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Full Paper

Selective Transport of Water-Soluble Proteins from Aqueous to Ionic Liquid Phase via a Temperature-Sensitive Phase Change of These Mixtures

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Mixtures of some ionic liquids (ILs) and water show reversible phase change between a homogeneous mixture and phase-separated state by a small change in temperature. Some water-soluble proteins have been migrated from the aqueous to the IL phase. When tetrabutylphosphonium 2,4,6-trimethylbenzenesulfonate was used as an IL, cytochrome *c* (Cyt.*c*) was found to be extracted from the water phase to the IL phase. Conversely, both horseradish peroxidase (HRP) and azurin remained in the aqueous phase. This selective extraction was comprehended to be due to the difference in solubility of these proteins in both phases. The separated aqueous phase contained a small amount of IL, which induced the salting-out of Cyt.*c*. On the other hand, condensed IL phase promoted the salting-in of Cyt.*c*. As a result, Cyt.*c* was preferably dissolved in the hydrated IL phase rather than aqueous phase. In the case of HRP, there was only a salting-out profile upon increasing the concentration of IL, which induced selective dissolution of HRP in the aqueous phase. These results clearly suggest that the profile of salting-out and salting-in for proteins is the key factor to facilitate the selective extraction of proteins from aqueous to the IL phase.

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Introduction

Phase separation of ionic liquids (ILs) and molecular solvents is of great interest not only in physical chemistry but also in chemical industry. In particular, interfaces formed by ILs and water offer a wide variety of applications for use of aqueous two phase systems (ATPSs) that act as candidates for most common ATPSs based on both aqueous salt solution and poly(ethylene glycol).[1,2] ILs have a potential to control the fluid properties through the suitable design of available ions,[3–5] so that IL-based ATPSs have gained much interest as solvent extraction media for many compounds including biopolymers.[6–11] There have been several reports of IL-based ATPSs, such as hydrophobic ILs/water systems[12,13] and ILs/inorganic salt solution systems.[14,15] Kragl et al. have investigated the partition coefficients of several proteins in IL/inorganic salt solution systems.[16] They suggested that the charges on the proteins are a major factor governing the dissolution of the proteins in the IL-rich phase.[16] There should be other factors influencing the partition coefficients of proteins including hydrophobic interaction[17] and the salting-out phenomenon.[10] However, the major driving force governing the extraction of proteins from aqueous to IL phase remains unclear.

We have studied remarkable properties of ILs upon mixing with water.[18,19] A few years ago, we reported the preparation of ILs with amino acid anions bearing a trifluoromethanesulfonyl group on the amino group.[18] These ILs underwent a temperature-sensitive phase change after mixing with water. For example, tetrabutylphosphonium *N*-trifluoromethanesulfonyl-leucine ($[P_{4444}][Tf\text{-}Leu]$) was found to be miscible with water, but a clear phase separation was observed in the mixture upon heating. Upon cooling it became miscible again.[18] This reversible phase behaviour, classified as a lower critical solution temperature (LCST)-type phase change of the IL/water mixture, had been reported by us.[18] The phase change between the homogeneous phase and the separated liquid/liquid biphases was controlled by changing the temperature a few degrees, so that the LCST-type phase transition of the IL/water mixture permits applications such as the extraction of biopolymers including proteins. We have used the $[P_{4444}][Tf\text{-}Leu]$/water mixture to extract several proteins. Many proteins have been extracted from the aqueous phase to the IL phase by exploiting the LCST-type phase transition.[20] Since the transport of proteins through the interface can be readily and rapidly conducted, several energy-saving systems could emerge by exploiting such temperature-sensitive phase changes. One of the possible systems should be enzymatic-reaction system.[21] In this system, several kinds of enzymes are set in the IL phase, and an aqueous solution containing substrates is added to the IL. The resulting mixture is then cooled down a few degrees to generate a homogeneous solution and the catalytic reaction is accelerated. After the reaction, the solution is heated again to undergo phase separation and the separated aqueous phase containing the products are separated while the enzymes remain in the IL phase. This process facilitates both product separation and recycling of enzymes, which would widen the possibility of ILs to contribute a sustainable world.

