Supramolecular Complex of Cytochrome *c* with Lariat Ether: Solubilization, Redox Behavior and Catalytic Activity of Cytochrome *c* in Methanol

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A variety of lariat ethers were employed to solubilize water-soluble cytochrome *c* in methanol, in which alcohol, ether, ester, amine, and amide functionalities were attached as cation-ligating side arms to 18-crown-6, 15-crown-5, and 12-crown-4 rings. Among these lariat ethers, the alcohol-armed 18-crown-6 derivative offered the highest solubilization efficiency for cytochrome *c* via supramolecular complexation. The resulting cytochrome *c*−lariat ether complexes were electrochemically and spectroscopically characterized and confirmed to have redox-active heme structures of 6-coordinate low-spin population in methanol. Some of them catalyzed the oxidation of pinacyanol chloride with hydrogen peroxide in methanol and exhibited higher activities than unmodified cytochrome *c* and its poly(ethylene glycolated) derivative. Since the supramolecular complexation between lariat ether and cytochrome *c* includes extremely simple procedures, it provides a facile preparation method of effective biocatalysts working in organic solvents from metalloproteins.

Introduction

Biocatalysts working in nonaqueous organic solvents have opened new fields in bioscience and biotechnology. Several kinds of enzymes are known to perform reactions which are impossible in aqueous solutions.[1] Peptide synthesis by proteases, transesterification by lipases, and biotransformation of water-insoluble substrates successfully proceeded in organic media. Heme proteins have the potential to be effective biocatalysts. Since most of them are insoluble in organic solvents, chemical modification and immobilization through covalent bonds are usually employed to solubilize them. Because these methods require a series of laborious procedures such as chemical derivatization, dialysis, and lyophilization and since they often lower the protein activities, there is a need for an alternative method of solubilization and functionalization of bioproteins.

Crown ethers and their derivatives can bind $-NH_3^+$, $-CO_2^-M^+$, or other functional moieties[2] exposed on the protein surface and form *n*:1 (crown ether:protein) types of supramolecular complexes. When the protein is wrapped by a number of small crown ethers, its solubility, stability, reactivity, and other functions can be modified.[3] Odell and Earlam demonstrated supramolecular complexation between water-soluble protein and crown ether.[4] They reported that 18-crown-6 and cryptand[2.2.2] allowed dissolution of several heme proteins in organic media but scarcely investigated the reactivities of the resulting supramolecular complexes. Reinhoudt et al. and our group recently employed crown ether derivatives in some enzymatic reactions and found remarkable enhancement of their reactivities.[5] Although only a limited number of crown ethers and related macrocycles have been examined in protein chemistry,[6] they are expected to modify the physical properties and to enhance the chemical reactivities of the biological metalloproteins upon noncovalent supramolecular complexation.

Here we present supramolecular complexes of water-soluble cytochrome *c* with alcohol-armed lariat ethers which work as effective biocatalysts in nonaqueous methanol.[7] Cytochrome *c* is a water-soluble heme protein which mediates electron transfer in the mitochondrial respiratory chain. This is not an enzyme in the biological processes but a promising candidate for an effective biocatalyst having great advantages over common heme enzymes: covalently protein-bound heme group and stable protein backbone.[8] There are a number of lysine residues ($-NH_3^+$) and other functional groups on its surface, which are useful for effective binding sites of crown ethers. We employed here a series of lariat ethers as complexing agents of cytochrome *c*. They are a family of crown ethers having functionalized side

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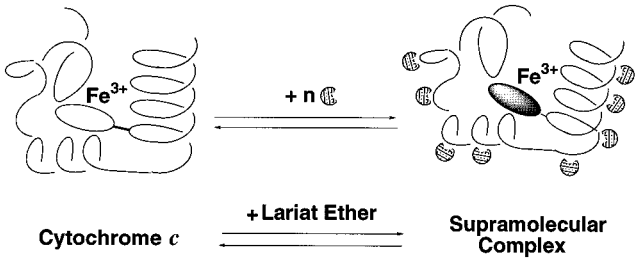
Scheme 1. Supramolecular Complexation between Cytochrome *c* and Lariat Ether

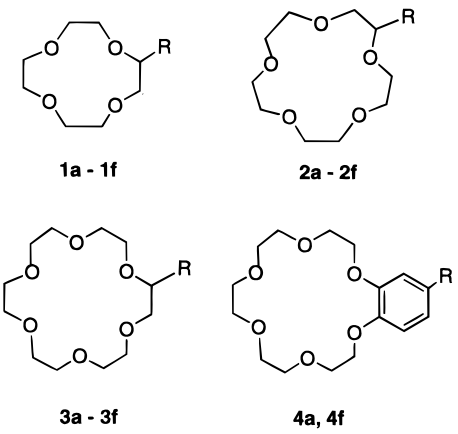


arms, and some of them were reported to operate as effective ligands for small and polymer guests. Since the native cytochrome *c* has several different conformations and properties which depend on microenvironments,[9] the supramolecular complexation with lariat ether can modify the solubility, redox behavior, and reactivity of cytochrome *c* (Scheme 1). The supramolecular complexes between cytochrome *c* and lariat ethers were obtained by simple procedures: mixing of a methanol solution containing lariat ether with solid cytochrome *c* gave a homogeneous brown-red solution of the supramolecular complex. Spectroscopic and electrochemical characterizations revealed that the supramolecular complexes have 6-coordinate low-spin-state hemes in methanol. Although cytochrome *c* itself does not work as a catalyst in the biological processes, the present supramolecular complexes exhibit interesting catalytic reactivities in the oxidation of pinacyanol chloride. Therefore, the present study describes a new possibility of the metalloprotein functionalization and also a useful method for the biocatalyst preparation.

