

# 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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1 A polar liquid zwitterion does not critically destruct cytochrome *c* at high  
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12

13 **Abstract**

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

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

8

## 9 Introduction

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

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

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

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

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

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

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

11

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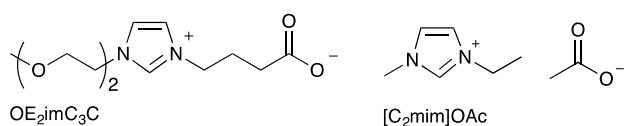
13

## 14 Results and Discussion

15 We used a ZI (OE<sub>2</sub>imC<sub>3</sub>C) shown in Fig. 1 in this study because it is  
16 the only ZI that is reported to dissolve cellulose so far.<sup>[25]</sup> It maintains liquid

1 state at room temperature due to the flexibility of its oligo-ether chain, while  
2 ZIs are typically solid. Therefore it can be used as a solvent to dissolve  
3 cellulose (solubility: 14 and 7 wt% at 120 °C with or without the co-solvent  
4 dimethylsulfoxide).<sup>[25, 26]</sup> OE<sub>2</sub>imC<sub>3</sub>C has high hydrogen bond basicity, and the  
5  $\beta$  value of Kamlet-Taft parameters is 1.12, which is similar to or somewhat  
6 higher than that of a typical polar IL (0.95 for 1-ethyl-3-methylimidazolium  
7 acetate ([C<sub>2</sub>mim]OAc, see Fig. 1)<sup>[27]</sup>). The high hydrogen bond basicity is the  
8 key factor for disrupting the hydrogen bond network of cellulose crystals to  
9 dissolve. We compared OE<sub>2</sub>imC<sub>3</sub>C with the typical cellulose-dissolving  
10 carboxylate-type IL, [C<sub>2</sub>mim]OAc.

11



13 **Fig. 1.** Structures of OE<sub>2</sub>imC<sub>3</sub>C and [C<sub>2</sub>mim]OAc.

14

15 We investigated the structure of ferric cyt. *c* in  
16 OE<sub>2</sub>imC<sub>3</sub>C/[C<sub>2</sub>mim]OAc solutions at various concentrations at room

1 temperature (Table S1 and Figs. S1–S4). A UV-vis spectrum of cyt. *c* in  
2 phosphate buffer (pH 7.0, 10 mM) was first measured as a reference: a sharp  
3 Soret band (408.9 nm), a broad Q band (529 nm), and a very small band  
4 derived from heme–Met80 bond (695 nm, absorbance: 0.005) were observed  
5 and it is confirmed to be native state, called III. In 30 wt% of OE<sub>2</sub>imC<sub>3</sub>C/water  
6 mixture, the Soret band shifted to 412.6 nm and the Q band was splitted at  
7 520.7 and 549.5 nm. It is similar to that of ferrous cyt. *c*. Such ferrous cyt. *c*  
8 like spectrum has been reported as state IV in alkali; the heme center  
9 undergoes at least partial autoreduction.<sup>[24]</sup> Carboxylate-type ZIs and ILs  
10 aqueous solutions are basically alkali (pH 7.7 for 30 wt% OE<sub>2</sub>imC<sub>3</sub>C solution),  
11 and there is no conflict to the previous report. The previous report<sup>[24]</sup> however  
12 mentioned that the transition to the state IV occurs at pH around 10, and  
13 thus the pH value is too low. It may involve ion concentration because 30 wt%  
14 OE<sub>2</sub>imC<sub>3</sub>C solution is very high concentration (~1.2 mol/L). In 50 wt%  
15 OE<sub>2</sub>imC<sub>3</sub>C, the same trend is observed. On the other hand, the Soret band of  
16 ferric cyt. *c* was observed at 408.4 nm, and a Q band was observed at 527.7