To construct the aforementioned systems, it is essentially important to investigate the basic physico-chemical analysis on affinity of several proteins in the IL/water mixtures. The distribution ratio (*D*) of many proteins in the IL/water mixture was then evaluated; it depended strongly on the isoelectric point (p*I*) of the corresponding proteins.[20] However, protein extraction with our biphasic system could not be explained by considering only the p*I* value. For instance, horseradish peroxidase (HRP), p*I* 7.2, was expected to have a high *D* value, but this is not the case. Some other proteins having quite similar p*I* to that of HRP (ex. hemoglobin and myoglobin) showed a high *D* value and were extracted mainly in the IL phase; the difference in the *D* value between HRP and other proteins has long been noted. In this study we analysed the relationship between the physico-chemical properties of IL/water mixtures and solubility of proteins to comprehend the factors governing the protein extraction from an aqueous to the IL phase. The present study would give an insight into the partition of proteins in IL-based ATPS.

Results and Discussion

Partition of Proteins between IL Phase and Aqueous Phase

We have recently analysed the phase behaviour of many ILs after mixing with water, and found a few ILs that undergo a temperature-sensitive LCST-type phase transition.[21–23] One of the ILs that undergo the LCST-type phase transition is a tetrabutylphosphonium 2,4,6-trimethylbenzenesulfonate ([P_{4444}][TMBS]; Fig. 1). The [P_{4444}][TMBS] is easy to prepare in large quantities, so that it is suitable for conducting detailed physico-chemical analysis of the extraction of proteins in IL/water mixtures. We first evaluated the *D* value of three proteins (cytochrome *c* (Cyt.*c*), HRP, and azurin) in a [P_{4444}][TMBS]/water mixture by measuring the absorbance of proteins in the aqueous phase and the IL phase. The *D* value of Cyt.*c* in the IL/water mixture was more than 0.99, meaning that more than 99 % of Cyt.*c* was extracted into the IL phase. No definite absorbance from Cyt.*c* was observed in the separated aqueous phase. The *D* value of both HRP and azurin was less than 0.01. Both HRP and azurin existed in the aqueous phase. Based on these results, we separated Cyt.*c* from mixtures that included them using the LCST phase change. An aqueous solution containing both Cyt.*c* and azurin was added to the IL at 35°C, and the solution was cooled to 30°C and stirred gently. The solution became homogeneous immediately. After phase separation was induced by raising the temperature to 35°C and keeping the solution for 10 min, Cyt.*c* was found to be extracted in the IL phase, but azurin remained in the aqueous phase (Fig. 1). These results demonstrate that selective extraction of water-soluble proteins from mixtures is readily carried out using these properties. The extracted Cyt.*c* in the IL phase can be re-extracted into the aqueous phase by adding hydrophobic organic solvents such as dichloromethane.[20] Mixing such organic solvents may act as effective additives that promotes both dissolution of Cyt.*c* in aqueous phase and recovery of IL in the organic phase.

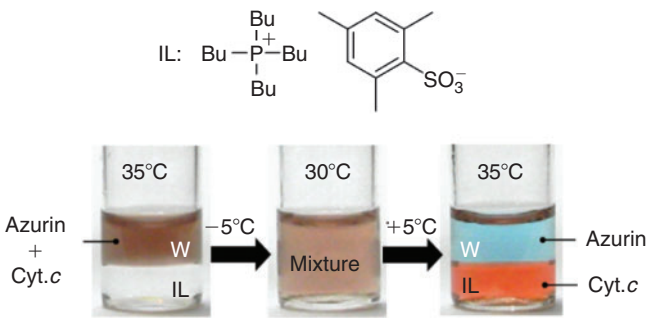

Fig. 1. Complete separation of Cyt.*c* and azurin from their mixture using LCST-type phase change of a [P_{4444}][TMBS]/water mixture. After the phase separation, right hand side, blue and red colour in an aqueous upper phase (W) and [P_{4444}][TMBS] bottom phase (IL) reflect a characteristic colour for azurin and Cyt.*c*, respectively.