Results and Discussion

1. Lariat Ethers. We examined a series of lariat ethers having alcohol, ether, ester, amine, and amide moieties on their side arms: 12-crown-4 (**1a**−**e**), 15-crown-5 (**2a**−**e**), 18-crown-6 (**3a**−**e**). Benzo-18-crown-6 having an alcohol-functionalized moiety (**4a**) was also examined for comparison (Figure 1). In this class of compounds, the parent crown ethers and side-arm functionalities act as binding sites "cooperatively" or "independently".[10] When we properly combine crown ring with side arm, the resulting lariat ether forms three-dimensional complexes with metal ions, ammonium groups, and water, which are common species in biological systems. This ether also may bind bifunctional guests at two points as a ditopic receptor and then may recognize amino acid sequence in the peptide backbone. Actually, the lariat ethers employed here bound both alkali-metal cations and organic cations. ^{13}C NMR binding experiments confirmed that the alcohol-armed 15-crown-5 **2a** typically bound methylammonium, imidazolinium, and methylguanidinium cations as well as Na^+ and K^+ ions in CD_3OD/D_2O (10/1, v/v).[11]

We have examined sid-arm effects of the lariat ethers on supramolecular complexation with cytochrome *c* as well as size effects of the parent crown rings. Cytochrome *c* from horse heart (M_w = 12 500) was chosen as a guest protein,[12] which has 19



-R	Substitutent	-R	Substitutent
a	$-CH_2OH$	d	$-CH_2NH_2$
b	$-CH_2O(CH_2)_3CH_3$	e	$-CH_2NHC(=O)CH_3$
c	$-CH_2OC(=O)CH_3$	f	-H

Figure 1. Employed lariat ethers and related polyethers.

Lys, 9 Glu, 3 Asp, and other ionic amino acids.[13] Since the lariat ether complexation modified the solubility and redox property of cytochrome *c* and also offered uncommon catalytic activity, a new series of biocatalysts working in organic solvents can be derived from water-soluble heme protein via supramolecular complexation.

2. Solubilization of Cytochrome *c* via Supramolecular Complexation. Supramolecular complexation was investigated by solid (cytochrome *c*)−liquid (lariat ether/methanol) solubilization experiments:[4] cytochrome *c* powder (0.4 μmol) was suspended in a methanol solution of lariat ether (160 μmol/1 mL). After 1.5 h of stirring, the suspension was centrifuged and a brown-red methanol solution was separated from cytochrome *c* powder. The methanol solution exhibited the characteristic UV spectrum of a porphyrin derivative: a strong Soret band at 407 nm and a weak Q-band at 528 nm. Since cytochrome *c* is insoluble in pure methanol, the amount of the solubilized cytochrome *c* is a good indication of the supramolecular complexation with lariat ether. Solubilization efficiency (%) of cytochrome *c* was estimated from the absorbance at the Soret band in methanol (Table 1). Among the employed lariat ethers, alcohol-armed lariat ethers **1a**−**3a** solubilized cytochrome *c* more effectively than ether-, ester-, amine-, and amide-armed lariat ethers **1b**−**3b**, **1c**−**3c**, **1d**−**3d**, and **1e**−**3e**. When 18-crown-6 derivatives were compared, the addition of 400 equiv of alcohol-armed lariat ether **3a** solubilized all the cytochrome *c* added, but considerable amounts of solid cytochrome *c* remained in the presence of 400 equiv of lariat ethers **3b**−**e**: solubilization efficiencies were determined as 100% for **3a**, 30% for **3b**, 32% for **3c**, 7% for **3d**, and 12% for **3e**. As reported earlier,[4a] the simple 18-crown-6 **3f** and cryptand[2.2.2] solubilized cytochrome *c* in methanol. The former exhibited

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(11) The ^{13}C NMR signal for the methine carbon in **2a** shifted in the presence of 3 equiv of guest salts: −0.76 ppm for methylammonium chloride; −0.12 ppm for guanidinium chloride; −0.12 ppm for imidazolinium chloride.

(12) We carried out several experiments with cytochrome *c* after dialysis and compared the results obtained with those without purification. However, no significant difference between them was observed.

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Table 1. Solubilization Efficiency (%)[a] of Cytochrome *c*

	se		se		se		se
1a	55	**2a**	90	**3a**	100	**4a**	79
1b	<3	**2b**	<3	**3b**	30		
1c	<3	**2c**	4	**3c**	32		
1d	<3	**2d**	33[b]	**3d**	7[b]		
1e	25	**2e**	<3	**3e**	12		
1f	<3	**2f**	<3	**3f**	100	**4f**	81

[a] Conditions: see text. The solubilization efficiency (se) is defined as [absorbance at 407 nm in the presence of 400 equiv of lariat ether]/[absorbance at 407 nm in the presence of 400 equiv of **3a**]. [b] Gelation occurred.

excellent solubilization efficiency (100%) under the employed conditions, while the latter formed gel products and offered only low efficiency (25%). The hydrophobicity of the crown ethers seemed to be one of the important factors determining the solubilization efficiency. We calculated the log *P* value for each 18-membered crown ether using the PALLAS for Windows program (version 3.0, CompuDrug International Inc., San Francisco, CA), which can be considered a measure of hydrophobicity of the crown ether: log $P = -1.73$ for **3a**, 0.38 for **3b**, −1.21 for **3c**, −1.91 for **3d**, −2.20 for **3e**, −1.01 for **3f**. Since this trend is apparently different from that of solubilization efficiency for cytochrome *c*, other factors should play more important roles in the supramolecular complexation. The alcohol-armed 15-crown-5 **2a** and 12-crown-4 **1a** solubilized cytochrome *c* much more effectively than unsubstituted crown ethers **2f** and **1f**: 90% for **2a**; <3% for **2f**; 55% for **1a**; <3% for **1f**. The alcohol-armed benzo-18-crown-6 **4a** also had high solubilization efficiency (79%), though 4-aminobenzo- and 4-acetamidobenzo-18-crown-6 derivatives rarely solubilized cytochrome *c* (solubilization efficiency <3%). Thus, the actions of an alcohol-functionalized side arm attached to the crown ether ring operated well in the supramolecular complexation and allowed effective dissolution of water-soluble cytochrome *c* in methanol.

