Calixarene-Assisted Protein Refolding via Liquid−Liquid Extraction

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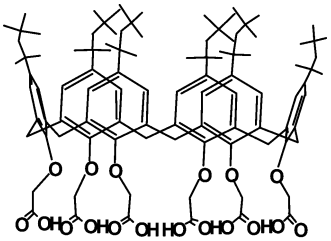
In this paper we report on protein refolding by means of a liquid−liquid transfer technique using a calixarene. We have found that a calix[6]areneacetic acid derivative forms a supramolecular complex with urea-denatured cytochrome *c* at the oil−water interface, which enables quantitative transfer of the protein from an 8 M urea aqueous solution into an organic phase through a proton-exchange mechanism. Denatured cytochrome *c* is completely separated from the denaturant and is isolated from other denatured cytochrome *c* molecules to suppress the generation of aggregates due to protein−protein interactions. The recovery of cytochrome *c* from the organic phase is successfully achieved under acidic conditions using an appropriate amount of 1-butanol. UV−vis, CD, and fluorescence spectroscopic characterizations demonstrate that cytochrome *c* transferred into a denaturant-free aqueous solution regains its native structure. The reduction kinetics of refolded cytochrome *c* using ascorbic acid indicates that the protein provides approximately 72% of native activity as an electron-transfer protein.

Introduction

Calix[*n*]arenes, which consist of phenol rings connected by methylene bridges, are one of the major classes of macrocyclic host compounds in supramolecular chemistry.[1,2] Calix[*n*]arenes possess a unique basketlike cavity that can be controlled by changing the number of phenol units. This feature enables the trapping of a target molecule by a size recognition effect in a three-dimensional network. The framework of calix[*n*]arenes is available for constructing a tailor-made recognition site with functional groups to create a receptor with specific affinity for a target molecule.[3−5] To date, numerous attractive calix[*n*]arene derivatives have been synthesized, and these are capable of recognizing and separating various metal ions[6−9] and small organic molecules.[10−14] Recently, application of calix[*n*]arenes as a recognition tool for biomacromolecules, such as proteins, has become a field of increasing interest and importance.[15,16] Hamilton et al.[17−20] have prepared protein surface receptors in which four peptide loops are attached to a central calix[4]arene scaffold. The receptors act as inhibitors by interacting with strongly cationic regions on cytochrome *c* (Cyt *c*) and chymotrypsin. Recently, we reported that a calix[6]areneacetic acid derivative (^{t}Oct[6]CH$_2$COOH, Figure 1) forms a supramolecular complex with lysine-rich proteins, such as Cyt *c*, at the oil−water interface, and provides high extraction performance.[21,22]

Advances in recombinant DNA technologies have made the production of a wide variety of proteins possible. However, the expression of proteins in bacteria often causes the formation of insoluble and biologically inactive aggregates, which are composed mainly of misfolded protein molecules. To regain biologically active forms of misfolded proteins, many studies on protein refolding have been performed.[23−26] In general, the refolding techniques consist of the following steps: isolation of insoluble proteins from bacterial cells, solubilization of the insoluble proteins using strong denaturants such as urea or guanidine hydrochloride, and renaturation through the removal of excess denaturants by dilution or dialysis with an appropriate buffer. Information encoded in the amino acid sequence of a denatured protein enables it to regain its native, three-dimensional structure. In many cases, however, instead of recovering a native form, irreversible aggregates are reobtained owing to hydrophobic interactions between partially folded intermediates. Protein refolding proceeds through a competitive process between correct intramolecular interaction and incorrect intermolecular hydrophobic interaction. To improve the efficiency of refolding by minimizing the formation of aggregates, suppression of unfavorable intermolecular interactions becomes key. Many approaches have involved the application of additives,[27,28] detergents,[29,30] reverse micelles,[31,32] liposomes,[33,34] size-exclusion chromatography,[35−37] immobilization onto a solid support,[38−40] polymeric nanogels,[41−43] artificial chaperones utilizing cyclodextrin,[44−46] and natural chaperones.[47−49]



^{t}Oct[6]CH$_2$COOH

Figure 1. Molecular structure and abbreviation of the calix[6]areneacetic acid derivative.