Concentration of IL (C_{IL}) in Both Aqueous and IL Phases After the Phase Separation

To determine the main factor influencing the protein distribution, we examined the basic physico-chemical properties of [P_{4444}][TMBS] after mixing with water. We have learned that a certain amount of water is in the separated IL phase even after the phase separation.[21] We therefore expect there to be a small amount of the IL in the aqueous phase. The temperature dependence of the concentration of the IL (C_{IL}/mol kg^{-1}) in both aqueous and IL phases was determined by the absorbance of [TMBS] anion ($\lambda = 280$ nm), as shown in Fig. 2. When [P_{4444}][TMBS] was mixed with water to reach the C_{IL} for 0.80 mol kg^{-1}, the phase separation temperature (T_c), at which the solution started turbid upon heating, was found to be 31°C. The C_{IL} value close to the T_c fluctuated and was not easy to determine. There was, however, a considerable change in C_{IL} from 30°C to 35°C, as seen in Fig. 2. We then determined C_{IL} value in each phase at and above 35°C. A relatively small C_{IL} value (0.24 mol kg^{-1}) was found in the upper aqueous phase. The lower IL phase of course consisted mainly of [P_{4444}][TMBS], but this phase contains some water. The C_{IL} in the lower IL phase was calculated as 1.5 mol kg^{-1} at 35°C; in other words, the phase contained 32 wt % water (~12 water molecules per ion pair). Surprisingly, the phase-separated IL phase contains a considerable amount of water. This should be helpful in dissolving some proteins.

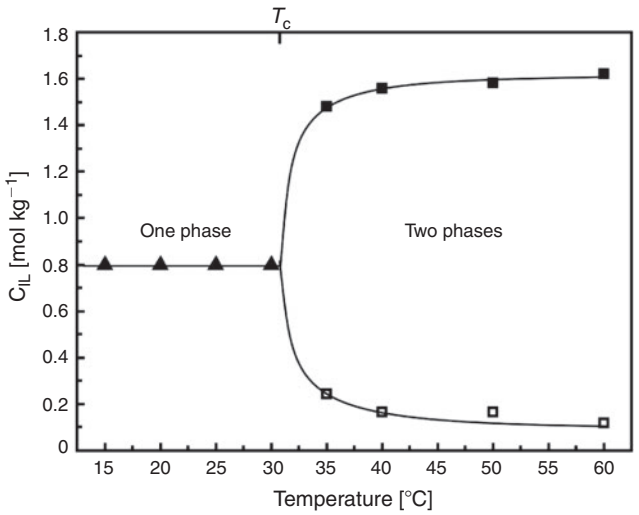

Fig. 2. Temperature dependence of the concentration of [P_{4444}][TMBS] (C_{IL}) between an aqueous phase (□), and an IL phase (■) after phase separation. As the [P_{4444}][TMBS]/water mixture is homogeneous below T_c, C_{IL} is a constant value (▲) regardless of temperature.

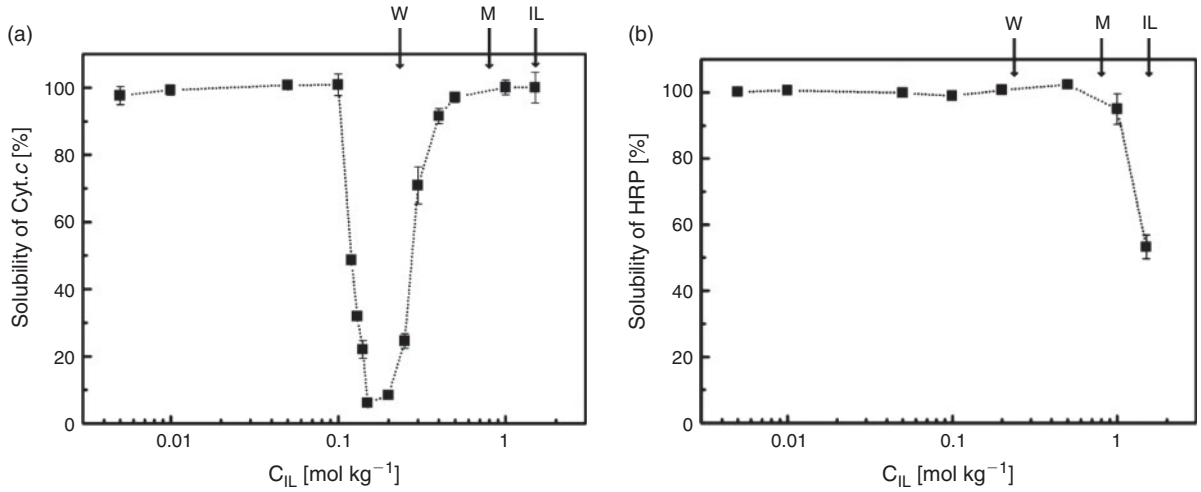

Fig. 3. Effect of C_{IL} on the solubility of (a) Cyt.*c* and (b) HRP. M, W, and IL respectively denote the C_{IL} of the homogeneous mixed phase, the separated aqueous phase, and the separated IL phase.