Solubilization efficiency is also dependent upon the size of the parent crown ring as well as the structure of the side-arm functionality. In general, 18-membered lariat ethers **3a**−**d** solubilized cytochrome *c* in methanol more effectively than corresponding 15- and 12-membered ethers **1a**−**d** and **2a**−**d**, indicating that the 18-crown-6 ring provides a better "fit" for the binding site of cytochrome *c* than the smaller 15-crown-5 and 12-crown-4 rings. A combination of 18-membered crown ring and alcohol-functionalized side arm probably promoted the supramolecular complexation. Polyether derivatives are known as effective solvating reagents for proteins.[14] Methoxypoly(ethylene glycol) (M_w = ca. 5000), poly(ethylene glycol) mono-*p*-isooctylphenyl ether (Triton X-100), and tris[2-(2-methoxyethoxy)ethyl]amine were examined, but they rarely solubilized cytochrome *c* under the employed conditions (<3%).

Figure 2 plots the absorbance of the solubilized cytochrome *c* at 407 nm vs the mole ratio of the added alcohol-armed lariat ether **1a**, **2a**, or **3a** to cytochrome *c*. When 15-membered lariat ether **2a** was employed, the dissolution of cytochrome *c* occurred in an allosteric manner: cytochrome *c* was scarcely solubilized at [lariat ether]/[cytochrome *c*] < 150, whereas it was acceleratively solubilized at [lariat ether]/[cytochrome *c*] > 150. Lariat ethers **1a** and **3a** behaved similarly, but the critical mole ratio was much lower in the case of 18-membered lariat ether **3a** than in the case of 12-membered **1a**: 5 for **3a** and 270 for **1a**. These results can be rationalized by assuming that cytochrome *c* is not solubilized by complexation with a few lariat ethers but becomes soluble when its surface is totally covered by a number of molecules.

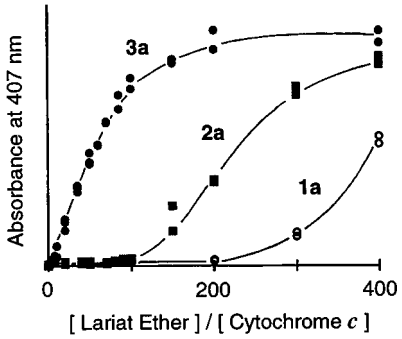

Figure 2. Solubilization profiles of cytochrome *c* with alcohol-armed lariat ethers **1a**−**3a**.

Variable-temperature NMR experiments provided more direct structural information about supramolecular complexation between cytochrome *c* and lariat ethers. For example, 90 equiv of alcohol-armed lariat ether **3a** completely solubilized cytochrome *c* in CD_3OD. The obtained solution gave a broad singlet signal for crown ring protons of $-CH_2-$ (δ_H 3.63 ppm) at room temperature which was not separated even at −80 °C. When the parent 18-crown-6 **3f** was employed, one set of signals for the complexed and free 18-crown-6 was observed at <−32 °C (δ_H 3.63 and 3.58 ppm). Although the stoichiometry of the cytochrome *c*−**3f** complex was not determined exactly, the shape analysis of the separated signals suggested that about 30 molecules of crown ether **3f** bound to one cytochrome *c* molecule. Both **3a** and **3f** exhibited similar solubilization efficiencies of cytochrome *c*, but there were marked differences in the NMR binding experiments. Thus, alcohol-armed lariat ether **3a** bound to cytochrome *c* more dynamically than parent crown ether **3f**.

3. CD Spectroscopic Characterization of Supramolecular Complexes. Figure 3 illustrates CD spectra of the cytochrome *c*−**3a** complex in methanol and cytochrome *c* itself in aqueous solutions (pH 10.3 and 4.6). Poly(ethylene glycolated) cytochrome *c* was employed for comparison.[1] As described in the Experimental Section, this has 7 or 8 chains of poly(ethylene glycol) (M_w = ca. 5000) and is readily soluble in methanol. All of them exhibited characteristic CD signals at the Soret band regions (ca. 407 nm), but with different shapes and intensities. Cytochrome *c* is known to exist in several pH-dependent conformational states[15] and to undergo a conformational change as the pH of the aqueous solution increases. In this conformational change, methionine-80 is displaced by lysine-79 at an axial ligand of heme and the heme crevice is opened. The shapes of the CD signals observed with the cytochrome *c* solubilized in methanol are similar to those observed at pH 10.3 rather than those observed at pH 4.6 (**[A]**, **[B]** vs **[C]**, **[D]** in Figure 3, top). Furthermore, the CD intensity at the Soret band of the cytochrome *c*−**3a** complex in methanol was 2.6 times larger than that of cytochrome *c* alone in alkaline aqueous solution, though that of poly(ethylene glycolated) cytochrome *c*[16] was 1.7 times larger. These spectra suggest that the solubilization of cytochrome *c* in methanol was accompanied by similar conformational changes to those observed at high pH, which probably include exchange of the axial ligand and the opening of the heme crevice.

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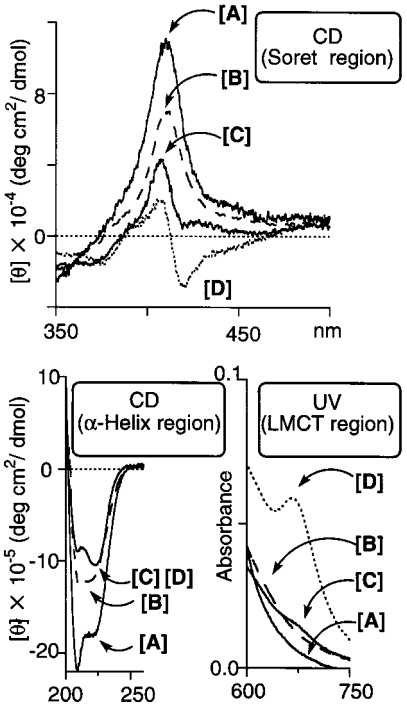

Figure 3. CD and UV spectra of cytochrome *c* derivatives: **[A]** cytochrome *c*−**3a** (MeOH); **[B]** poly(ethylene glycolated) cytochrome *c* (MeOH); **[C]** cytochrome *c* (pH 10.3); **[D]** cytochrome *c* (pH 4.6).