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In the present study, we aimed to achieve protein refolding by means of a liquid−liquid transfer technique using ^{t}Oct[6]-CH_2COOH. The refolding process is explained as follows: Denatured protein is separated from the denaturant by transfer into an organic phase. Formation of a supramolecular complex between the protein and multiple ^{t}Oct [6]CH_2COOH isolates denatured the protein molecules and dissociated them from each other, which suppresses the generation of aggregates due to protein−protein interactions. That is, calix[6]arene is expected to function like a chaperone, assisting protein folding in vivo. Back-extraction of a protein into a denaturant-free aqueous solution assures spontaneous refolding into its native conformation. We employed Cyt *c* from horse heart as a model protein; Cyt *c* is a representative heme protein that plays an important role in the biological electron-transfer system. Cyt *c* is a convenient model protein for folding and unfolding studies, because the folding pathway has been thoroughly characterized by various spectroscopic techniques.[50−52] In addition, Cyt *c* has a covalently protein-bound heme group that can be used as a probe for the local structure of the protein.[53−58] The heme active site in Cyt *c* consists of the six-coordinate, low-spin, heme-binding His 18 and Met 80 in the axial ligands. We describe herein that denatured Cyt *c* can be quantitatively extracted from a high-concentration urea solution into an organic solution containing ^{t}Oct[6]CH_2COOH and can successfully regain its native structure through back-extraction into a denaturant-free aqueous solution. The structural characterization of refolded Cyt *c* is probed through various spectroscopic methods and reduction kinetics. The structure of the heme vicinity of Cyt *c* is fully characterized by UV−vis, CD, and fluorescence spectroscopic methods. To the best of our knowledge, this is the first study showing that a calixarene assists protein refolding.

Experimental Section

Materials. The *p-tert*-octylcalix[6]arenehexacarboxylic acid derivative [37,38,39,40,41,42-hexakis(carboxymethoxy)-5,11,17,23,29,35-hexakis(1,1,3,3-tetramethylbutyl)calix[6]arene] (^{t}Oct[6]CH_2COOH) was synthesized according to the procedure described in a previous paper.[59] The final product was purified by recrystallization and identified by FT-IR, ^{1}H NMR, MALDI-TOF-MS, and elemental analysis measurements.

Cyt *c* from horse heart was purchased from Sigma Co. (St. Louis, MO) and used without further purification. A urea nitrogen assay kit was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of commercially available analytical grade and were used as received.

Liquid−Liquid Extraction of Denatured Cyt *c* Using Calixarene. Native Cyt *c* (10 μM) was dissolved in denaturant solutions, the pH of which was adjusted from 2 to 7 using 10 mM Tris buffer, 10 mM MES buffer, and HCl solution containing 8 M urea,[60−62] and incubated overnight at room temperature. An extracting phase was prepared by dissolving ^{t}Oct[6]CH_2COOH (1 mM) in isooctane containing 10 vol % 1-octanol, which assists the solubilization of ^{t}Oct[6]CH_2COOH.[63] Equal volumes of the aqueous and organic solutions were mixed and gently shaken at 30 °C for 12 h to attain equilibrium. After phase separation, the concentrations of Cyt *c* in both phases were determined by the absorbance of the Soret band peak using a UV−vis spectrophotometer (JASCO U-best 570), which then allowed calculation of the extractability ($E = [\text{Cyt } c]_{org,eq}/[\text{Cyt } c]_{aq,ini}$; org, aq, eq, and ini denote the organic phase, the aqueous phase, the equilibrium state, and the initial state, respectively). The concentration of urea extracted in the organic phase was directly measured by the urease−indophenol assay using a urea nitrogen assay kit.

