Crown Ether Strategy Toward Chemical Activation of Biological Protein Functions

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The chemical activation of biological proteins is outlined, in which small molecules are used to alter the chemical and physical properties of biological proteins through direct or indirect interactions. Crown ethers have the potential to modulate the protein functions by supramolecular complexations, because they bind alkylammonium and other ionic residues of the proteins as well as ionic components in their systems. Two interesting examples are described in which crown ether derivatives improved the protein functions: (1) enhancement of reactivity and enantioselectivity in lipase-catalyzed asymmetric reactions; and (2) generation of catalytic activity in the oxidation with cytochrome c. This chemical activation based on crown ether chemistry can be viewed as a complementary method to biological mutation in modifying the biological protein functions.

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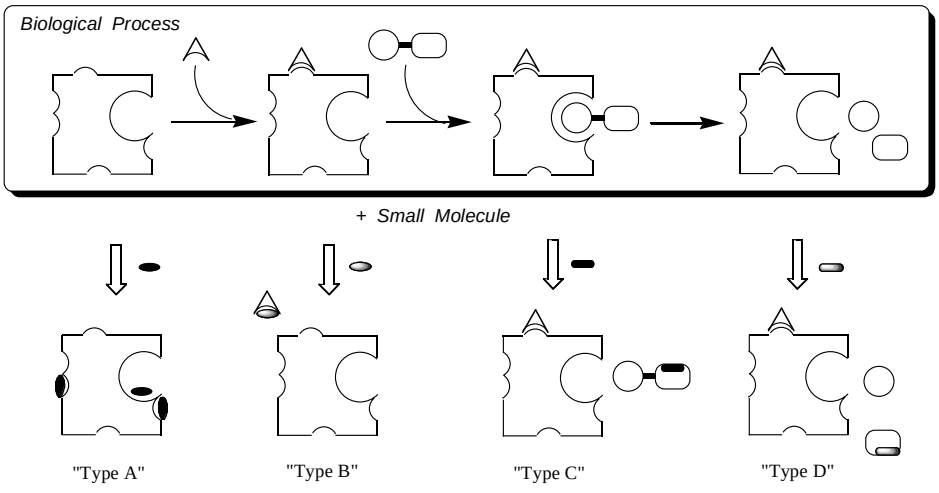
Introduction.

Molecular recognition plays an important role in biological enzyme and protein systems. Crown ethers and related macrocycles mimic some parts of biological molecular recognition and mediate subsequent chemical processes. Since they bring several ions and/or molecules together around the crown rings, they can catalyze reactions and facilitate membrane transport in a biomimetic manner [1]. In addition to amino acids and common biological substrates, crown ethers can bind $-NH_3^+$, $-CO_2^-M^+$ or other functional moieties of the biological proteins. Thus, they can form supramolecular complexes with proteins, even though these guests are too large to form 1:1 complexes [2]. Once the protein is wrapped by a number of crown ethers, its solubility, stability, reactivity and further functions can be regulated by non-covalent interaction.

Protein engineering has provided a practical technique to improve the functions of biological proteins. Since this has been restricted to natural amino acid replacements, chemical modification techniques have been combined with site-directed mutagenesis to address this limitation [3]. A new complementary approach has recently been developed from a chemical stand-point, in which small molecules were used to activate or inactivate protein systems by non-covalent interactions [4,5]. Biological proteins have several different structures and functions depending on solvent, temperature and other environmental factors, and certain small molecules can modify the microenvironments and hence protein functions. Chart 1 schematically illustrates the chemical activation of protein functions, in which small molecules interact with proteins ("Type A") and other components ("Type B", "Type C" and "Type D") and offer both direct and indirect perturbations onto the protein reactivity. This approach usually involves the search for small molecules that modulate protein activity or reaction pathway. The geneticist generates a large number of mutants, and selects the desired mutations from the mutant library. Likewise, we can construct the library