The supramolecular cytochrome *c*−**3a** complex also exhibited largely enhanced CD signals around 209 nm, which is a good indication of α-helix contents in the protein backbone. Since poly(ethylene glycolated) cytochrome *c* gave a modestly intensified CD signal in this region, the α-helix structure of cytochrome *c* was more stabilized by lariat ether complexation than by poly(ethylene glycolation) (**[A]** vs **[B]** in Figure 3, bottom left). The native cytochrome *c* has a 6-coordination heme group in the neutral aqueous solution, in which methionine acts as the second axial ligand. This was clearly supported by the fact that the LMCT band was observed at 690 nm.[17] When cytochrome *c* was solubilized in alkaline aqueous solution, the methionine was displaced by lysine and the LMCT band at 690 nm disappeared (**[C]** vs **[D]** in Figure 3, bottom right). Similar UV changes were observed when cytochrome *c* derivatives were solubilized in methanol (**[A]** and **[B]** in Figure 3, bottom right). Thus, the cytochrome *c*−lariat ether **3a** complex was suggested to have a structurally ordered protein structure in methanol, where the heme was accommodated in an unnatural fashion.

4. Resonance Raman and ESR Spectroscopic Characterization of Supramolecular Complexes. Resonance Raman spectroscopy has been an effective method to characterize the nature of heme derivatives.[18] Figure 4 shows the resonance Raman spectra of supramolecular complexes with alcohol-armed lariat ether **3a** and parent crown ether **3f**, together with that of poly(ethylene glycolated) cytochrome *c*, all of which were measured in methanol. The Raman bands in the high-frequency region (1300−1700 cm^{-1}) are assigned to porphyrin in-plane vibrational modes that are sensitive to the electron density in the porphyrin ring and also to the coordination and spin state

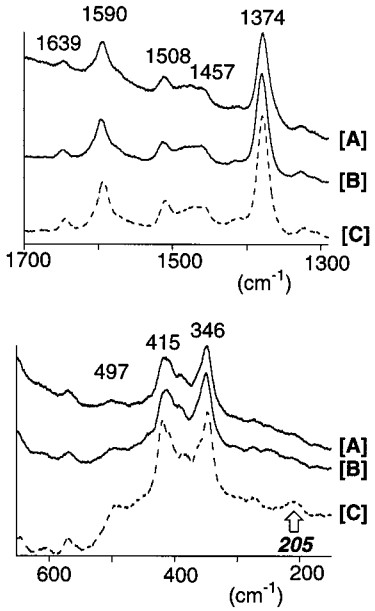

Figure 4. Resonance Raman spectra of **[A]** cytochrome *c*−**3a**, **[B]** cytochrome *c*−**3f**, and **[C]** poly(ethylene glycolated) cytochrome *c* in methanol.

of the iron atom. The electron density marker band ν_4 at 1374 cm^{-1} in all spectra indicates that these cytochrome *c* derivatives have ferric heme groups (**[A]**, **[B]**, and **[C]** in Figure 4, top). The spin- and coordination-sensitive band ν_3 at 1508 cm^{-1} confirms that they are in 6-coordinate ferric low-spin states. Since the bands ν_2 and ν_{10} were also observed at 1590 and 1639 cm^{-1}, the examined cytochrome *c* derivatives were supported to have 6-coordinated low-spin heme groups. In the low-frequency region (200−600 cm^{-1}), there are some differences between supramolecular complexes and poly(ethylene glycolated) cytochrome *c* (**[A]** and **[B]** vs **[C]** in Figure 4, bottom): the band at 205 cm^{-1} was observed only in the case of poly(ethylene glycolated) cytochrome *c*. Since this region provides useful information about axial coordination of the heme group, poly(ethylene glycolated) cytochrome *c* may have somewhat different modes of axial ligation from those of the two supramolecular complexes.

We applied ESR spectroscopy in nonoriented rigid glass to identify the ground-state spin multiplicity of the cytochrome *c*−**3f** complex. A mixture of ESR spectra with salient features of ground-state low spin and high spin (sextet) due to heme iron was observed from 2.5 to 35 K. The loww-spin-state signals dominated despite the minority species due to ferric iron. Only the signals attributed to the low-spin state showed a rapid passage at 2.5 K, indicating that the low-spin state is isolated in terms of electronic structure and there is no apparent correlation between the low-spin and high-spin states. On the basis of ESR spectroscopic characterization, Bowyer and Odell concluded that the cytochrome *c*−**3f** complex contained at least three different states for the heme iron.[4b] Our ESR results suggest that there is no evidence of the occurrence of a strong quantum-mechanical spin mixing and thermal mixing. Thus, these supramolecular complexes were demonstrated to mainly have the 6-coordinated low-spin population in methanol at room temperature.

5. Electrochemical Characterization of Supramolecular Complexes. Cytochrome *c* has a redox-active heme and mediates electron transfer in biological systems. Because of very slow electron transfer between protein and electrode, the indirect electrochemical method has been developed to determine its

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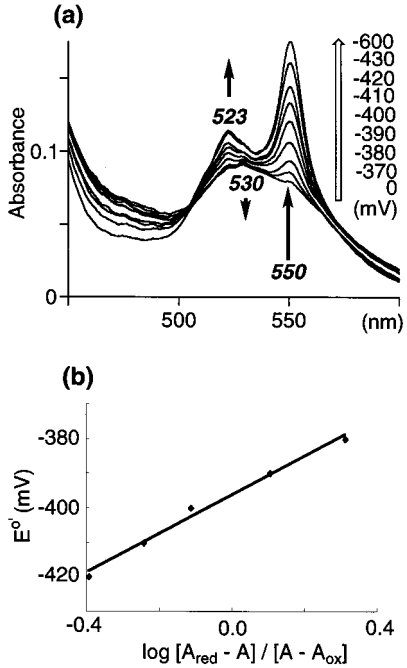

Figure 5. **(a)** Spectra of cytochrome *c*−**3a** in methanol at different potentials and **(b)** its Nernst plot.

redox potential in aqueous solution, where the redox-active mediator promoted the interfacial electron-transfer process.[19] We applied this method to characterize the supramolecular complexes in methanol. By a thin-layer spectroscopic technique,[20] their redox processes could be followed using methylviologen as a mediator. When the methylviologen was reduced in a methanol solution of the cytochrome *c*−**3a** complex at a controlled potential of −0.6 V vs Ag/AgCl, new absorptions for the reduced species were observed at 523 and 550 nm in the visible spectrum with disappearance of a small absorption band at 530 nm for the fully oxidized species[21] (Figure 5a). Since the reduced cytochrome *c* species formed during the controlled-potential electrolysis, this supramolecular complex was confirmed to have the redox-active heme structure in methanol.