1 nm in the 70 wt% OE<sub>2</sub>imC<sub>3</sub>C solution at room temperature. They were also  
2 observed at 408.7 and 527.0 nm, respectively, in 80 wt% OE<sub>2</sub>imC<sub>3</sub>C solution.  
3 The results suggest that cyt. *c* is similar to native (III) structure in the 70 and  
4 80 wt% solutions of OE<sub>2</sub>imC<sub>3</sub>C. Here we checked the band at 695 nm, which  
5 is attributed to the bond between Met80 and heme, and it is not observed in  
6 these solutions. It indicates the bond between Met80 and heme is not  
7 maintained in these solutions. The bond is very weak and it is known to be  
8 broken at pH 8.8. The OE<sub>2</sub>imC<sub>3</sub>C solutions show the pH of 10.1 and 10.9 at  
9 70 and 80 wt%, respectively. From this fact, the state of cyt. *c* in 70 and 80  
10 wt% solutions can be estimated as 3.5 (3.5 means a state between III and  
11 IV<sup>[24]</sup>), but it is not 3.5 because the cyt. *c* does not possess the characteristics  
12 of state 3.5: a flat Q band and the signal at 550 nm (the spectrum is similar  
13 to 30 wt% solution shown in Fig. S2). The cyt. *c* in 70 and 80 wt% is thus in  
14 state between III and 3.5 (defined as III–3.5 in this study) As a summary, cyt.  
15 *c* in 70 and 80 wt% solutions was not completely native but maintains major  
16 part of the structure at heme vicinity. It is noted that showing state IV and

1 3.5 at lower and higher OE<sub>2</sub>imC<sub>3</sub>C concentration, respectively, is unexpected  
2 and interesting. It also may involve with ion concentration because these  
3 solutions are extremely concentrated, 3–4 mol/L. In 90 wt% solution, Soret and  
4 Q bands shifted to 411.2 and 524.6 nm, respectively, and the absorbance of  
5 the bands significantly decreased. The state itself is still in III–3.5 based on  
6 the Q and 695 nm bands but clearly denatured, compared to the samples  
7 mentioned above.

8 To investigate the thermal stability of cyt. *c* in the OE<sub>2</sub>imC<sub>3</sub>C  
9 solutions, the samples were incubated at 80 °C. We first a cyt. *c* solution in  
10 buffer and the irreversible aggregation and visible precipitation of cyt. *c* were  
11 observed. On the other hand, the non-aggregated cyt. *c*, which was obtained  
12 through filtration, showed state III–3.5 because the band at 695 nm vanished  
13 (see Table S1). In all OE<sub>2</sub>imC<sub>3</sub>C solutions, there was no irreversible  
14 aggregation: OE<sub>2</sub>imC<sub>3</sub>C mitigated the heat shock. While the state in 70, 80,  
15 and 90 wt% solutions did not change after heat shock, the state changes from  
16 IV to 3.5 in 30 and 50 wt% solutions. It is interesting because of the opposite

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

2 UV-vis spectra of cyt. *c* in [C<sub>2</sub>mim]OAc solutions was measured at

3 room temperature to compare. In the 30 wt% solutions, Soret and Q band

4 were observed at 409.0 and 528.7 nm, and the signal at 695 nm was slightly

5 observed: it is state III rather than 3.5. It suggests [C<sub>2</sub>mim]OAc does not

6 much perturb cyt. *c* at 30 wt%. The Soret band shifts to downside and the

7 absorbance of Q band decreases with increasing the concentration of

8 [C<sub>2</sub>mim]OAc: Soret band at 406.8, 407.6, 406.3, and 401.9 nm (native: 408.9

9 nm) and Q band for absorbance of 0.043, 0.027, 0.027, and 0.017 (native:

10 0.037) for 50, 70, 80, 90 wt%, respectively. They, especially in 70–90 wt%

11 solution, are thus far from native state at least at the vicinity of heme,

12 compared to the cases of OE<sub>2</sub>imC<sub>3</sub>C, although they are still in state III–3.5

13 based on the Q and 695 nm bands. As a further proof of partial denaturation,

14 new signal appeared at around 615 nm. The new signal is assigned to partial

15 denaturation of cyt. *c* itself or ligation by [C<sub>2</sub>mim]OAc because imidazolium

16 cation and acetate anion can play a role as a ligand for heme<sup>[28]</sup>. Here we

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

7           Stability against heat shock in the [C<sub>2</sub>mim]OAc solutions was  
8 investigated. There are almost no changes in the spectra after 80 °C heating  
9 and thus cyt. *c* in 70–90 wt% solutions are far from native state. From these  
10 results, cyt. *c* is relatively stable and become thermally stable in the  
11 concentrated OE<sub>2</sub>imC<sub>3</sub>C solutions (70 and 80 wt%), compared to that in  
12 [C<sub>2</sub>mim]OAc solutions, while the state is not completely native.