*'Salting-In' of Cyt.*c *Upon Increasing* C_{IL}

We propose that the difference in the C_{IL} between the aqueous phase and the IL phase governs the solubility of proteins in both phases. As $[P_{4444}][TMBS]$ is freely miscible with water below the T_c, it is possible to analyse the effect of C_{IL} on the solubility of proteins. A large amount of protein is required to determine the solubility in the IL/water mixture over a wide range of C_{IL}. We used Cyt.*c* and HRP because of their availability in large amounts and relatively low cost. Fig. 3a shows the solubility of Cyt.*c* as a function of C_{IL}. Cyt.*c* dissolved completely in a $[P_{4444}][TMBS]$/water mixture when C_{IL} was between 0 and $0.10\,mol\,kg^{-1}$. The solubility of Cyt.*c* fell exponentially, however, to less than 10 % just above $0.10\,mol\,kg^{-1}$. Cyt.*c* began to dissolve again in the mixture at $0.20\,mol\,kg^{-1}$, and finally reached a fully soluble state when C_{IL} was ~0.50 to $1.00\,mol\,kg^{-1}$. Upon comparing the data in Fig. 3a with C_{IL} in both phases, it is obvious that Cyt.*c* is almost insoluble at the C_{IL} in the aqueous phase (Fig. 3a, **W**). On the other hand, Cyt.*c* was fully soluble in the mixture at the C_{IL} for the IL phase (Fig. 3a, **IL**) and for the homogeneously mixed phase (Fig. 3a, **M**). In view of the relationship between C_{IL} and the solubility of Cyt.*c*, it is apparent that Cyt.*c* dissolves in the IL phase rather than in the aqueous phase. The solubility of HRP in the $[P_{4444}][TMBS]$/water mixture was also determined by the same procedure as for Fig. 3a. As shown in Fig. 3b, the solubility of HRP in the mixture fell below 60 % at $C_{IL} = 1.50\,mol\,kg^{-1}$. This result shows that HRP is not extracted in the IL phase, but remains in the aqueous phase, by considering the difference in the solubility of HRP in the aqueous phase (**W** in Fig. 3b) and in the IL phase (**IL** in Fig. 3b).

Fig. 3 clearly shows that there are two independent profiles, a salting-out and a salting-in profile. In general, the salting-out phenomenon of proteins takes place in a concentrated salt solution, and the salting-in phenomenon is observed in a dilute aqueous salt solution.[24] Because dehydration of water molecules from the protein surfaces is an important step towards the salting-out of proteins,[25] the salting-out profile of proteins depends strongly on protein species. In the case of HRP, there was only a salting-out profile (Fig. 3b). HRP is known to have hydrophilic glycoside chains on the surface, which should inhibit the salting out of HRP. On the other hand, salting-out of a Cyt.*c* in $[P_{4444}][TMBS]$/water mixture was found in a relatively low C_{IL} between 0.10 and $0.20\,mol\,kg^{-1}$, followed by the salting-in of Cyt.*c* ($C_{IL} > 0.20\,mol\,kg^{-1}$). These results show that the $[P_{4444}][TMBS]$/water mixture is capable of shifting C_{IL} for both the salting-out and salting-in of proteins. We have reported previously the extraordinary stability of proteins in ILs containing small amounts of water, known as hydrated ILs.[26,27] The hydrated ILs are characterised by water molecules bound to the ions. On the basis of our study of hydrated ILs, the unusual solubility of Cyt.*c* in condensed $[P_{4444}][TMBS]$/water mixture should be due to the hydration state of $[P_{4444}][TMBS]$.

