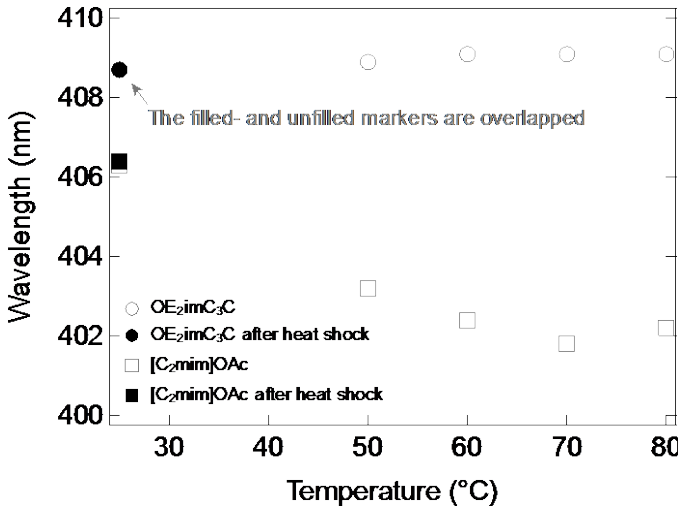

Fig. 3. Wavelength at the maximum absorbance of the Soret band of cyt. *c* in 80 wt% solutions of OE_2imC_3C or $[C_2mim]OAc$ at a variety of temperatures. The filled markers indicate those measured at room temperature after heated at 80 °C. The Soret band in phosphate buffer was observed at 408.9 nm.

We again discuss the structure of cyt. *c* at room temperature after heating at 80 °C to confirm that cyt. c was resistant to heat shock. There was no change in the structure of cyt. *c* in the 70 and 80 wt% OE_2imC_3C solutions (Soret and Q bands: 408.3 and 526.2 nm, respectively (70 wt%); 408.7 and 526.7 nm, respectively (80 wt%), see Figs. 2 and 3). There were no significant changes in absorbance and the 615 nm band, either. It was confirmed that heating did not damage cyt. *c* in hydrated OE_2imC_3C solutions. In the case of 70 wt% $[C_2mim]$OAc solution, the Soret and Q bands after heating were observed at 407.2 and 527.1 nm, respectively, and were similar to those before heating (407.6 and 528.5 nm, see Fig. 2) whereas these bands were found at 403.0 and 528.1 nm, respectively, at 80 °C. The band at 615 nm also returned from 0.003 (80 °C) to less than 0.001 (room temperature after heat shock). This indicated that the vicinity of cyt. *c* was returned, which is not conflict with previous studies reporting that cyt. *c* can reversibly unfolded and refolded without irreversible denaturation and/or aggregation.[23] The same trend was observed in the 80 wt% $[C_2mim]$OAc solution, although their

structure was far from native state even at room temperature. The Soret and Q bands were observed at 406.3 and 528.3 nm, respectively, before heating, and 406.4 and 526.5 nm, respectively, after heating, whereas these bands were found at 402.2 and 526.4 nm, respectively, at 80 °C. The band at 615 nm also returned from 0.004 (80 °C) to 0.001 (room temperature after heat shock).

The redox ability of cyt. *c* in 80 wt% of OE_2imC_3C solution was confirmed. Cyt. *c* is a typical redox-active protein, and its activity can be evaluated with UV-vis spectroscopy. When cyt. *c* was dissolved in 80 wt% OE_2imC_3C solution, cyt. *c* was observed in an oxidized state. An UV-vis absorption spectrum of the cyt. *c* solution was measured after the addition of a small excess amount of the reducing agent, sodium hydrosulfite (Fig. S10). In the spectrum, the Soret band was observed at 418.2 nm, and the sharp α- and β bands in the Q-band region were observed at 551.3 and 522.5 nm, respectively, indicating reduced cyt. *c*. This clearly shows that cyt. *c* maintained the redox activity in the 80 wt% of OE_2imC_3C solution. It is noted that almost the same bands were found at 80 °C (Soret band, 418.2 nm; α-

and β bands, 552.3 and 523.3 nm, respectively). Whereas almost the same spectrum was found in 80 wt% [C_2mim]OAc solution after addition of sodium hydroxysulfite, it is sure that cyt. *c* in concentrated OE_2imC_3C solutions maintains redox ability at least with a strong reducing agent.

Conclusion

We investigated the stability of cyt. *c* in a polar ZI, OE_2imC_3C, with a small volume of water because general ZIs tend to stabilize proteins compared to ILs. Cyt. *c* was not far from native structure in the 80 wt% OE_2imC_3C solution because the Soret band did not shift and the band at 615 nm, which may be attributed to free heme, was not observed. On the other hand, cyt. *c* was far from the native state in the 80 wt% [C_2mim]OAc solution as indicated by the Soret band shift from 408.9 to 406.3 nm and the band at 615 nm was observed. OE_2imC_3C improved the thermal stability of cyt. *c*. The Soret band did not shift at temperatures up to 80 °C in the both 70 and 80 wt% OE_2imC_3C solutions and the band at 615 nm was not observed even at

80 °C. On the other hand, the Soret band shifted significantly to 403.0 and 402.2 nm at 80 °C in the 70 and 80 wt% $[C_2mim]OAc$ solutions, respectively, and the band at 615 nm increased with elevating temperature up to 80 °C. These results indicate that cyt. *c* was critically disrupted in $[C_2mim]OAc$ solutions, and OE_2imC_3C solutions were better solutions for proteins compared to $[C_2mim]OAc$ solutions.

Experimental

UV-vis spectroscopy of cyt. *c* in OE_2imC_3C and $[C_2mim]OAc$ solutions

Cyt. *c* from equine heart, oxidized state, (Sigma-aldrich) was dissolved in a certain amount of pure water, and the resulting liquid was mixed with OE_2imC_3C[25, 32] or $[C_2mim]OAc$ (purchased form BASF, used after drying). The final concentration of cyt. *c* was 1 mg/g. UV-vis absorption spectra were measured using quartz cells with a 0.5 mm light-path length. To heat the samples, we used a cell heating system (CoolSpeK USP-203, Unisoku Co., Ltd.). Phosphate buffer (pH 7.0, 10 mM) was prepared and used for reference.

Supplementary Material

Spectra of cyt. *c* in OE_2imC_3C or $[C_2mim]OAc$ solutions, summary of wavelength at the maximum absorbance and the absorbance of the Soret-, Q-,

695 nm-, 615 nm bands of cyt.*c* in OE_2imC_3C or $[C_2mim]OAc$ solutions are available on the journal's website.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgments

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