Two-dimensional infrared spectroscopic study of cytochrome c peroxidase activity in deep eutectic solvent

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ABSTRACT
Deep eutectic solvents (DESs) prepared by mixing hydrogen-bond donor and acceptor molecules have been found to be of use in several applications. Recently, it was shown that DESs can enhance the peroxidation activity of cytochrome c. Here, to elucidate the effects of DESs on the peroxidase activity of cytochrome c, we carried out linear and nonlinear infrared spectroscopic studies of the CO stretch mode of carbon monoxide cytochrome c (COCyt c) in ethylammonium chloride (EAC)/urea DES. The FTIR spectrum of COCyt c shows a significant spectral shift upon addition of the DES. The broadening and red-shifting of the CO band are observed in both urea and DES solutions, which are induced by the change of the distal ligands around the heme. Although the FTIR study is sensitive to structural changes in the active site, it does not provide quantitative information about structural dynamics related to the catalytic activity itself. Thus, we carried out two-dimensional IR spectroscopy of the CO mode, which suggests that there is a different conformer that could be related to the enhanced catalytic activity in DES. In particular, the spectral diffusion dynamics of that conformer exhibits quite different behavior. The experimental results lead us to propose a hypothesis that the DES increases the population of the conformer with distal ligand lysines close to the reaction center through the combining effect of urea and EAC, which results in the enhancement of the peroxidase activity of cytochrome c. We anticipate that the present experimental work stimulates future investigations of the effects of DES on biocatalysis.

INTRODUCTION
Cytochrome c (Cyt c) is an electron transfer hemoprotein comprising 104 amino acids, and it plays a key role in the life-supporting synthesis of adenosine triphosphate (ATP) in the mitochondria. Interestingly, Cyt c is known as a multifunctional enzyme because it can adopt several different conformers with various different biological functions beyond respiration.1–3 It turned out that pH has a huge impact on the structure of Cyt c, and five distinct spectroscopic forms, states I–V, of this protein were observed with the variation of pH between pH = 1 and pH = 12.4 State III, the dominant one at neutral pH, is considered as the native conformation of the protein, and an alkaline conformer, state IV, is formed at pH above ~8.5–9.5. The alkaline conformer of the Cyt c exhibits a weak peroxidase activity1 and functions as an electron transfer gate as well as a binary molecular switch where the reduction potential of the protein is strongly dependent on pH.5 The other proapoptotic conformer with enhanced peroxidase activity is capable of catalyzing the peroxidation of a mitochondria-specific phospholipid, cardiolipin, which is essential for the release of proapoptotic factors from the mitochondria to initiate the subsequent apoptotic processes, the formation of the apoptosome, and the progression of apoptosis.5–9 Recently, four phosphorylation sites on Cyt c were identified, which indicates that its multiple functions are regulated by cell signaling pathways.10–12 Consequently, the focus has been placed on Cyt c as an interesting heme-based biocatalyst.13–15 Papadopoulou et al.16 recently investigated the effect of a choline chloride and ethylammonium chloride-based
deep eutectic solvent (DES) formulated with three hydrogen-bond donors (HBDs), i.e., urea, ethylene glycol, and glycerol, on the peroxidation activity of Cytc and horseradish peroxidase. It is noted that DESs can be easily prepared by mixing hydrogen-bond acceptor (HBA) and hydrogen-bond donor (HBD) molecules. One of the most popular HBA molecules for DESs are ammonium salts such as choline chloride, and the commonly used HBDs include sugars, polyols, and amino acids. DESs adopt a liquid state at room temperature and are generally cheap, nontoxic, nonvolatile, nonflammable, thermally stable, and even biodegradable solvents, which make them advantageous over ionic liquids. Therefore, DESs have recently been studied as excellent solvents for various biocatalytic reactions.20-22 Although the presence of DES was shown to affect the peroxidase activity of Cytc, it does not influence that of horseradish peroxidase, where the activity enhancement depends on the concentration of the DES and the type of ammonium salt and hydrogen-bond (H-bond) donor molecules used. Interestingly, the addition of 30% v/v ethylammonium chloride (EAC)/urea DES to the reaction mixture increases the peroxidase activity to almost 100-fold compared to that in a buffer solution. This effect was not observed when either EAC or urea was added to the Cytc/buffer solution separately.

Intrigued by the highly enhanced activity of Cytc peroxidase in the EAC/urea DES solutions, we herein evaluate the structure and dynamics of carbon monoxide Cytc (COCytc) in the buffer, DES, EAC, and urea solutions using FTIR and 2D IR spectroscopy. The aim of this research is to gain structural information around the heme domain and ultimately to understand the molecular origin of the enhanced biocatalytic activity of the peroxidase activity of the Cytc induced by the DES.