We simply considered that the transport of Cyt.*c* from aqueous phase to IL phase should be based on the equilibrium.[20] So that, Cyt.*c* was considered to migrate from aqueous to IL phase under clear phase-separated state after enough storage time. However, in the present paper, we have revealed that the Cyt.*c* transport is driven by their salting-in and salting-out phenomena. According to this, the hydrated IL phase cannot be a good solvent for the Cyt.*c* even after mixing them for a long time. To confirm this, an aqueous solution containing Cyt.*c* was slowly added onto hydrated $[P_{4444}][TMBS]$, and the resulting solution was stored at 35°C. The solubilising behaviour was monitored for 24 h at this temperature without stirring. At this temperature, the solution remained in the phase-separated state. As shown in Fig. 4, Cyt.*c* started to concentrate at the IL/water interface and all the Cyt.*c* were accumulated at the interface after 12h. The Cyt.*c* aggregate was found at the bottom of the solution after storing the solution for 24h. The aggregated Cyt.*c* was not soluble in the hydrated IL phase even after stirring the solution vigorously. This behaviour is consistent with the present study that the small amount of IL induces the salting-out of Cyt.*c* (see Fig. 3a). These results show that the LCST-type phase transition is an effective method to transport some water-soluble proteins from aqueous to IL phase without serious change in their higher-ordered structure.

*Redox Activity of Cyt.*c *in IL Phase*

Spectroscopic analysis has been undertaken to determine the dissolved state and stability of Cyt.*c* in the hydrated $[P_{4444}][TMBS]$. Cyt.*c* is known as a typical redox active protein, so that

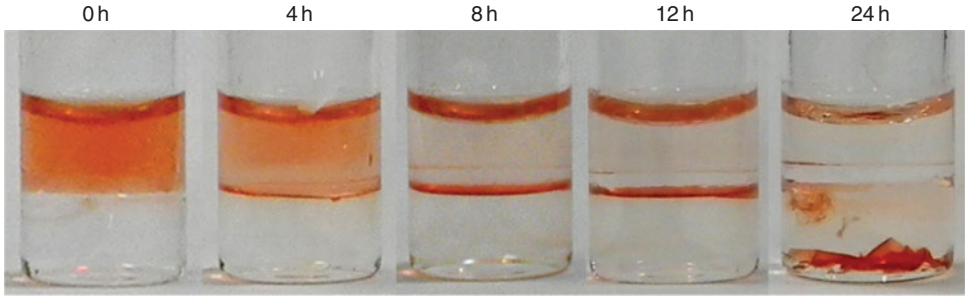

Fig. 4. Time course of the dissolved Cyt.*c* in the phase-separated $[P_{4444}][TMBS]$/water two phases.

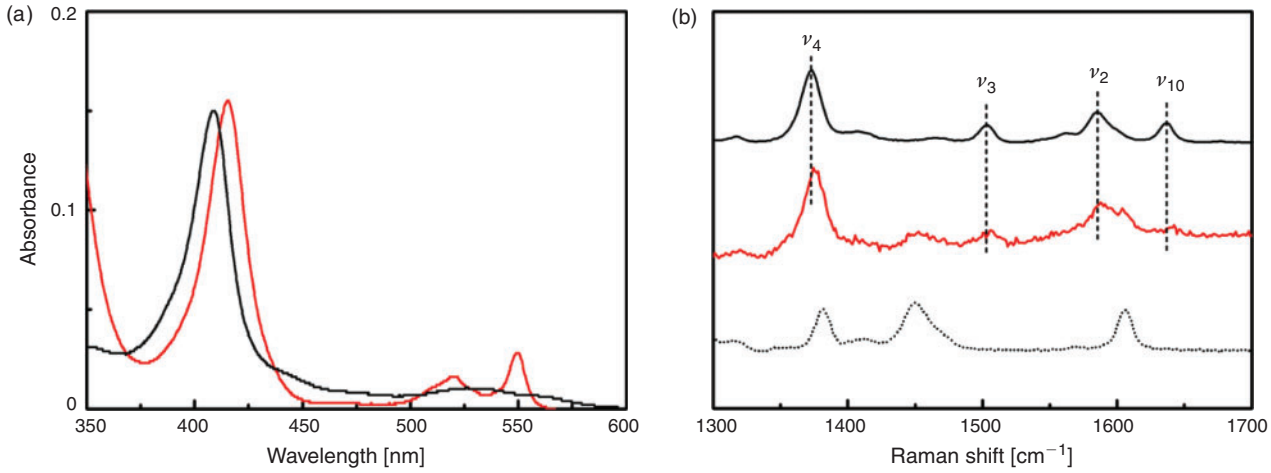

Fig. 5. Spectroscopic characterisation of dissolved Cyt.*c* in hydrated $[P_{4444}][TMBS]$ phase. (a) UV-vis spectra of oxidised Cyt.*c* (black line) and reduced Cyt.*c* after treated with dithionite (red line) in $[P_{4444}][TMBS]$ phase; (b) resonance Raman spectra of Cyt.*c* in an aqueous solution (black solid line), in the $[P_{4444}][TMBS]$ phase (red solid line), and in the $[P_{4444}][TMBS]$ phase without Cyt.*c* (black dotted line).

