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Cytochrome c–crown ether complexes as a new type of biocatalysts effective in methanol

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Abstract

Water-soluble cytochrome c was readily solubilized in methanol by addition of several crown ethers, and the resulting complexes catalyzed oxidation of pinacyanol chloride with hydrogen peroxide more effectively than polyethylene glycolated cytochrome c in the organic solvent.

Keywords: crown ethers; proteins; porphyrins and analogues; catalysts.

Biocatalysts working in non-aqueous organic solvents have opened a new field in the biotechnological applications of proteins. Although the number of effective biocatalysts remains limited, some enzymes are known to perform reactions which are impossible in aqueous solutions.[1] Peptide synthesis by proteases, trans-esterification by lipases and biotransformation of water-insoluble substrates successfully proceeded in organic media. Cytochrome c is a water-soluble heme protein and mediates electron transfer in the mitochondrial respiratory chain. This is not an enzyme but a promising candidate for an effective biocatalyst having great advantages over common heme enzymes: a covalently protein-bonded heme group and stable protein backbone.[2] Polyethylene glycolated cytochrome c was typically reported to catalyze the oxidation of organosulfides and aromatic hydrocarbons in the aqueous acetonitrile.[3,4] This exhibited higher catalytic activities than those of cytochrome c alone, but the increase in content of organic solvent drastically lowered its activities.

Here, we demonstrate that cytochrome c–crown ether complexes are readily obtained and work as effective biocatalysts in a 'non-aqueous' solvent. Because cytochrome c has 19 lysine residues ($-NH_3^+$) and other ionic moieties on the surface, these groups can function as effective binding sites of crown ethers. Odell and Earlam first reported that unsubstituted 18-crown-6 interacted with cytochrome c and solubilized it in a methanol.[5,6] Since the resulting complex is easily prepared by mixing cytochrome c (powder) and crown ether in the methanol, this solubilization method can offer a facile approach to biocatalyst preparation which does not require chemical derivatization, dialysis, lyophilization or other laborious procedures. We applied below a new series of crown ether derivatives having functionalized

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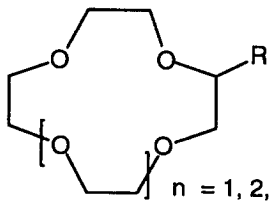
sidearms in the solubilization of cytochrome c and successfully carried out catalytic oxidation of pinacyanol chloride with their cytochrome c complexes: 12-crown-4s **1b–1d**; 15-crown-5s **2b–2d**; 18-crown-6s **3b–3d**; and unsubstituted crown ethers **1a**, **2a** and **3a**.



-R \ n	1	2	3
-H	**1a**	**2a**	**3a**
$-CH_2OH$	**1b**	**2b**	**3b**
$-CH_2OCOCH_3$	**1c**	**2c**	**3c**
$-CH_2O(CH_2)_3CH_3$	**1d**	**2d**	**3d**

Cytochrome c powder (0.4 mmol) was suspended in a methanol solution of crown ether (160 mmol/2 mL). After 1.5 h of stirring, the suspension was centrifuged and purple/red methanol solution was obtained (Table 1). Since cytochrome c itself is insoluble in the methanol, the cytochrome c is believed to be solubilized via crown ether complexation.[7] Solubilization efficiency (%) of the cytochrome c in the methanol is apparently dependent upon two structural factors of the employed crown ether: size of parent crown ring and structure of sidearm functionality. Typically, alcohol-armed crown ethers **1b**, **2b** and **3b** more effectively solubilized the cytochrome c than corresponding crown ethers having ester- and ether-functionalized sidearms (**1b** vs **1c**, **1d**: **2b** vs **2c**, **2d**: **3b** vs **3c**, **3d**). Addition of 200 equivalents of 18-crown-6 **3b** completely solubilized all the cytochrome c added, while large amounts of the solid cytochrome c remained in the presence of 400 equivalents of 18-crown-6s **3c** and **3d**. When a linear polyethylene glycol (Mw=ca. 5000) was employed instead of crown ethers, no cytochrome c was solubilized in the methanol under the employed conditions.

Table 1
Solubilization efficiency of cytochrome c via crown ether complexation

	Solubilization Efficiency (%)[a]			
	R = -H	$-CH_2OH$	$-CH_2OCOCH_3$	$-CH_2O(CH_2)_3CH_3$
12-Crown-4	**1a:** < 3	**1b:** 55	**1c:** < 3	**1d:** < 3
15-Crown-5	**2a:** 6	**2b:** 90	**2c:** < 3	**2d:** 4
18-Crown-6	**3a:** 100	**3b:** 100	**3c:** 32	**3d:** 30

[a] [Absorbance at 407 nm in the presence of 400 equivalents of crown ether] / [Absorbance at 407 nm in the presence of 400 equivalents of **3a**] x 100. Cytochrome c from horse heart was employed. Other conditions: see text.