A Nernst plot of absorbance at 550 nm for the ferricytochrome *c*/ferro-cytochrome *c* couple according to eq 1 gave the redox potential $E^{\circ\prime} = -0.396$ V vs Ag/AgCl for the cytochrome *c*−**3a** complex (Figure 5b)), which corresponds to −0.360 V vs SHE (see Experimental Section). The supramolecular complex with **3f** and poly(ethylene glycolated) cytochrome *c* were similarly reduced in methanol, and their reduction potentials were estimated as −0.393 and −0.439 V vs SHE. Table 2 compares the reduction potentials of several cytochrome *c* derivatives observed in methanol with those reported in aqueous solutions,[21] indicating that the former exhibited more negative reduction potentials. The reduction potentials of the supramolecular complexes observed in methanol should be affected by several factors, such as self-assembling and conformational change of cytochrome *c* and the natures of coexisting environmental species. Among them, the methanol-induced conformational change of cytochrome *c* must first be considered. When the electrochemical reduction was carried out at pH 9.0, cytochrome *c* exhibited a reduction potential (0.060 V) more negative than that observed at pH 6.0 (0.260 V).[21] As described above, the solubilization of cytochrome *c* into methanol induces changes in the ligation mode of heme and protein conformations which are similar to those observed in alkaline aqueous solution. Thus, the cytochrome *c* solubilized in methanol is thought to exhibit a negatively shifted reduction potential upon such conformational changes. Simple solvation generally influences the reduction potentials of redox-active species. For example, the reduction potential of methylviologen was estimated to be more negative by 50 mV in methanol than that in aqueous solution.[22] Other factors giving negative reduction potentials of cytochrome *c* are the presence of perchlorate electrolyte and a high concentration of cytochrome *c*.[17,21] When cytochrome *c* was solubilized in methanol by supramolecular complexation, its solubility was determined by concentrations of cytochrome *c*, lariat ether, and coexisting perchlorate anion. We employed 1 mM of cytochrome *c* and 0.05 M of perchlorate to obtain reproducible data in the electrochemical experiments, which are much higher than those usually reported in aqueous solution systems.[21] Therefore, the observed negative reduction potentials of the supramolecular complexes support that they have ordered structures in methanol as observed in alkaline aqueous solutions.

$$E = E^{\circ\prime} + \frac{0.059}{n} \log \left[\frac{A_{red} - A}{A - A_{ox}} \right] \qquad (1)$$

Table 2. Reduction Potentials of Cytochrome *c* Derivatives

cytochrome *c*	λ_{max} (nm)	*n* (mV)	$E^{\circ\prime}$ (V vs SHE)	remarks
cytochrome *c*			0.260	H_2O[a] (pH 6.0)
cytochrome *c*			0.060	H_2O[a] (pH 9.0)
cytochrome *c*−**3a**	550	57	−0.360	MeOH[b] (0.05 M Et_4NClO_4)
cytochrome *c*−**3f**	550	53	−0.393	MeOH[b] (0.05 M Et_4NClO_4)
poly(ethylene glycolated) cytochrome *c*	550	47	−0.439	MeOH[b] (0.05 M Et_4NClO_4)

[a] Conditions: cytochrome *c*, 3.6×10^{-5} mol/L. For other conditions, see ref 21. [b] Conditions: cytochrome *c*, 10^{-3} mol/L; **3a**, 0.5 mol/L; **3f**, 0.5 mol/L; methylviologen, 10^{-4} mol/L.

Table 2 also indicates that three cytochrome *c* derivatives have different reduction potentials, though these included similar 6-coordination heme moieties: −0.360 V for the **3a** complex, −0.393 V for the **3f** complex, and −0.439 V for poly(ethylene glycolated) cytochrome *c*. Since the charge distribution on the protein surface was reported to have a great influence on the reduction potential of cytochrome b_5,[23] the coordination of lariat ethers or poly(ethylene glycol) to the cationic sites of the cytochrome *c* should modify charge distribution on the protein surface and subsequently shift the reduction potential.

6. Catalytic Oxidation of Pinacyanol Chloride. Since the cytochrome *c*−lariat ether complexes have unique heme properties in methanol, they are expected to offer uncommon activities as biocatalysts. We employed them in the oxidation of pinacyanol chloride with hydrogen peroxide. Pinacyanol chloride (1,1′-diethyl-2,2′-carbocyanine chloride) has a maximum absorbance at 603 nm and has frequently been used as a useful substrate to spectroscopically determine activities of manganese peroxidase and related model catalysts in the organic media.[24] Although cytochrome *c* was reported to catalyze the oxidation

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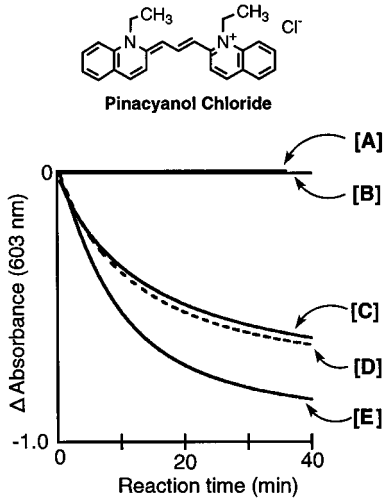