the stability of the Cyt.*c* in the IL phase is easily detectable by analysing the redox activity. In UV-vis spectra of Cyt.*c* in the IL phase, both strong Soret band and broad Q-band were observed at 409 and 530 nm, respectively (Fig. 5a, black line). These bands indicate that Cyt.*c* exists as an oxidised state in the IL phase similar to that in an aqueous solution. It should be mentioned that the molar extinction coefficient ($\varepsilon_M/M^{-1}\,cm^{-1}$) of Cyt.*c* changed after dissolving in the IL phase. We determined the ε_M value of oxidised Cyt.*c* at 409 nm after the extraction as $9.2 \times 10^4\,M^{-1}\,cm^{-1}$; this value is slightly larger than that in a buffer solution ($8.2 \times 10^4\,M^{-1}\,cm^{-1}$). When a small excess amount of sodium dithionite was added as a reducing agent to the hydrated IL, an obvious spectrum for reduced Cyt.*c* was observed. As shown in Fig. 5a (red line), the spectrum showed a sharp α-band at 550 nm, a sharp β-band at 521 nm, and a Soret band at 415 nm, which is typical in reduced Cyt.*c* in an aqueous solution (See Supplementary Material; Fig. S1). This means that Cyt.*c* retains the redox activity even after treated with the IL. Resonance Raman spectroscopy also shows little change in the structure in the vicinity of the haem (Fig. 5b). The ν_4 band at 1503 cm^{-1} represents the valency of the haem iron. Both ν_3 and ν_2 bands are sensitive to coordination number and spin state. These bands indicate that Cyt.*c* in the IL phase exhibits a six-coordinate geometry and low spin state (6cLS). The structure in the vicinity of the haem moiety of Cyt.*c* in the IL phase was strongly suggested to be similar to that in an aqueous solution.

All of the results discussed above have a bearing on protein extraction in IL based ATPS, based on the differential concentration of salts between the aqueous and IL phases. Furthermore, the IL/water mixture with a temperature-sensitive LCST-type phase change between the homogeneous phase and the liquid/liquid phase separation would provide novel processes for biopolymers such as proteins. We know that a significant amount of further work should be required for proving the effectiveness of our IL/water system. The further work includes the stability and activity of many proteins dissolved in both aqueous and IL phase, and concrete comprehension of the fundamental properties that govern the difference in profiles of protein salting-out and salting-in. These future works would open novel applications not only for extraction solvents during manufacture of biological products but also for efficient systems that facilitate both enzymatic reaction and product separation. We are now confident that achievements in this study would offer novel challenges to existing protein chemistry. The great diversity of ILs promises that this technique should be widely deployable in both basic science and practical applications.

Conclusion

Significant difference in solubility of proteins in the IL/water mixtures reveals to govern the partition of the proteins between IL phase and aqueous phase. The difference in the solubility of Cyt.*c* was found to be driven by a salting-out, followed by the

salting-in of Cyt.*c* with increasing C_{IL}. Since the profile of the salting-out and the salting-in for proteins deeply depends on the protein species, a target protein is readily extracted from their mixtures. Moreover, Cyt.*c* retained the redox function even after treated with the hydrated IL.

Experimental

Materials

Sodium 2,4,6-trimethylbenzenesulfonate (Na[TMBS]) was purchased from Tokyo Chem. Ind. Co., and hydrochloric acid (HCl) from Wako Chem. Co. Cytochrome *c* from *horse heart* and azurin from *Pseudomonas aeruginosa* were purchased from Sigma Aldrich. Horseradish peroxidase was donated by Toyobo Co. and tetrabutylphosphonium hydroxide ($[P_{4444}]OH$) by Hokko Chem. Co. All of the chemicals and proteins were used as received.