METHODS
Materials
Cytochrome c from the equine heart (>95% protein content), urea, ethylammonium chloride, monobasic and dibasic potassium phosphate, sodium dithionite, and D2O were purchased from Sigma-Aldrich and used as received. The pH 7 potassium phosphate buffer of urea-ethylammonium chloride DES was described elsewhere.20 In chrome DES was mixed with phosphate buffer to achieve the desired concentration. Therefore, DESs have recently been studied as excellent and even biodegradable solvents, which make them advantageous over traditional solvents. 2D-IR spectroscopy was 16 mM for buffer, EAC/buffer, and urea/buffer solutions using FTIR and 2D IR spectroscopy. The aim of this research is to gain structural information around the heme domain and ultimately to understand the molecular origin of the enhanced biocatalytic activity of the peroxidase activity of the Cytc induced by the DES.

RESULTS AND DISCUSSION

FTIR and 2D IR spectroscopy

Figure 1 shows the FTIR spectra of carbon monoxide cytochrome c (COCytc) in buffer solution, 1.9 M EAC/buffer solution, 2.9 M urea/buffer solution, and 30% v/v DES/buffer solution. The concentrations of EAC and urea in the 30% v/v DES/buffer solution are also 1.9 M and 2.9 M, respectively. In the buffer solution [Fig. 1(a)], the CO stretch band appears at around 1970 cm−1 with a clear shoulder peak in the high frequency side. When the EAC is added to the buffer solution [Fig. 1(b)], the shoulder peak intensity decreases and the CO stretch band becomes significantly narrower than that in the pure buffer solution. Previously, Jaganathan et al. showed that the addition of ethylammonium based ionic liquid (ethylammonium nitrate) stabilizes the protein structure of Cytc. They suggested that the stabilization of the Cytc structure could be attributed to the electrostatic interaction between ethylammonium nitrate and the charged groups in Cytc, as well as the interaction between the ethyl group of the ethylammonium cation and the hydrophobic part of Cytc.23 The observed spectral narrowing of the CO stretch band of COCytc in the EAC/buffer solution could result from the reduction of the population of COCytc conformers with high CO frequency. In contrast, in the urea/buffer and DES/buffer solutions, the CO stretch band appears to be significantly broadened and is red-shifted. Kim et al. observed a similar red-shifting and broadening of the CO stretch band upon addition of guanidium hydrochloride (GuHCl), a denaturant like urea.24 Thus, the observed CO peak broadening and red-shift in DES/buffer and urea/buffer solutions could result from a perturbation of the protein structure by denaturants. In all solutions, the FTIR spectra exhibit a complicated line shape, but they can be well-fitted with two Voigt functions as shown in Fig. 1. The detailed fitting results are listed in Table S1 in the supplementary material. In the case of carbon monoxide myoglobin (MbCO), another hemoprotein, which similarly to the Cytc contains iron-bound heme in its structure and can bind small ligands such as O2, CO, and NO, the CO stretch band was used to identify different conformers that are denoted as A0–A3, differing from one another by

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the detailed conformations of neighboring histidines and heme-bound CO. In addition to the frequency shift of the CO stretch band, the intensity ratios of the involved states associated with different conformers was also found to be dependent on the solvent type, temperature, and pH. Therefore, we expected that the detailed FTIR analyses of the COCytC CO stretch bands provide the structural information in various solvents. Based on the fitting results (Fig. 1 and Table S1), we can assign three spectroscopically distinguishable peaks of COCytC as the C1 state, observed at a higher frequency in EAC/buffer and buffer solutions, C2 state, observed in all the solutions, and C3 state, observed at a lower frequency in DES/buffer and urea/buffer solutions), which are assumed to be associated with different conformers. The C3 conformer may be related to the catalytic activity enhancement of CytC because it is observed only in DES/buffer and urea/buffer solutions. Choi et al. showed that, at the A3 state of MbCO, the distance between the distal Histidine64 H atom and the CO oxygen atom is shorter than those of the other states, which results in a stronger H-bond between them. Such a H-bonding interaction induces an increase in the CO bond strength and a red-shift of the CO stretch band. It was further shown that the histidine plays an important role in the peroxidase activity of hemoglobin in which the rotation of distal histidine was restricted. In the present case of COCytC in a buffer solution, the CO binds to the heme iron of CytC and it replaces the bond between the Methionine (Met80) sulfur and the iron, where Met80 is the nearest ligand. Previous studies showed that the addition of 3 M urea induces the exchange of Met80 with the Lys79 and Lys72/73 which are in equilibrium within the compact conformation of cytochrome c. In such a state, urea induces the increased dynamics of the heme region of CytC and makes substrates more accessible to the heme iron. It is possible that C3 corresponds to this state. The second possibility is that the EAC induces significant changes in the heme proximity structure. Like in the case of the 6 M urea, histidine (His33) gets close to the CO.
and forms a H-bond with the CO. In other words, the neighboring ligand is replaced from lysines to histidine due to the addition of EAC to the urea/buffer solution. Thus, the enhanced catalytic activity in the DES/buffer solution in this case results from the positioning of the histidine (His33) in the active site. It should be noted that Papadopoulou et al. measured the circular dichroism (CD) spectra of Cytc in several DES/buffer solutions, and the observed spectral differences in the CD Soret region indicate that the heme plane undergoes a reorientation in the active site pocket. Such a structural change can contribute to both scenarios.