13           The difference in the cyt. *c* structures in the OE<sub>2</sub>imC<sub>3</sub>C and  
14 [C<sub>2</sub>mim]OAc solutions should be attributed to the specific hydrated state of  
15 ZIs. ZIs exhibit a specific hydrated state<sup>[30]</sup> that improves the stable  
16 dissolution of proteins as mentioned above;<sup>[17]</sup> it has been reported that

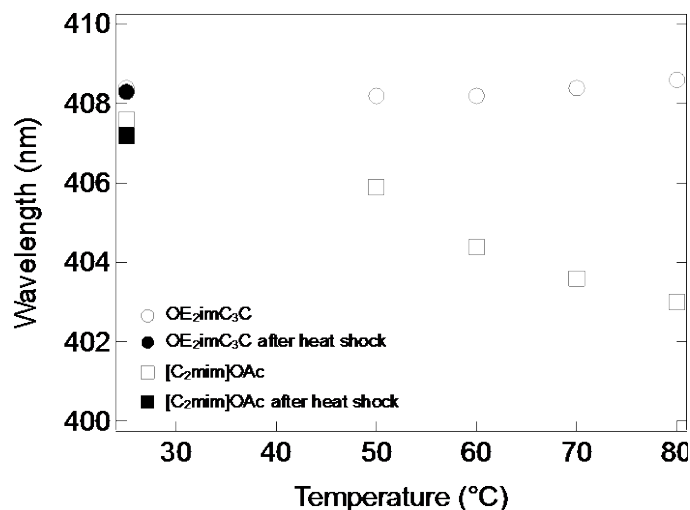
1 carboxylate-type ZIs also have the specific hydrated state<sup>[31]</sup> as well as  
2 biocompatible ZIs (sulfonate-type),<sup>[16, 17]</sup> ILs,<sup>[16]</sup> and polymers<sup>[18]</sup>. On the other  
3 hand, it is known that polar ILs, including carboxylate-type ILs, strip water  
4 molecules around proteins, which has been attributed to their extremely high  
5 hydrogen bond basicity; resulting in protein denaturation.<sup>[11, 12]</sup> The difference  
6 in cyt. *c* in the solutions are not attributed to the hydrogen bond basicity  
7 because OE<sub>2</sub>imC<sub>3</sub>C has higher  $\beta$  values in Kamlet-Taft parameters (1.12)  
8 than [C<sub>2</sub>mim]OAc (0.95).<sup>[25, 27]</sup> It is noted that the pH values of the solutions  
9 may not be a critical factor of the difference on cyt. *c* in the OE<sub>2</sub>imC<sub>3</sub>C and  
10 [C<sub>2</sub>mim]OAc solutions, either, because there was not significant difference on  
11 pH values of OE<sub>2</sub>imC<sub>3</sub>C and [C<sub>2</sub>mim]OAc solutions (see Table S1). Although  
12 there is difference only in the pH of the 30 wt% solutions (pH 7.7 and state  
13 IV for OE<sub>2</sub>imC<sub>3</sub>C solution, pH 7.7 and state III(-3.5) for [C<sub>2</sub>mim]OAc  
14 solution), it might not be the reason for the difference of the state of cyt. *c*  
15 because the state was also different in the 50 wt% solutions despite almost  
16 the same pH (pH 8.2 and state IV for OE<sub>2</sub>imC<sub>3</sub>C solution, pH 8.0 and state