Back-Extraction of Cyt *c* from the Organic Phase. Forward extraction of denatured Cyt *c* (10 μM) using ^{t}Oct[6]CH_2COOH (1 mM) was performed following the same procedure as described above. The organic phase was divided into aliquots. Each aliquot was added to a denaturant-free HCl solution containing a small amount of 1-butanol. Both phases were mixed and shaken at 30 °C for 12 h. After phase separation, the pH of the refolded Cyt *c* solutions was neutralized with Na_2HPO_4 buffer (0.2 M) to prevent spectral change due to the difference of pH condition, and the concentrations of Cyt *c* in both phases were quantified to evaluate the degree of back-extraction ($=[\text{Cyt } c]_{aq,eq}/[\text{Cyt } c]_{org,ini}$).

UV−Vis, CD, and Fluorescence Spectroscopic Experiments. Denatured Cyt *c* solutions were prepared by dissolving native Cyt *c* in 10 mM MES buffer containing 8 M urea (pH 5.5). Cyt *c* organic solutions were prepared according to the same extraction procedure as described above. Refolded Cyt *c* was obtained by transfer into a HCl solution containing 30 vol % 1-butanol (pH 2.5). After lyophilization of the refolded Cyt *c* solutions, the resulting powders were dissolved in Tris buffer (pH 7.5). UV−vis absorption spectra (350−800 and 650−750 nm) of native Cyt *c* in deionized water, of urea-denatured Cyt *c*, of Cyt *c* in the organic phase, and of refolded Cyt *c* in Tris buffer were measured at 30 °C using a UV−vis spectrophotometer (JASCO U-best 570). Measurement conditions were as follows: [Cyt *c*] = 10 μM, [^{t}Oct[6]CH_2COOH] = 1 mM (350−800 nm); [Cyt *c*] = 100 μM, [^{t}Oct[6]CH_2COOH] = 5 mM (650−750 nm). The optical path length was 1 cm.

In CD spectrum measurement, the far-UV CD spectra (205−250 nm) and Soret region CD spectra (350−450 nm) of native Cyt *c* in deionized water, of urea-denatured Cyt *c*, of Cyt *c* in the organic phase, and of refolded Cyt *c* in Tris buffer were obtained at 30 °C using a CD spectropolarimeter (JASCO J-820). Measurement conditions were as follows: [Cyt *c*] = 1 μM (205−250 nm); [Cyt *c*] = 10 μM, [^{t}Oct-[6]CH_2COOH] = 1 mM (350−450 nm). The optical path length was 1 cm.

Fluorescence spectra of urea-denatured Cyt *c* and of refolded Cyt *c* in Tris buffer were measured at 30 °C using a spectrofluorometer (JASCO FP-6500). The excitation wavelength was 295 nm (5 nm band-pass), which eliminated emission from amino acids other than tryptophan, and emission spectra were recorded from 300 to 500 nm (5 nm band-pass). The concentration of Cyt *c* was 10 μM.

Reduction Kinetics of Cyt *c* Using Ascorbic Acid. Cyt *c* is reduced from the ferric state to the ferrous state by reacting with ascorbic acid;[64−66] consequently, reduced Cyt *c* exhibits a new absorption peak at 550 nm. Refolded Cyt *c* in Tris buffer (pH 7.5) was prepared according to the same procedure as described above. Native or refolded Cyt *c* solutions (10 μM, 2.45 mL) were added to a quartz cell, and subsequently, the reductive reaction was initiated by the addition of ascorbic acid (25 mM, 50 μL). The reaction was followed spectroscopically by monitoring the absorbance at 550 nm. The initial reduction rate coefficient, *k*, was evaluated by eq 1,[22,67] where *A* denotes the absorbance at 550 nm and A_{max} and A_{min} are the maximum and the minimum absorbances at 550 nm during the assay, respectively.