It is unfortunate that one cannot distinguish the two cases, i.e., the H-bonds between the NH group of His33 and CO and between the NH of Lys79 (or Lys72/73) and CO because they both induce a similar frequency shift of the CO stretch mode. This is why the FTIR analyses cannot provide sufficient information for explaining the structural changes around heme.

2D-IR spectroscopy, as explained elsewhere, has widely been used to study the structural evolutions of a variety of proteins. Briefly, 2D-IR spectroscopy can monitor picosecond time scale equilibrium dynamics by vibrationally labeling molecules or chemical groups with their initial frequencies \(\omega_i\) and then registering the final frequencies \(\omega_f\) of the initially labeled molecules after a finite waiting time \(T_w\). The changes in the peak shapes with increasing \(T_w\) are directly related to the structural evolution of the protein. In the present work, we use the CO stretch mode of COCytc as the IR probe of the local environment around the heme group. Figure 2 shows the 2D IR vibrational echo spectra collected at four different waiting times for COCytc in the urea/buffer and the DES/buffer solutions. The other two series of the 2D-IR spectra of COCytc in the buffer and the EAC/buffer solutions are shown in Fig. S4 in the supplementary material. In all the 2D-IR spectra, there are two dominant peaks, where the positive (red) peaks along the diagonal \((\omega_i = \omega_f)\) arise from the ground state bleaching and stimulated emission contributions that involve the fundamental vibrational transition \((\nu = 0 \leftrightarrow \nu = 1)\). The negative peaks (blue) below the diagonal line result from the excited state absorption from \(\nu = 1 \leftrightarrow \nu = 2\) states. The 2D-IR spectra of COCytc show two positive peaks on the diagonal line at the same positions identified with the fitting analyses of the FTIR spectra. The excited state absorption contributions also appear as two negative peaks that are red-shifted from the fundamental frequencies along the detection frequency axis. Here, it should be mentioned that unlike MbCO, the CO ligand is difficult to be introduced into the heme pocket of Cytc (see Fig. S2; supplementary material). Therefore, only a small amount of COCytc could be prepared for the present experimental studies. Nonetheless, the high power and the improved sensitivity of our laser system allowed us to measure high-quality 2DIR spectra for really low-concentration COCytc solutions due to the difficulty of the CO ligation to the heme reaction center. Note that the absorbance of COCytc in the DES/buffer solution is as small as 0.01 and that in other solutions, it is about 0.02 after the background subtraction.

At a short waiting time of 0.2 ps, inhomogeneous line-broadening makes the 2D-IR peak diagonally elongated, which results from the interactions between the CO and the heterogeneous environments within the heme pocket. With increasing \(T_w\) up to \(\sim 40\) ps, the structural evolution causes a spectral diffusion of the transition frequency, which, in turn, results in a change in the line shape of the 2D-IR peak, i.e., rounding of the diagonal peak in the 2D-IR spectrum. As can be found in all the 2D-IR spectra in Fig. 2, a weak negative peak is down-shifted by approximately \(\sim 20\)–25 cm\(^{-1}\) relative to the main
negative peak. This additional negative peak originates from either the higher-order field-matter interactions, e.g., fifth-order process or the transition from the overtone state to the second overtone state due to the two-photon transition by a strong pump pulse. However, the weak negative peaks are of no importance because we focus on the spectral features of the main diagonal peaks only.