1 III–3.5 for [C<sub>2</sub>mim]OAc solution).

2 Thermal stability of cyt. *c* was investigated in detail in 70 and 80  
3 wt% OE<sub>2</sub>imC<sub>3</sub>C/[C<sub>2</sub>mim]OAc solutions. The wavelength of the Soret band at  
4 various temperatures in 70 wt% OE<sub>2</sub>imC<sub>3</sub>C/[C<sub>2</sub>mim]OAc solutions is shown  
5 in Fig. 2 (the spectra are shown in Figs. S6 and 7, and detailed data set is also  
6 summarized in Table S2). In the 70 wt% OE<sub>2</sub>imC<sub>3</sub>C solution, the Soret band  
7 was observed at 408.4, 408.2, 408.2, 408.4, and 408.6 nm at room temperature,  
8 50, 60, 70, and 80 °C, respectively; the Soret band barely shifted with the  
9 increasing temperatures. The Q band was also barely affected by the  
10 increasing temperature (527.7, 528.3, 527.9, 528.8, and 528.7 nm at room  
11 temperature, 50, 60, 70, and 80 °C, respectively). Here, there were no  
12 aggregates of cyt. *c*, and these observations demonstrate that cyt. *c* maintains  
13 state III–3.5, even at high temperatures while cyt. *c* aggregated in phosphate  
14 buffer at 80 °C. Here, the band at 615 nm was not observed even at high  
15 temperature; indicating cyt. *c* is not far from native state. In the 70 wt%  
16 [C<sub>2</sub>mim]OAc solution, the Soret band was observed at 407.6, 405.9, 404.4,

1 403.6, and 403.0 nm at room temperature, 50, 60, 70, and 80 °C, respectively;  
2 the Soret band was significantly shifted with increasing temperatures (see  
3 Fig. 2). This shows that the structure of cyt. *c* was disrupted, although the Q  
4 band did not change with increasing temperature (528.5, 528.4, 528.4, 528.4,  
5 and 528.1 nm at room temperature, 50, 60, 70, and 80 °C, respectively). These  
6 results indicate the state of cyt. *c* itself maintained III–3.5. However, the  
7 absorbance of Q band became very small with increasing temperature: 0.027  
8 and 0.018 at room temperature and 80 °C, respectively (cf. 0.037 in buffer at  
9 room temperature, 0.042 and 0.034 in OE<sub>2</sub>imC<sub>3</sub>C solution at room  
10 temperature and 80 °C, respectively). The absorbance of the band at 615 nm  
11 increased from 0.001 (room temperature) to 0.003 (80 °C); presumably  
12 indicating that heme displacement was enhanced by heating (see Table S2).  
13 In addition, it was noted that the solution turned from red to somewhat green  
14 at higher temperatures, although there were no irreversible aggregates of cyt.  
15 *c*. The green color is a typical color of disrupted cyt. *c*.

1



2

3 **Fig. 2.** Wavelength at the maximum absorbance of the Soret band of cyt. *c* in  
4 70 wt% solutions of OE<sub>2</sub>imC<sub>3</sub>C or [C<sub>2</sub>mim]OAc at various temperatures. The  
5 filled markers indicate those measured at room temperature after heated at  
6 80 °C. The Soret band in phosphate buffer was observed at 408.9 nm.

7

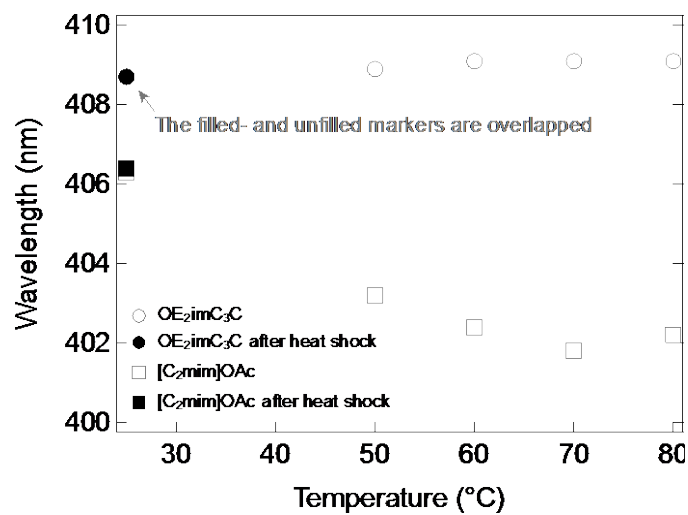
8 A similar trend was observed in 80 wt% solutions (Fig. 3). Regarding  
9 OE<sub>2</sub>imC<sub>3</sub>C, the Soret band was observed at 408.7, 408.9, 409.1, 409.1, and  
10 409.1 nm at room temperature, 50, 60, 70, and 80 °C, respectively. The Q band  
11 did not shift either; 527.0, 528.5, 528.5, 527.8, and 528.3 nm at room  
12 temperature, 50, 60, 70, and 80 °C, respectively. These results showed that