$$\log\{(A_{max} - A)/(A_{max} - A_{min})\} = -kt \qquad (1)$$

Results and Discussion

Calixarene-Mediated Extraction of Denatured Cyt *c* from a Urea Solution into an Organic Solution. In general, liquid−liquid transfer of native proteins into organic solutions is achieved using a reversed micellar system. On the basis of this system for native proteins, Hagen et al.[68,69] first reported protein refolding in reversed micelles utilizing the liquid−liquid transfer technique. However, the extraction efficiency of a denatured protein is extremely low because of inhibition by a high concentration of denaturant (extractability at 1 or 0.5 M denaturant concentration is 20% or 60%, respectively.). As shown in Figure 2, we have apparently overcome this previously reported problem through calixarene-mediated extraction of

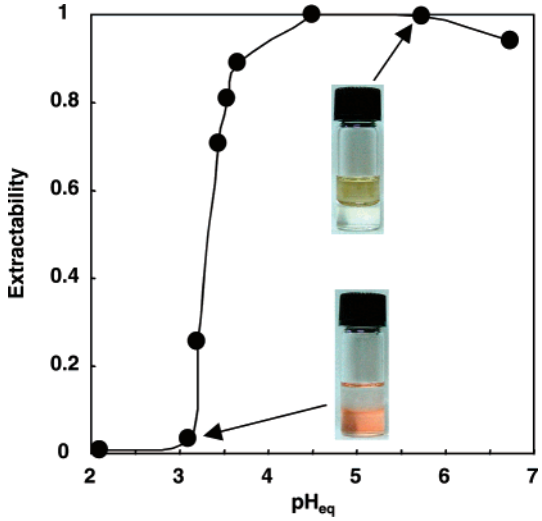

Figure 2. Extraction behavior of denatured Cyt *c* with ^{t}Oct[6]CH$_2$COOH: [Cyt *c*] = 10 μM, [urea] = 8 M, [^{t}Oct[6]CH$_2$COOH] = 1 mM. Inset: Pictures of denatured Cyt *c* transfer.

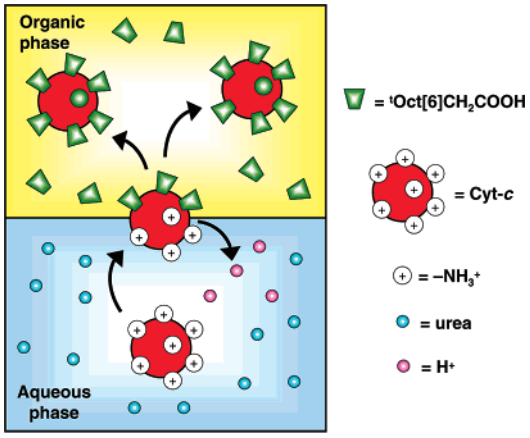

Figure 3. Schematic illustration showing the extraction of denatured Cyt *c* from a urea aqueous solution into the organic phase using ^{t}Oct[6]CH$_2$COOH.

Cyt *c*, in spite of the sample containing 8 M urea. The transfer of denatured Cyt *c* from the denaturant solution into the organic phase using ^{t}Oct[6]CH$_2$COOH is promoted with increasing pH. Finally, quantitative extraction is achieved at neutral conditions, which is also apparent to the naked eye, since the red color of Cyt *c* visually displays its distribution between the phases. Cyt *c* from horse heart has 19 lysine residues. The isoelectric point (p*I*) value of Cyt *c* is 10.6, indicating that Cyt *c* exists as a cationic species under the conditions tested in the present study. On the other hand, ^{t}Oct[6]CH$_2$COOH is an anionic extractant, and its cavity fits the size and the symmetric property (C_3) of the ammonium cation ($-NH_3^+$).[12] Therefore, ^{t}Oct[6]CH$_2$COOH has a strong affinity for the $-NH_3^+$ group within the lysine residues on the protein surface, owing to an electrostatic interaction, an inclusion effect, and a tripodal hydrogen-bonding interaction.[21,22] We confirmed that the pH value is reduced after extraction because of the release of a proton from ^{t}Oct[6]CH$_2$COOH. This result indicates that the transfer of Cyt *c* using ^{t}Oct[6]CH$_2$COOH proceeds through a proton-exchange mechanism. This extraction phenomenon is shown in Figure 3. Multiple ^{t}Oct[6]CH$_2$COOH molecules can strongly coordinate with the lysine residues of Cyt *c* at the oil−water interface and can form a supramolecular complex with Cyt *c*. Simultaneously, a proton belonging to ^{t}Oct[6]CH$_2$COOH is exchanged for cationic Cyt *c* and is released into the aqueous phase. This