Figure 6. Time course of catalytic oxidation of pinacyanol chloride by cytochrome *c*−lariat ether complexes: **[A]** cytochrome *c*; **[B]** cytochrome *c*−**1a**; **[C]** cytochrome *c*−**2a**; **[D]** poly(ethylene glycolated) cytochrome *c*; **[E]** cytochrome *c*−**3a.**

of aromatic hydrocarbons and organosulfides in aqueous methanol solution, this did not work well in nonaqueous methanol.[25] We carried out the oxidation of pinacyanol chloride in nonaqueous methanol and compared the catalytic activities of cytochrome *c*−lariat ether complexes with that of poly(ethylene glycolated) cytochrome *c*: pinacyanol chloride, 25 μmol; H_2O_2, 480 μmol; cytochrome *c*, 2.5 μmol; lariat ether, 500 μmol; MeOH, 2.01 mL. When alcohol-armed lariat ether **1a**, **2a**, or **3a** was added to the reaction solution, disappearance rates of the pinacyanol chloride were apparently dependent on the solubilized amounts of cytochrome *c* in methanol (Figure 6). Lariat ethers **2a** and **3a** readily solubilized cytochrome *c* (91 and 100%) and offered high catalytic activities (29 and 37%). In the case of lariat ether **1a**, all the cytochrome *c* added was suspended as a powder and oxidation rarely occurred. Figure 6 also illustrates that the activity of each cytochrome *c* catalyst decreased after 20 or 30 min. Although native cytochrome *c* is known to be rapidly decomposed in the presence of hydrogen peroxide,[26] cytochrome *c*−**2a** and −**3a** complexes retained high catalytic activities for a relatively long period. Thus, the supramolecular complexation not only modified the solubility of cytochrome *c* but also offered nonbiological catalytic reactivity.

Table 3 summarizes the catalytic activities of various cytochrome *c*−lariat ether complexes, together with their solubilization efficiencies determined under the oxidation conditions. When alcohol-armed lariat ether **3a** was employed, all the cytochrome *c* added was solubilized in the reaction mixture, and its supramolecular complex exhibited slightly higher catalytic activity (37%) than the cytochrome *c*−crown ether **3f** complex (35%). Since our electrochemical and NMR experiments suggested that these two ethers bound to cytochrome *c* in different fashions, the structure of the lariat ether influenced the catalytic activity of cytochrome *c* as well as its solubility and redox behavior. Other 18-membered crown ethers, **3b**,**e** and **4a**,**f**, modestly solubilized cytochrome *c* under the employed conditions and promoted the oxidation of pinacyanol chloride with activities lower than those of **3a** and **3f**. The attachment of poly(ethylene glycol) has been widely used to solubilize the proteins in organic media,[1a,25] but this usually includes a series of laborious procedures. Actually, the total preparation of poly(ethylene glycolated) cytochrome *c* requires more than 3 days: activation of poly(ethylene glycol) (1 day), reaction with poly(ethylene glycol) and cytochrome *c* (3 h), dialysis (1 day), and lyophilization (1 day). Fedorak and colleagues reported that poly(ethylene glycolated) cytochrome *c* catalyzed some oxidation reactions in THF/H_2O (90/10).[25] This was readily soluble in nonaqueous methanol but exhibited catalytic activity (30%) in the oxidation of pinacyanol chloride that was lower than those of cytochrome *c*−**3a** and −**3f** complexes (37 and 35%). Since 2 hours is enough for the present complexation method, the preparative period of biocatalysts was remarkably shortened and their catalytic activities were satisfactorily high.

Table 3. Oxidation of Pinacyanol Chloride with Cytochrome *c*−Lariat Ether Complexes

lariat ether	conversion (%)[a]	se (%)[b]
1a	0	<5
2a	29	91
2f	0	<5
3a	37	100
3b	2	28
3c	0	8
3d	0	<5
3e	0	10
3f	35	100
4a	20	73
4f	19	77
poly(ethylene glycolated)	30	100[c]
unmodified	0	0

[a] On the basis of absorbance at 603 nm after 40 min. For oxidation conditions, see the Experimental Section. [b] Since the added cytochrome *c* was completely solubilized in the presence of 200 equiv of **3a**, the solubilization efficiency (se) of cytochrome *c* was defined as follows: [absorbance at 407 nm in the presence of 200 equiv of lariat ether]/[absorbance at 407 nm in the presence of 200 equiv of **3a**] × 100. Note the added amount of cytochrome *c* was different from that reported in Table 1. [c] The concentration of cytochrome *c* in the reaction solution was adjusted to be same as that of the **3a** complex, based on absorbance at 407 nm.

Conclusion

We have demonstrated that some lariat ethers formed supramolecular complexes with water-soluble cytochrome *c* and effectively solubilized it in methanol. In particular, the supramolecular complex with alcohol-armed lariat ether **3a** had redox-active heme in the ordered protein matrices and worked as an effective catalyst in the oxidation of pinacyanol chloride with hydrogen peroxide. The solubilization experiments revealed that the attachment of an alcohol-functionalized side arm to the crown ring effectively promoted supramolecular complexation with cytochrome *c*. The spectroscopic and electrochemical characterizations confirmed that the supramolecular complexes had structures and redox properties similar to those of cytochrome *c* in alkaline aqueous solution. Since supramolecular complexation with alcohol-armed lariat ethers offered higher catalytic activities than poly(ethylene glycolation) of cytochrome *c*, this has advantages over chemically modified proteins of easy solubilization procedure, structural versatility, and high catalytic activity. Further combinations of synthetic receptors and metalloproteins can offer a facile preparation method of a new series of biocatalysts working in organic media.

Experimental Section

General Considerations. ^{1}H and ^{13}C NMR spectra were recorded on JEOL LA-300 and GX-400 spectrometers. IR, mass, and CD spectra

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were obtained on Jasco FT/IR-420, JEOL AX500, and Jasco J-720 spectrometers, respectively. ESR spectra were obtained from liquid helium temperature to 35 K with a Bruker ESR 300 spectrometer. Raman scattering was excited by a 413.1 nm Kr ion laser (Spectra Physics, Model 2580). The resonance Raman light was dispersed with a JEOL 400D spectrometer equipped with a photomultiplier.