1 cyt. *c* maintained state III–3.5, in 80 wt% OE<sub>2</sub>imC<sub>3</sub>C solutions even at high  
2 temperatures. The band at 615 nm was not observed even at high  
3 temperature; indicating cyt. *c* is not far from native state. On the other hand,  
4 we investigated the structure of cyt. *c* in the 80 wt% [C<sub>2</sub>mim]OAc solution at  
5 higher temperatures, although the Soret band shifted even at room  
6 temperature. The Soret band was observed at 406.3, 403.2, 402.4, 401.8, and  
7 402.2 nm at room temperature, 50, 60, 70, and 80 °C, respectively, showing a  
8 shift as the temperature increased, similar to that with the 70 wt% solution.  
9 The Q band was observed at 528.3, 528.6, 526.1, 526.5, and 526.4 nm at room  
10 temperature, 50, 60, 70, and 80 °C, respectively; even the Q band shifted at  
11 higher temperatures. Although the state of cyt. *c* in the 80 wt% [C<sub>2</sub>mim]OAc  
12 solution itself is also categorized into III–3.5 from the shape of Q band, the  
13 absorbance of Q band became very small with increasing temperature: 0.027  
14 and 0.020 at room temperature and 80 °C, respectively (cf. 0.037 in buffer at  
15 room temperature, 0.039 and 0.035 in OE<sub>2</sub>imC<sub>3</sub>C solution at room  
16 temperature and 80 °C, respectively). The absorbance of the band at 615 nm

1 increased from 0.002 (room temperature) to 0.004 (80 °C); presumably  
2 indicating that heme displacement enhanced by heating (see Table S2). From  
3 these results, cyt. *c* is durable to heat in the OE<sub>2</sub>imC<sub>3</sub>C solutions while the  
4 disruption of cyt. *c*, at least at the vicinity of heme, was enhanced by heating  
5 in the [C<sub>2</sub>mim]OAc solutions.

6



7

8 **Fig. 3.** Wavelength at the maximum absorbance of the Soret band of cyt. *c* in  
9 80 wt% solutions of OE<sub>2</sub>imC<sub>3</sub>C or [C<sub>2</sub>mim]OAc at a variety of temperatures.

10 The filled markers indicate those measured at room temperature after heated  
11 at 80 °C. The Soret band in phosphate buffer was observed at 408.9 nm.

12

1           We again discuss the structure of cyt. *c* at room temperature after  
2 heating at 80 °C to confirm that cyt. *c* was resistant to heat shock. There was  
3 no change in the structure of cyt. *c* in the 70 and 80 wt% OE<sub>2</sub>imC<sub>3</sub>C solutions  
4 (Soret and Q bands: 408.3 and 526.2 nm, respectively (70 wt%); 408.7 and  
5 526.7 nm, respectively (80 wt%), see Figs. 2 and 3). There were no significant  
6 changes in absorbance and the 615 nm band, either. It was confirmed that  
7 heating did not damage cyt. *c* in hydrated OE<sub>2</sub>imC<sub>3</sub>C solutions. In the case of  
8 70 wt% [C<sub>2</sub>mim]OAc solution, the Soret and Q bands after heating were  
9 observed at 407.2 and 527.1 nm, respectively, and were similar to those before  
10 heating (407.6 and 528.5 nm, see Fig. 2) whereas these bands were found at  
11 403.0 and 528.1 nm, respectively, at 80 °C. The band at 615 nm also returned  
12 from 0.003 (80 °C) to less than 0.001 (room temperature after heat shock).  
13 This indicated that the vicinity of cyt. *c* was returned, which is not conflict  
14 with previous studies reporting that cyt. *c* can reversibly unfolded and  
15 refolded without irreversible denaturation and/or aggregation.<sup>[23]</sup> The same  
16 trend was observed in the 80 wt% [C<sub>2</sub>mim]OAc solution, although their