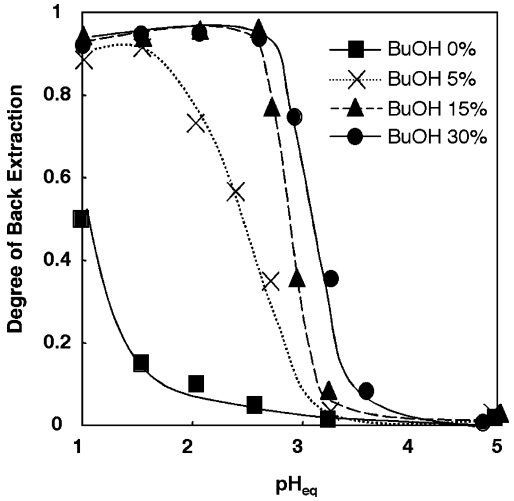

Figure 4. Back-extraction profile of denatured Cyt *c* from the organic phase: [Cyt *c*] = 10 μM, [urea] = 8 M, [^{t}Oct[6]CH$_2$COOH] = 1 mM, 0% (■), 5% (×), 15% (▲), and 30% (●) 1-BuOH.

complex formation offsets the positive charge on the surface of Cyt *c* and enhances the hydrophobicity of Cyt *c*, which enables its transfer into the organic phase. Since the extraction performance of ^{t}Oct[6]CH$_2$COOH is controlled by electrostatic interaction, the extraction behavior of denatured Cyt *c* with ^{t}Oct[6]CH$_2$COOH strongly depends on the pH conditions in the aqueous phase. Unfortunately, we could not increase the pH value to more than 7, since gelation in the organic phase occurs at high pH.

It was confirmed that only 0.05% of the urea in the aqueous phase was extracted into the organic phase, indicating that denatured Cyt *c* was successfully separated from the high-concentration urea solution. Furthermore, no aggregate of Cyt *c* was observed at the oil−water interface. This result suggests that ^{t}Oct[6]CH$_2$COOH enables isolation of denatured protein molecules from one another to suppress protein−protein interactions. The extractant ^{t}Oct[6]CH$_2$COOH has a rather simple structure but provides extremely high coordination ability, selectivity, and a chaperone-like function for Cyt *c*.

Recovery of Cyt *c* from the Organic Phase. The back-extraction of target proteins from an organic phase into an aqueous stripping phase is a very important process for the recovery of proteins. Since the driving force for the Cyt *c* transfer using ^{t}Oct[6]CH$_2$COOH is an electrostatic interaction, the back-extraction of unfolded Cyt *c* in the organic phase was performed by controlling the pH of the aqueous stripping phase (Figure 4). The conventional back-extraction method involving acid solutions results in a low recovery ratio of Cyt *c*, although ^{t}Oct[6]CH$_2$COOH declines in its coordination ability under acidic conditions. We attempted a recovery operation by the addition of a polar organic solvent into the extracting phase to reduce the interaction between ^{t}Oct[6]CH$_2$COOH and Cyt *c*.[22] We employed 1-butanol (log *P* = 0.88), which distributes into both the aqueous and organic phases, as a polar cosolvent. As shown in Figure 4, the recovery of Cyt *c* was greatly promoted with increasing amounts of 1-butanol in the extracting phase. Cyt *c* is quantitatively recovered into acidic solutions with a pH less than 2.5 when these solutions contain 30 vol % 1-butanol, and no aggregation of Cyt *c* occurs at the oil−water interface. This result implies that this system has an advantage over reversed micellar systems, in which the recovery of proteins without the formation of irreversible precipitates is very difficult.