It is clear that the 2D-IR spectroscopy has an enhanced frequency resolvability compared to the linear FTIR spectroscopy. In the urea/buffer and DES/buffer solutions, diagonal peaks corresponding to the C2 and C3 states are observed separately with an almost equal intensity (Fig. 2). In the corresponding FTIR spectra [Figs. 1(c) and 1(d)], however, the high frequency peak (C2) appears to be more intense than the low frequency one (C3). This indicates that the transition dipole strength of the CO stretching mode of the C3 conformer is larger than that of the C2 state. This again supports our assignment that the C3 state is associated with the conformer with an H-bond between the CO oxygen atom and the NH2 group of lysines. This is also the case of MbCO, where the H-bonding interaction between the CO and the ligand increases the bond length of CO, which results in a frequency red-shift of the CO stretching mode and an increase in the corresponding vibrational transition dipole moment. The spectral diffusions of the two states can be extracted by analyzing the time-dependent 2D-IR spectra with the centerline slope method (CLS; see the supplementary material).36–38 Since there exist two conformer states for COCyt in the DES/buffer and urea/buffer solutions, the CLS analyses are performed for the low and high frequency peaks separately, which provide information about the spectral diffusion dynamics of the C3 and C2 states (Fig. S5, supplementary material).

The CLS data and the exponential decay fits are shown in Fig. 3. A single exponential function with a constant offset is found to fit the CLS data well (see Table I). Figure 3(a) shows the CLS data of the C1 state observed in the COCyt in the buffer and EAC/buffer solutions. As listed in Table I, the CLS decay time constant becomes fast with the addition of EAC in the buffer solution. However, there is a large increase in the offset value in the EAC/buffer solution. A similar increase in the offset value is observed in the CLS data of the C2 state which is plotted in Fig. 3(b). Interestingly, the effect of EAC is quite different in the EAC/buffer solution and DES/buffer solution. When added to the buffer solution, EAC produces an increase in the offset value of the CLS data of the C2 state, which is almost the same as one in the C1 state. However, the EAC in the DES solution does not produce the increase in the CLS offset value in the C2 state. It indicates that the urea affects the structural variation around the heme pocket more directly than EAC. This difference may also be related to the eutectic properties of DESs in the aqueous solution, in which the solvent structures can uniquely change by the concentration of DESs.39,40

In Fig. 3(c), the CLS data and fitting results of the C3 states of the COCyt in DES/buffer and urea/buffer solutions are shown. The C3 conformer in the DES/buffer solution is likely to be related to the enhanced catalytic activity of the COCyt. The CLS decay time constants of the two C3 states show a significant difference between urea and DES, which are 2.3–4.0 ps, respectively. The decay time for the C3 conformer also increases from 3.4 ps to 5.3 ps with the addition of EAC. Comparing the spectral diffusion dynamics between the C2 and C3 states in the DES/buffer and urea/buffer solutions, we found that the FFCF of the C3 state decays faster than that of the C2 state. This increased FFCF decay rate can be caused by the H-bonding interaction...

![FIG. 3. Center line slope (CLS) data (points) for the carboxycytochrome c in the phosphate buffer, EAC, urea, and 30% v/v DES/buffer solutions for (a) high frequency peaks in the buffer and in a 1.9 M EAC solution corresponding to the C1 state, (b) low frequency peaks in the buffer and in a 1.9 M EAC solution and high frequency peaks in a 2.9 M urea solution and in a 30% v/v DES/buffer solution correspond to the C2 state, and (c) low frequency peaks in a 2.9 M urea solution and in a 30% v/v DES/buffer solution correspond to the C3 state. The fits, shown as solid curves, are to an exponential decay function with an offset.](image-url)
between lysines and CO in the C\textsubscript{3} state. Another notable difference observed in Fig. 3 is that the ultrafast decay of the FFCF, which is associated with the so-called homogeneous dephasing component, depends on the presence of DES. The initial CLS values for both the C\textsubscript{2} and C\textsubscript{3} states of COCyt in the DES/buffer solution show an appreciable deviation from unity at $T_w = 0$ ps, where the initial CLS value was obtained from the extrapolation of the CLS data with the exponential fit functions. The homogeneous dephasing component arises mainly from the inherently faster motion, which cannot be resolved with our approximately 100 fs time resolution.

The observed differences in the FFCF decay times depending on solvents may be attributed to their viscosities, even though the structural fluctuations of folded proteins were shown to be weakly dependent on the solvent viscosity. Considering the large difference in the viscosities of the urea/buffer and the DES/buffer solutions, such macroscopic solvent property might be of use for explaining the increase in the spectral diffusion time constants in DES. However, the viscosity dependence of the structural fluctuations cannot be the source of the increased homogeneous component observed for different conformers of COCyt in the DES/buffer solution.