Materials. Alcohol- and amine-armed lariat ethers **1a**−**3a** and **1d**−**3d** were obtained from Aldrich as well as unsubstituted crown ethers **1f**−**4f** and employed as received. Cryptand[2.2.1], methoxypoly(ethylene glycol) (M_w = ca. 5000), poly(ethylene glycol) mono-*p*-isooctylphenyl ether (Triton X-100), tris[2-(2-methoxyethoxy)ethyl]-amine, and 4-aminobenzo-18-crown-6 were also purchased from Merck, Sigma, Nakalai Tesque, Fluka, and Acros. Ether-armed lariat ethers **1b**−**3b** were prepared by reaction of butyl bromide and corresponding crown ethers **1a**−**3a** in the presence of NaH.[27] Ester-armed lariat ethers **2c** and **3c** were synthesized as described for compound **1c**.[28] Amide-armed lariat ethers **1e**−**3e** were derived from amine-armed lariat ethers **1d**−**3d**. Alcohol-armed benzo-18-crown-6 **4a** was also obtained from its carboxyl derivative,[29] which was purchased from Acros. All of them were chromatographed (silica gel; ethyl acetate/hexane) and had the correct elemental compositions determined by microanalysis and high-resolution mass spectroscopy (EI mode). The purity of all new compounds was established by ^{1}H and ^{13}C NMR spectroscopy. The newly obtained materials were oils, except for **3e**, and their selected spectroscopic data are summarized below.

1,4,7,10-Tetraoxacyclododecane-2-methyl butyl ether (1b) was prepared from crown ether **1a** and *n*-bromobutane: δ_H ($CDCl_3$) 0.91 (3H, t, CH_3), 1.36 (2H, sex, C*H*$_2$CH$_3$), 1.54 (2H, qui, CH$_2$C*H*$_2$CH$_2$), 3.37−3.88 (19H, m, 9 × OCH_2, OCH); δ_C ($CDCl_3$) 14.15, 19.52, 31.97, 70.53, 70.68, 71.00, 71.12, 71.25, 71.66, 72.14, 78.95; ν (KBr) 1127 cm^{-1}. High-resolution MS (*m*/*z*): M^+, 262.1780; calcd for $C_{13}H_{26}O_5$, 262.1822.

1,4,7,10,13-Pentaoxacyclopentadecane-2-methyl butyl ether (2b) was prepared from crown ether **2a** and *n*-bromobutane: δ_H ($CDCl_3$) 0.91 (3H, t, CH_3), 1.35 (2H, sex, C*H*$_2$CH$_3$), 1.54 (2H, qui, CH$_2$C*H*$_2$-CH$_2$), 3.44−3.88 (23H, m, 11 × OCH_2, OCH); δ_C($CDCl_3$) 13.92, 19.29, 31.74, 70.25, 70.56, 70.60, 70.77, 70.93, 71.02, 71.04, 71.35, 71.66, 78.75; ν (KBr) 1117 cm^{-1}. High-resolution MS (*m*/*z*): M^+, 306.2042; calcd for $C_{15}H_{30}O_6$, 306.2061.

1,4,7,10,13,16-Hexaoxacyclooctadecane-2-methyl butyl ether (3b) was prepared from crown ether **3a** and n-bromobutane: δ_H ($CDCl_3$) 0.92 (3H, t, CH_3), 1.35 (2H, seq, C*H*$_2$CH$_3$), 1.54 (2H, qui, CH$_2$C*H*$_2$-CH$_2$), 3.41−3.81 (27H, m, 13 × OCH_2, OCH); δ_C ($CDCl_3$) 13.88, 19.25, 31.70, 69.91, 70.64, 70.71, 70.78, 70.86, 71.29, 71.83, 78.43; ν (KBr) 1108 cm^{-1}. High-resolution MS (*m*/*z*): M^+, 350.2304; calcd for $C_{17}H_{34}O_7$, 350.2343.

1,4,7,10,13-Pentaoxacyclopentadecane-2-methyl acetate (2c) was prepared from crown ether **2a**: δ_H ($CDCl_3$) 2.08 (3H, s, CH_3), 3.55−4.12 (21H, m, 10 × OCH_2, OCH); δ_C ($CDCl_3$) 20.91, 64.29, 70.24, 70.45, 70.51, 70.58, 70.78, 70.87, 70.90, 71.12, 77.45, 170.08; ν (KBr) 1737 and 1117 cm^{-1}. High-resolution MS (*m*/*z*): M^+, 292.1522; $C_{13}H_{24}O_7$, 292.1579.

1,4,7,10,13,16-Hexaoxacyclooctadecane-2-methyl acetate (3c) was prepared from crown ether **3a**: δ_H ($CDCl_3$) 2.08 (3H, s, CH_3), 3.59−4.24 (25H, 12 × OCH_2, OCH); δ_C ($CDCl_3$) 20.95, 63.93, 69.85, 70.62, 70.66, 70.75, 70.83, 70.93, 71.02, 77.07, 170.96; ν (KBr) 1736 and 1108 cm^{-1}. High-resolution MS (*m*/*z*): M^+, 336.1784; $C_{15}H_{28}O_8$, 336.1781.

1,4,7,10-Tetraoxacyclododecane-2-methylacetamide (1e) was prepared from crown ether **1d**: δ_H($CDCl_3$) 1.99 (3H, s, CH_3), 3.27−3.80 (17H, m, 7 × OCH_2, NCH_2, CH), 5.91 (1H, br, NH); δ_C ($CDCl_3$) 23.16, 40.63, 69.49, 70.20, 70.47, 70.65, 70.75, 72.13, 76.61, 170.52; ν (KBr) 1650 and 1128 cm^{-1}. High-resolution MS (*m*/*z*): M^+, 247.1420; $C_{11}H_{21}NO_5$, 247.1446.

1,4,7,10,13-Pentaoxacyclopentadecane-2-methylacetamide (2e) was prepared from crown ether **2d**: δ_H ($CDCl_3$) 2.01 (3H, s, CH_3), 3.25−3.79 (21H, m, 9 × OCH_2, NCH_2, CH), 6.30 (1H, br, NH); δ_C ($CDCl_3$) 23.19, 41.07, 69.53, 70.29, 70.39, 70.48, 70.68, 70.74, 71.05, 72.08, 77.48, 170.57; ν (KBr) 1658 and 1120 cm^{-1}. High-resolution MS (*m*/*z*): M^+, 291.1682; $C_{13}H_{25}NO_6$, 291.1670.