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

6           The redox ability of cyt. *c* in 80 wt% of OE<sub>2</sub>imC<sub>3</sub>C solution was  
7 confirmed. Cyt. *c* is a typical redox-active protein, and its activity can be  
8 evaluated with UV-vis spectroscopy. When cyt. *c* was dissolved in 80 wt%  
9 OE<sub>2</sub>imC<sub>3</sub>C solution, cyt. *c* was observed in an oxidized state. An UV-vis  
10 absorption spectrum of the cyt. *c* solution was measured after the addition of  
11 a small excess amount of the reducing agent, sodium hydrosulfite (Fig. S10).  
12 In the spectrum, the Soret band was observed at 418.2 nm, and the sharp  $\alpha$   
13 and  $\beta$  bands in the Q-band region were observed at 551.3 and 522.5 nm,  
14 respectively, indicating reduced cyt. *c*. This clearly shows that cyt. *c*  
15 maintained the redox activity in the 80 wt% of OE<sub>2</sub>imC<sub>3</sub>C solution. It is noted  
16 that almost the same bands were found at 80 °C (Soret band, 418.2 nm;  $\alpha$

1 and  $\beta$  bands, 552.3 and 523.3 nm, respectively). Whereas almost the same  
2 spectrum was found in 80 wt% [C<sub>2</sub>mim]OAc solution after addition of sodium  
3 hydroxysulfite, it is sure that cyt. *c* in concentrated OE<sub>2</sub>imC<sub>3</sub>C solutions  
4 maintains redox ability at least with a strong reducing agent.

5

## 6 Conclusion

7           We investigated the stability of cyt. *c* in a polar ZI, OE<sub>2</sub>imC<sub>3</sub>C, with  
8 a small volume of water because general ZIs tend to stabilize proteins  
9 compared to ILs. Cyt. *c* was not far from native structure in the 80 wt%  
10 OE<sub>2</sub>imC<sub>3</sub>C solution because the Soret band did not shift and the band at 615  
11 nm, which may be attributed to free heme, was not observed. On the other  
12 hand, cyt. *c* was far from the native state in the 80 wt% [C<sub>2</sub>mim]OAc solution  
13 as indicated by the Soret band shift from 408.9 to 406.3 nm and the band at  
14 615 nm was observed. OE<sub>2</sub>imC<sub>3</sub>C improved the thermal stability of cyt. *c*. The  
15 Soret band did not shift at temperatures up to 80 °C in the both 70 and 80  
16 wt% OE<sub>2</sub>imC<sub>3</sub>C solutions and the band at 615 nm was not observed even at

1 80 °C. On the other hand, the Soret band shifted significantly to 403.0 and  
2 402.2 nm at 80 °C in the 70 and 80 wt% [C<sub>2</sub>mim]OAc solutions, respectively,  
3 and the band at 615 nm increased with elevating temperature up to 80 °C.  
4 These results indicate that *cyt. c* was critically disrupted in [C<sub>2</sub>mim]OAc  
5 solutions, and OE<sub>2</sub>imC<sub>3</sub>C solutions were better solutions for proteins  
6 compared to [C<sub>2</sub>mim]OAc solutions.

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1 **Experimental**

2 UV-vis spectroscopy of cyt. *c* in OE<sub>2</sub>imC<sub>3</sub>C and [C<sub>2</sub>mim]OAc solutions

3 Cyt. *c* from equine heart, oxidized state, (Sigma-aldrich) was dissolved in a  
4 certain amount of pure water, and the resulting liquid was mixed with  
5 OE<sub>2</sub>imC<sub>3</sub>C<sup>[25, 32]</sup> or [C<sub>2</sub>mim]OAc (purchased from BASF, used after drying).  
6 The final concentration of cyt. *c* was 1 mg/g. UV-vis absorption spectra were  
7 measured using quartz cells with a 0.5 mm light-path length. To heat the  
8 samples, we used a cell heating system (CoolSpeK USP-203, Unisoku Co.,  
9 Ltd.). Phosphate buffer (pH 7.0, 10 mM) was prepared and used for reference.

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13 **Supplementary Material**

14 Spectra of cyt. *c* in OE<sub>2</sub>imC<sub>3</sub>C or [C<sub>2</sub>mim]OAc solutions, summary of  
15 wavelength at the maximum absorbance and the absorbance of the Soret-, Q-,

1 695 nm-, 615 nm bands of *cyt.c* in OE<sub>2</sub>mC<sub>3</sub>C or [C<sub>2</sub>mim]OAc solutions are  
2 available on the journal's website.

3

#### 4 **Conflict of Interest**

5 The authors declare no conflicts of interest.

6

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