Structural Characterization of Refolded Cyt *c*. The structural analysis of refolded Cyt *c* was probed using

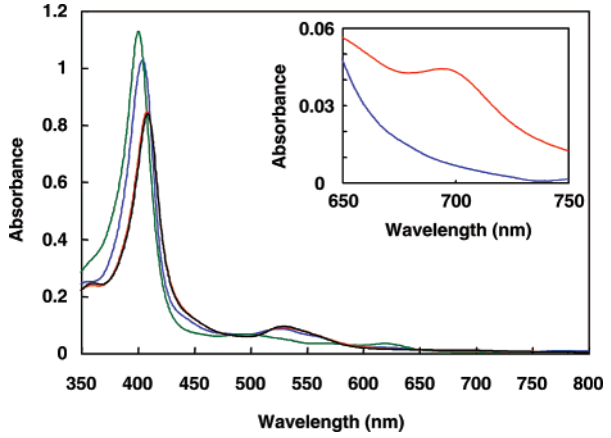

Figure 5. UV−vis spectra (350−800 nm) of Cyt *c*: [Cyt *c*] = 10 μM; black line, native Cyt *c*; blue line, urea-denatured Cyt *c*; green line, Cyt *c* in the organic phase with ^{t}Oct[6]CH_2COOH (1 mM); red line, refolded Cyt *c*. Inset: Absorption spectra of Cyt *c* at the LMCT band; [Cyt *c*] = 100 μM; blue line, urea-denatured Cyt *c*; red line, refolded Cyt *c*.

UV−vis, CD, and fluorescence spectroscopic methods. The spectra of refolded Cyt *c* were obtained by dissolving Cyt *c*, which had been lyophilized to remove the small amount of 1-butanol, in a Tris solution (pH 7.5) and measuring the resultant solution. Figure 5 shows the UV−vis spectra of refolded Cyt *c*, native Cyt *c*, urea-denatured Cyt *c*, and Cyt *c* in the organic phase. The oxidized (Fe(III)) form of native Cyt *c*, which has a six-coordinate low-spin ferric heme coordinated by His 18 and Met 80 in the axial ligands, displays a sharp Soret band at 409 nm, a broad Q-band at 530 nm, and a very weak charge-transfer (LMCT) band from the sulfur atom of Met 80 to the heme Fe(III) at 695 nm. Slight blue shifts of the Soret band (405 nm) and Q-band (526 nm), a slight increase in the absorbance at the Soret band, and the complete disappearance of the LMCT band were observed for urea-denatured Cyt *c*, consistent with disruption of Met 80 ligation to the heme iron and the formation of a characteristic misligated bis-histidine-coordinated heme.[51,54,70−72] Cyt *c* in the organic phase exhibits a further blue shift and an increase in the absorbance at the Soret band (401 nm), a blue shift and a decrease in the absorbance at the Q-band (495 nm), and, in particular, the appearance of a new peak at 620 nm, which implies transformation into a high-spin heme complex.[54,61,73] On the other hand, the spectrum of refolded Cyt *c* is identical to that of native Cyt *c*, indicating the recovery of the structure of the heme vicinity in Cyt *c*.

Figure 6a compares the far-UV CD spectrum of refolded Cyt *c* with that of native Cyt *c*. The far-UV CD spectrum of native Cyt *c* shows negative bands at around 222 nm (n−π* amide transitions) and 208 nm (π−π* amide transitions), which is typical of proteins containing mainly α-helical structure. The far-UV CD spectrum of refolded Cyt *c* is very similar to that of native Cyt *c*, suggesting that refolded Cyt *c* possesses a nativelike secondary structure. The slight spectral changes around the minimum at 208 nm may arise from spectral contributions of other secondary structural components or may be due to the presence of optically active heme transitions other than those associated with the amide transitions of the polypeptide chain.[53,55] Therefore, an unambiguous interpretation of the spectral changes in this region of the spectrum is not possible. Unfortunately, we could not take an accurate measurement of the far-UV CD spectrum for Cyt *c* in the organic phase because of the impermeability of ^{t}Oct[6]CH_2COOH for short-wavelength light.