1,4,7,10,13,16-Hexaoxacyclooctadecane-2-methylacetamide (3e) was prepared from crown ether **3d** and recrystallized from EtOAc−hexane: mp 56−57 °C; δ_H ($CDCl_3$) 2.00 (3H, s, CH_3), 3.32−3.82 (25H, m, 11 × OCH_2, NCH_2, CH), 6.77 (1H, br, NH); δ_C ($CDCl_3$) 23.03, 40.78, 68.84, 70.23, 70.33, 70.39, 70.42, 70.65, 70.75, 70.93, 71.91, 170.83; ν (KBr) 1656 and 1108 cm^{-1} Anal. Found: C, 53.57; H, 8.92; N, 4.13. Calcd for $C_{15}H_{29}NO_7$: C, 53.72; H, 8.72; N, 4.18.

2,3-Benzo-1,4,7,10,13,16-hexaoxacyclooctadecan-2-ene-4′-methylacetamide (4-acetamidobenzo-18-crown-6) was prepared from 4-aminobenzo-18-crown-6 and recrystallized from CH_2Cl_2−diethyl ether: mp 114 °C; δ_H ($CDCl_3$) 2.14 (3H, s, CH_3), 3.68−3.89 (12H, m, 6 × CH_2), 3.90 (4H, t, PhOCH$_2$C*H*$_2$), 4.13 (4H, t, PhOC*H*$_2$), 6.79 + 7.28 (1H, d, 2 × Ph H), 6.85 (1H, dd, Ph H), 7.30 (1H, br, NH); δ_C ($CDCl_3$) 24.44, 68.90, 69.47, 69.51, 69.66, 70.68, 70.71, 70.76, 70.77, 107.15, 112.40, 114.59, 132.06, 145.57, 149.09, 168.16; ν (Nujol) 1656, 1129, 840 cm^{-1}. Anal. Found: C, 57.13; H, 7.46; N, 3.70. Calcd for $C_{18}H_{27}O_7N\cdot 2H_2O$: C, 57.50; H, 7.45; N, 7.45.

Poly(ethylene glycolated) cytochrome *c* was prepared using activated methoxypoly(ethylene glycol) (M_w = ca. 5000; Sigma-Aldrich) with cyanuric chloride,[30] the polydispersity index of which is approximately 1.2. To 20 nmol of cytochrome *c*, in 3 mL of 40 mM borate buffer (pH 10), was added 10 M excess (over amino groups in the cytochrome *c*) of activated poly(ethylene glycol). The resulting mixture was gently stirred at room temperature for 1 h and then dialyzed at 4 °C against phosphate buffer (pH 6.1) using an ultrafiltration cell fitted with an Amicon PM 30 membrane.[16] The average number of introduced poly(ethylene glycol) groups was estimated as 7.3 per cytochrome *c* by measuring the number of the unreacted amino groups with trinitrobenzenesulfonate.[31] The concentration of this cytochrome *c* derivative was adjusted to be the same as that of the cytochrome *c*−lariat ether complex, based on the absorbance at 407 nm.

Solubilization Experiment. The solubilization experiments were carried out by adding a methanol solution of lariat ether (1 mL, 80 μmol) to cytochrome *c* solid (0.4 μmol). After the mixture had been stirred for 1.5 h, the methanol phase was separated by centrifuge and characterized using UV and CD spectroscopy. The solubilization efficiency (%) was estimated on the basis of the absorbance at 407 nm of the methanol solution after appropriate dilution. The obtained methanol solutions of supramolecular complexes were essentially stable at 4 °C and exhibited exactly the same UV and CD spectra after 10 days.

CD, Raman, and ESR Experiments. The methanol solutions of supramolecular complexes and poly(ethylene glycolated) cytochrome *c* were prepared as described above.

CD experiments for measurement of the Soret region (350−500 nm): cytochrome *c*, 0.4 mmol; lariat ether, 80 mmol; MeOH, 4 mL. CD experiments for measurement of α-helix region (200−250 nm): cytochrome *c*, 0.04 mmol; lariat ether, 8 mmol; MeOH, 4 mL. The pH values of aqueous solutions were adjusted using phosphate buffers.

Raman experiments: cytochrome *c*, 0.2 mmol; lariat ether, 40 mmol; MeOH, 2 mL.

ESR experiments: cytochrome *c*, 0.5 mmol; lariat ether, 100 mmol; MeOH, 4 mL.

Electrochemical Experiments. Methylviologen dichloride (reagent grade) was recrystallized from methanol and dried in vacuo. The electrochemical reduction of cytochrome *c* derivative was carried out in an air-tight thin-layer spectroelectrochemical cell: the working electrode is gold gauze and is sandwiched by a pair of quartz plates with a Teflon spacer. Another platinum gauze is used as an auxiliary electrode, while Ag/AgCl is employed as reference electrode. The redox

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potential of the ferrocenium/ferrocene couple was observed as +0.364 V vs Ag/AgCl in methanol. Since this was reported as +0.400 V vs SHE,[32] the redox potential $E^{\circ\prime} = -0.396$ V vs Ag/AgCl of cytochrome *c*−**3a** complex typically corresponds to −0.360 V vs SHE. The conditions are shown in Table 2.

The reduction potentials of methylviologen were determined both in methanol and aqueous solution via cyclic voltammetry (Bioanalytical Systems Inc. CV-50W): methylviologen, 0.1 mM; tetraethylammonium perchlorate, 0.05 M.

Oxidation of Pinacyanol Chloride with Hydrogen Peroxide. The reaction mixture (1 mL) contained 20 μM pinacyanol chloride (1,1′-diethyl-2,2′-carbocyanine chloride) and 5 μM cytochrome *c*−lariat ether complex (cytochrome *c*:lariat ether = 1:200) in methanol. The oxidation was performed at room temperature and started by adding 1 mM hydrogen peroxide. The reaction progress was monitored spectroscopically: the decrease in the amount of pinacyanol chloride was determined by measuring the decrease in the absorbance at 603 nm.

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