The 2D-IR spectra of COCyt in the EAC/buffer and the buffer solution were also measured (Fig. S3), and the spectral diffusion dynamics were analyzed to understand the effect of EAC on the dynamics of COCyt [Figs. 3(a) and 3(b)]. As shown in Table I, the FFCF decay rate becomes fast with the addition of EAC to the buffer solution. These results indicate that the viscosity increase cannot explain the slow-down of the spectral diffusion dynamics in the DES/buffer solution, because the EAC also increases the solvent viscosity but simultaneously increases the FFCF decay rate. From the comparisons between the experimental results shown in Fig. 3 and summarized in Table I, EAC causes a significant increase in the offset value, which is related to static inhomogeneity. This experimental observation is consistent with the previous work showing that the structural stabilization of the Cytc protein by ethylammonium based ionic liquids like EAC is caused by the direct binding of ionic liquids with Cytc. The binding of EAC to the COCyt can increase the static inhomogeneity, which makes the FFCF approach to an offset constant within the experimental time window. However, the fast CLS decay rate observed in the EAC/buffer solution indicates that the same structural stabilization effect by EAC molecules does not impact on the local dynamics of amino-acid residues close to the heme buried inside the protein. In relation to this, Finkelstein et al. measured the FTIR and 2D-IR spectra of the CO ligated Horseradish peroxidase (HRP-CO) with and without the substrate benzohydroxamic acid (BHA). They showed that the presence of BHA causes the corresponding CO stretch IR spectrum to be a single peak, which is in contrast to the doublet spectrum of HRP-CO in the buffer solution without BHA. Furthermore, the decay of the FFCF for the BHA-bound HRP is much faster than the free HRP, but the amplitude of decay is smaller within the 2D-IR time window ($\sim40$ ps). The results indicate that the BHA binding significantly reduces the ultrafast protein fluctuations within the 2D-IR time window. Their experimental observation that the substrate binding to HRP dynamically restrains the structural flexibility of HPR-CO is quite similar to ours for the EAC solution in which the line shape of the corresponding FTIR spectrum becomes narrow and the CLS data suggest an increased structural heterogeneity due to the increase in potential energy barriers between different conformers.

In summary, we here propose that the C\textsubscript{3} state is associated with the catalytically active conformer that is related to the enhanced peroxidase activity. Furthermore, from the present IR spectroscopic studies of the effects of urea and EAC in the COCyt DES/buffer solution, we found that the 2.9 M urea increases the population of the C\textsubscript{3} state that has lysines close to the heme. In the COCyt protein, the H-bonding interactions between CO and NH\textsubscript{2} in lysines cause a frequency red-shift of the CO stretch mode in the C\textsubscript{3} conformer. Second, the added EAC molecules increase the population of the C\textsubscript{3} conformer by their binding to COCyt without causing any significant change of the heme structure. Now, it becomes clear that the spectral diffusion dynamics of IR probe frequency, which can only be extracted from time-resolved 2D-IR spectroscopy, provides direct information about structural dynamics within the enzyme active site as well as the role of solvents in protein functions.

**CONCLUSIONS**

The effect of ethylammonium chloride/urea DES on the local environment around the heme site of Cytc has been studied by using FTIR and 2D-IR spectroscopy of the CO stretching mode of COCyt. The addition of EAC induces both a frequency red-shift of the CO stretch mode and narrowing of the band, which results from the protein structure stabilization due to the direct interaction between EAC and Cytc. On the other hand, the addition of urea and DES to the Cytc solution induces a much larger frequency red-shift and notable broadening of the CO stretch band, which can be attributed to the change of the distal ligand directly interacting with the CO bound to the heme of Cytc. The $T_w$-dependent line shape analyses of the 2D-IR spectra provide information about the structural fluctuation dynamics of COCyt in urea/buffer and DES/buffer solutions. From the analyses of the FTIR and 2D-IR results, we conclude that the most plausible hypothesis is that the C\textsubscript{3} conformer is the active conformer for the peroxidase reaction with lysines close to the heme pocket and that the urea increases the population of this conformer and the EAC molecules in the DES further stabilize it, which results in the enormous enhancement of the catalytic activity of the Cytc. To confirm the hypothesis proposed here using the experimental results obtained with FTIR and 2D-IR spectroscopic methods, detailed molecular dynamics simulation studies in the future would be necessary.
SUPPLEMENTARY MATERIAL
See the supplementary material for the details on FTIR analysis, UV-vis and CD spectroscopy, and CLS analysis.

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