The resulting cytochrome c–crown ether complexes were characterized by several spectroscopic methods. Compared with the UV spectrum of cytochrome c in an aqueous solution, disappearance of a charge-transfer band from the sulfur atom of Met 80 to heme iron(III) at 695 nm was observed. Since Raman spectral data supported that these complexes had 6-coordination heme moieties, the axial coordinating Met 80 residue may be replaced by another amino acid.[8] Circular dichroism bands of the cytochrome c–crown ether complexes were intensified in the methanol but their patterns were similar to those of cytochrome c in water: a negative (419 nm) and a positive (406 nm) around the Soret region and a negative (209 nm) around the α-helix region. Thus, the cytochrome c–crown ether complexes have redox active heme moieties in the ordered protein matrices.[9]

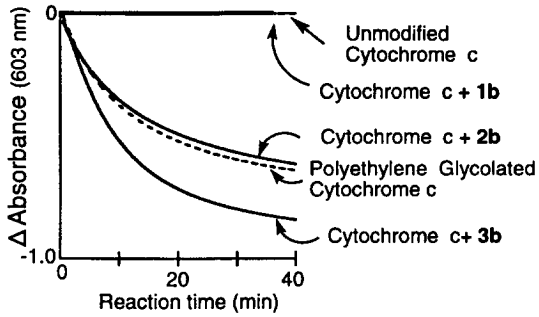


Figure 1. Catalytic oxidation of pinacyanol chloride by cytochrome c–crown ether complexes

The cytochrome c–crown ether complexes catalyzed the oxidation of pinacyanol chloride with hydrogen peroxide in methanol. Pinacyanol chloride has a maximum absorbance at 603 nm and has frequently been a useful substrate to spectroscopically determine activities of manganese peroxidase and related model catalysts in organic media.[10] We employed cytochrome c–crown ether complexes as biocatalysts in the non-aqueous methanol and compared their activities with that of cytochrome c itself: pinacyanol chloride, 25 μmol; H_2O_2, 480 μmol; cytochrome c, 2.5 μmol; crown ether, 500 μmol; methanol, 2.01 mL. Typically, 18-crown-6 **3b** and 15-crown-5 **2b** complexes offered excellent catalytic activities, though **1b** and unmodified cytochrome c rarely enhanced the reaction rates (Fig. 1). These catalytic behaviors are apparently dependent on the solubilized amounts of cytochrome c in the reaction solutions (Table 2). 18-Crown-6 **3b** and 15-crown-5 **2b** exhibited high solubilities of cytochrome c in the methanol (100% and 91%), while 12-crown-4 **1b** rarely solubilized it (0%). When other crown ethers **1a**, **1c**, **1d**, **2a**, **2c**, **2d**, **3c** and **3d** were employed, most of the cytochrome c added was suspended as powder and could not accelerate the oxidation. Polyethylene glycolated cytochrome c was examined,[11] which was reported to catalyze the oxidation of aromatic hydrocarbons in THF/H_2O (90/10).[2a] Its total preparation requires more than 3 days: activation of polyethylene glycol (1 day), reaction with polyethylene glycol and cytochrome c (3 h), dialysis (1 day) and lyophilization (1 day). This cytochrome c derivative was well soluble in the methanol, but exhibited lower catalytic activity than cytochrome c-**3b** complex. Since 2 hours are enough for crown ether complexation, this provides a remarkably shortened method to prepare effective biocatalysts. Table 2 also shows that cytochrome c-**3b** complex catalyzed the oxidation of pinacyanol chloride more effectively than both polyethylene glycolated cytochrome c and 18-crown-6 **3a** complex, though all the cytochrome c added was completely solubilized in these cases. Since simple heme derivatives were readily decomposed by hydrogen peroxide, the crown ether-bound protein can offer a suitable microenvironment in which the heme groups are catalytically active and protected against decomposition. Therefore, the cytochrome c-crown ether complexes can be seen as a new type of effective biocatalyst which has advantages over chemically modified proteins of easy solubilization procedure, structure versatility and high catalytic activity. Further combinations of synthetic receptors and bioproteins may have many extensions in various fields of organic chemistry, bioscience and biotechnology.

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Table 2
Catalytic oxidation of pinacyanol chloride by cytochrome c–crown ether complexes

Crown Ether	Conversion of Substrate (%) after 40 minutes[a] [Solubilization Efficiency (%)][b]			
	R = -H	$-CH_2OH$	$-CH_2OCOCH_3$	$-CH_2O(CH_2)_3CH_3$
12-Crown-4	**1a**: 0 [0]	**1b:** 0 [0]	**1c:** 0 [0]	**1d:** 0 [0]
15-Crown-5	**2a**: 0 [0]	**2b:**29 [91]	**2c:** 0 [0]	**2d:** 0 [0]
18-Crown-6	**3a**:35 [100]	**3b:**37 [100]	**3c:** 0 [0]	**3d:** 2 [3]
Polyethylene glycolated cytochrome c		30 [100][c]		
Unmodified cytochrome c		0 [0]		

[a] Based on absorbance changes at 603 nm. [Substrate]/[Cytochrome c]=10/1. Other conditions: see text.
[b] These were estimated for cytochrome c under oxidation conditions and different from those in Table 1.
[c] The concentration of cytochrome c in the reaction solution was adjusted to be same as those of **3a** and **3b** complexes, based on absorbance at 407 nm.

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