Synthesis of Tetrabutylphosphonium 2,4,6-Trimethylbenzenesulfonate ($[P_{4444}][TMBS]$)

An aqueous solution of $[P_{4444}]OH$ was first neutralised with hydrochloric acid, to prepare $[P_{4444}]Cl$. Next, $[P_{4444}]Cl$ was dissolved in water, and a slight excess of Na[TMBS] was added. This solution was stirred for 24 h at room temperature. The product was extracted with dichloromethane, and the dichloromethane layer was washed repeatedly with water. The dichloromethane layer was evaporated and the product was dried under reduced pressure for 24 h at 70°C. δ_H (400 MHz, $CDCl_3$) 0.95 (t, *J* 14.4, 12H, CH_2CH_3), 1.49–1.50 (m, 16H, CH_2), 2.30 (s, 3H, $ArCH_3$), 2.32–2.38 (m, 8H, PCH_2), 2.70 (s, 6H, $ArCH_3$), 6.79 (s, 2H, ArH). Anal. Calc. for $C_{25}H_{47}O_3PS$: C 65.46, H 10.33, N 0. Found: C 65.31, H 10.43, N 0 %.

Determination of the Concentration of $[P_{4444}][TMBS]$ (C_{IL})

The concentration of $[P_{4444}][TMBS]$ (C_{IL}) was defined as a following equation: C_{IL} (mol kg^{-1}) $= N_{IL}/(W_{IL} + W_{water})$, where N_{IL}, W_{IL}, and W_{water} respectively denote number of ion pairs, weight of IL, and weight of water. Pure water was added to the $[P_{4444}][TMBS]$ to reach the C_{IL} for 0.8 mol kg^{-1}. The solution thus prepared was stirred vigorously, and heated to induce phase separation. The C_{IL} value in both aqueous phase and IL phase after the phase separation was determined by an absorbance of [TMBS] anion ($\lambda = 280$ nm, Shimadzu UV-2550).

Determination of the Distribution Ratio (D) of Proteins

Each protein was dissolved in pure water, and the resulting solution was added to the $[P_{4444}][TMBS]$/water mixture (the final concentration of the $[P_{4444}][TMBS]$ was 0.80 mol kg^{-1}). The mixture formed a homogeneous phase immediately after stirring followed by the water bath. This homogeneous solution was then heated to 35°C and left to stand until the separated phases became clear. After keeping it for 10 min, equal volumes of aqueous and IL phases were seen in the mixture, and each phase was pipetted out carefully. The absorbance of both the aqueous and IL phases was then measured by UV-vis spectroscopy. The *D* value was calculated from the absorbance of proteins in each phase, using the following equation: $D = Abs_{IL}/|Abs_{IL} - Abs_{water}|$, where Abs_{IL} and Abs_{water} denote the absorbance of the IL phase and the aqueous phase. The absorption of the Q-band at 530 nm was used for Cyt.*c*, and the Soret band at 403 nm was used for HRP. For azurin, the absorption at 620 nm derived from type 1 copper ion was used.

Calculation of the Solubility of Proteins in $[P_{4444}][TMBS]$/Water Mixture

Protein solutions (5.0 mg g^{-1}) containing different concentrations of $[P_{4444}][TMBS]$ were prepared by the same procedure as that for determining the value of *D*. These homogeneous solutions were mixed vigorously and the precipitates were centrifuged at 6.2×10^3 rpm. The resulting clear upper solution was pipetted out, to measure the absorbance of the proteins. The ratio of dissolved proteins to added amount was calculated using the following equation: Solubility (%) $= (C_{after}/5.0) \times 100$, where C_{after} denotes the concentration of proteins after centrifugation.

Determination of Redox Activity of Cyt.c in the IL Phase

Cyt.*c* was extracted in the $[P_{4444}][TMBS]$ phase by using the same procedure as described above. A 100 μL of the IL phase was pipetted out and the 400 μL of phosphate buffer (100 mM, pH = 7.0) was added to the solution. The final concentration of Cyt.*c* was 0.20 mg mL^{-1} (16 μM). An excess amount of sodium dithionite was then added to the solution and the resulting mixture was stirred gently. The absorption of Cyt.*c* in the mixture before and after addition of the sodium dithionite was determined by UV-vis spectroscopy. As a control, the redox activity of Cyt.*c* in phosphate buffer was also confirmed with UV-visible spectrometry (Supplementary Material; Fig. S1).

Supplementary Material

The redox activity of Cyt.*c* in a phosphate buffer solution as a reference is available as Supplementary Material on the Journal's website.

Acknowledgements

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