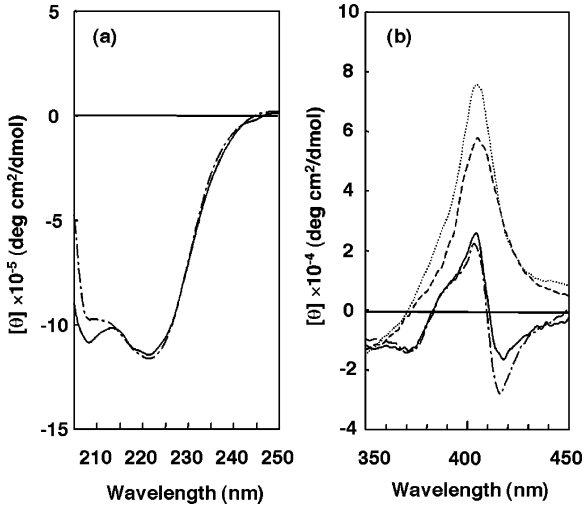

Figure 6. CD spectra of Cyt *c*. (a) Far-UV CD spectra (205−250 nm): [Cyt *c*] = 1 μM, native Cyt *c* (-·-), refolded Cyt *c* (—). (b) Soret region CD spectra (350−450 nm): [Cyt *c*] = 10 μM, native Cyt *c* (-·-), urea-denatured Cyt *c* (···), Cyt *c* in the organic phase with ^{t}Oct-[6]CH_2COOH (1 mM) (---), refolded Cyt *c* (—).

The Soret region CD spectrum can provide further insight into the integrity of the heme crevice. Optical activity in the Soret region of heme proteins is generated through the coupling of heme π−π* electric dipole transition moments with those of nearby aromatic residues in the protein.[53,55,74] As shown in Figure 6b, the Soret region CD spectrum of native Cyt *c* exhibits a negative peak (416 nm) and a positive peak (403 nm) produced due to a split Cotton effect, primarily as a result of heme−polypeptide interactions. The CD spectrum of urea-denatured Cyt *c* is characterized by the disappearance of the negative Cotton effect and a concomitant increase in the intensity of the positive Cotton effect at 405 nm, analogous to that of Cyt *c* in the organic phase. These changes indicate a disruption of the coupling between the heme π−π* transitions and those of nearby aromatic residues[53,55] and an increase in the planarity of the ferric heme moiety.[75] In refolded Cyt *c*, no substantial changes are observed in the CD spectrum when compared with the CD spectrum of native Cyt *c*, suggesting that there are no significant changes in the structure of the heme vicinity.

We also employed fluorescence spectroscopy to aid the characterization of the structure of the heme vicinity of refolded Cyt *c* (Figure 7). The tryptophan fluorescence of Cyt *c* is a sensitive probe for monitoring conformational changes. A single tryptophan (Trp 59) in native Cyt *c* is buried in the hydrophobic core, and the fluorescence emission of Trp 59 is completely quenched through Förster energy transfer from Trp 59 to the heme.[53−58] Urea-denatured Cyt *c* shows a drastic increase in fluorescence intensity at 345 nm. This change is consistent with an increase in the energy-transfer distance between Trp 59 and the heme, owing to a highly expanded conformation of the polypeptide chain. In refolded Cyt *c*, no significant changes in the overall fluorescence intensity are observed when compared with the fluorescence intensity of native Cyt *c*. This result suggests that refolded Cyt *c* regains the native structure of the heme vicinity and supports the interpretations of the UV−vis and CD spectra of refolded Cyt *c*.

Reduction Kinetics of Refolded Cyt *c*. We evaluated the biological function of refolded Cyt *c* in addition to its structural characterization mentioned above. Ascorbic acid is an efficient reductant of Cyt *c*, and conformational changes in Cyt *c* greatly affect the reductive reaction using ascorbic acid.[64−66] As shown in Figure 8, kinetics plots on the basis of eq 1 gave satisfactory

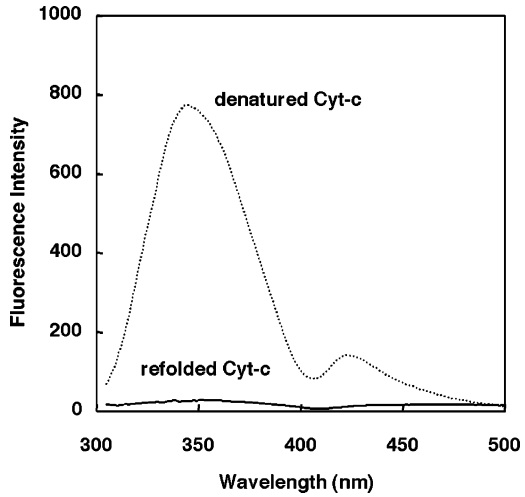

Figure 7. Fluorescence spectra (300−500 nm) of Cyt *c*: [Cyt *c*] = 10 μM, urea-denatured Cyt *c* (···); refolded Cyt *c* (—), excitation wavelength λ_{ex} = 295 nm.

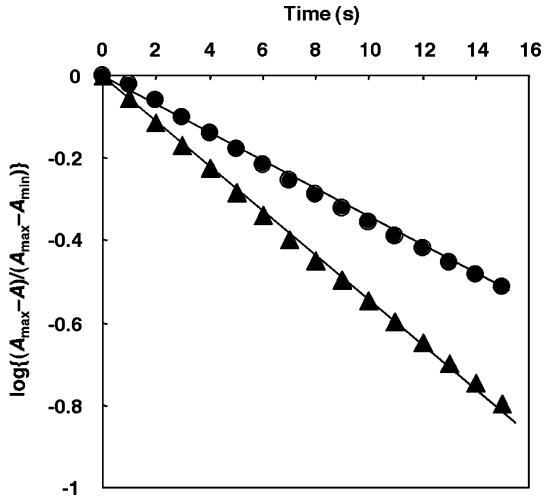

Figure 8. Reduction kinetics of refolded Cyt *c* using ascorbic acid: [Cyt *c*] = 10 μM, [ascorbic acid] = 0.5 mM, native Cyt *c* (▲), refolded Cyt *c* (●).

linear relationships for both native and refolded Cyt *c*, and their slopes provided the initial reduction rate coefficients, *k* (0.049 for native Cyt *c* and 0.035 for refolded Cyt *c*). These results indicate that refolded Cyt *c* regains around 72% of its native activity as an electron-transfer protein. A small decline in the activity of refolded Cyt *c* may be attributed to slight, undetected conformation changes in the polypeptide chain other than the α-helical structure and the structure of the heme vicinity.

Conclusions

In the present study, protein refolding by means of liquid−liquid extraction using ^{t}Oct[6]CH$_2$COOH was investigated. ^{t}Oct[6]CH$_2$COOH provides high coordination ability, selectivity, and a chaperone-like function for Cyt *c*. Denatured Cyt *c* is selectively extracted from the denaturant solution into the organic phase using ^{t}Oct[6]CH$_2$COOH, via supramolecular complexation and is successfully isolated from other denatured Cyt *c* molecules, thereby suppressing the generation of aggregates due to protein−protein interactions. Back-extraction of Cyt *c* is accomplished by contacting the organic phase with acidic solutions containing an appropriate amount of 1-butanol. Cyt *c* recovered into the denaturant-free aqueous solution regains a nativelike structure and provides around 72% of its native activity in the reductive reaction using ascorbic acid. Furthermore, this novel system incorporating a calixarene is expected to simultaneously handle protein refolding and the separation of proteins. The present findings suggest that application of simple but suitable extractants can broaden the potential utility of liquid−liquid transfer in the field of bioengineering. In the future, modification of appropriate functional groups at the lower and/or upper rims in calixarenes will expand the frontiers in the recognition of biomacromolecules using synthetic receptors.

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