Chart 1



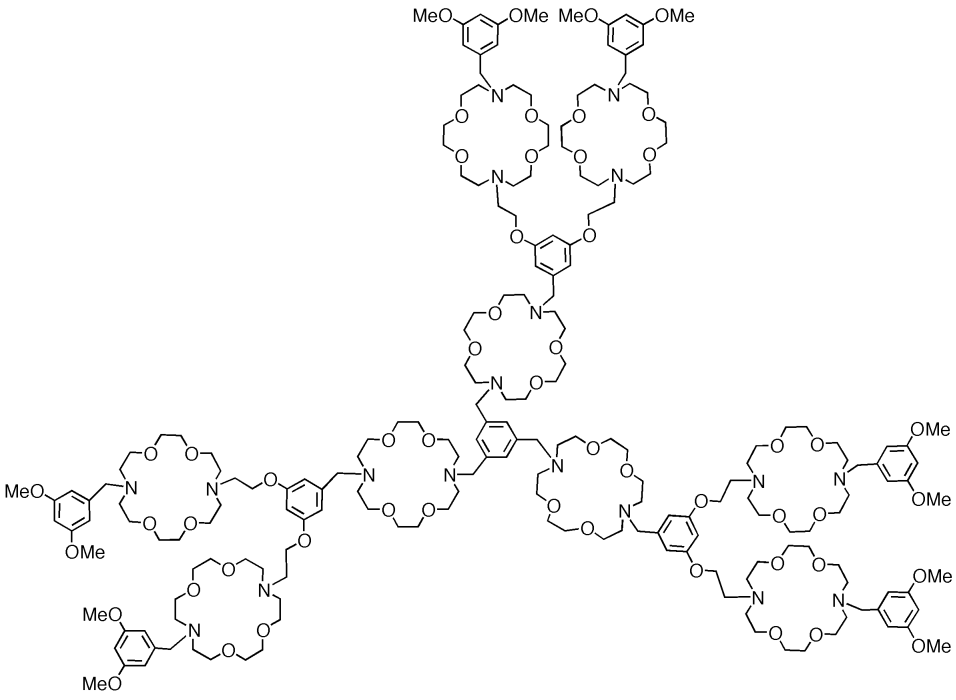
Schematic Illustration of Chemical Activation of Protein Function with Small Molecule

of small molecules to interact with proteins and select those desired through the use of combinatorial screens. Several natural products and their derivatives were successfully discovered using this method which modified the protein functions [6].

This review describes recent advances in chemical activation of biological protein functions based on crown ether chemistry. The basic concept and typical examples are briefly summarized, in which several kinds of crown ether derivatives effectively enhance the protein reactivity by non-covalent interaction. They readily form complexes with various guest species, and no laborious experimental procedures are required to discover effective crown ethers for a particular protein partner. Two of our original efforts to activate the proteins using crown ether derivatives are further described: (1) enhancement of reactivity and enantioselectivity in lipase-catalyzed asymmetric reactions [7]; and (2) generation of catalytic activity in the oxidation with cytochrome c [8]. Since crown ethers and their derivatives are demonstrated below as a new class of small molecules to activate proteins, crown ether strategy provides promising applications in chemical activation of biological protein functions based on supramolecular complexation [9].

Crown Ethers as Synthetic Receptors of Biological Proteins.

Crown ethers are known to bind biological proteins, because most of them possess alkylammonium, guanidinium, and carboxylate salts on their surfaces. Odel and Earlam first reported weak association of protein and crown



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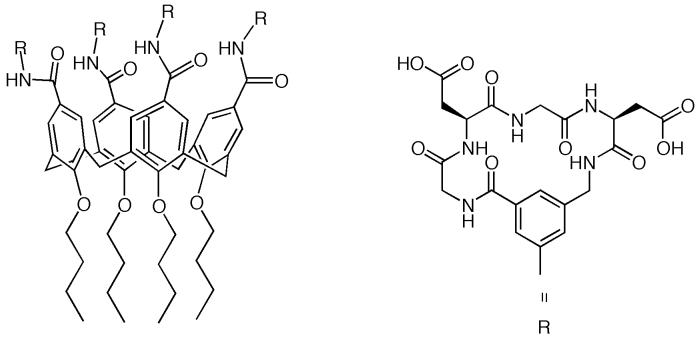

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ether in methanol (K_{ass} = 1 - 3 M^{-1}) [10]. This type of complexation resulted in the solubilization of water-soluble proteins in organic solvents, which included bovine serum, cytochrome c, lysozyme, myoglobin and α-chymotrypsin. Table 1 lists the molar ratios of 18-crown-6 to protein required for dissolution of these proteins: the protein - crown ether complexation was significantly dependent on a number of factors such as ring size of the crown ether, nature of solvent and property of the protein guest: 18-crown-6 provided a better fit for several proteins than either smaller or larger membered crown ethers; methanol, ethanol and dimethylformamide were suitable solvents to dissolve water-soluble proteins; and the primary, secondary and tertiary structures of protein also influenced the dissolution process. Cytochrome c and lysozyme were dissolved more easily than α-chymotrypsin and bovine insulin. Reinhoudt *et al.* reported that crown ether derivatives solubilized α-chymotrypsin in organic solvents and had a remarkable influence on its reactivity [11]. Nagasaki *et al.* employed crown ether dendrimers such as **1** in the solubilization of myoglobin in organic media [12]. Although only a few systematic investigations have been undertaken, crown ether derivatives may have wide applications in the modification of protein solubility and reactivity.

Table 1

Solubilization of Biological Proteins by Crown Ether Complexation

Protein	[Protein]:[Crown Ether]		
	15-Crown-5	18-Crown-6	21-Crown-7
Bovine insulin	insoluble	1:1200	
Cytochrome c	1:1200	1:120 – 300	1:800
Bovine serum		1:430	1:6000
Lysozyme		1:150	
Myoglobin	insoluble	1:400	
α–Chymotrypsin	insoluble	insoluble	

Activation of Protein Functions with Small Molecules.

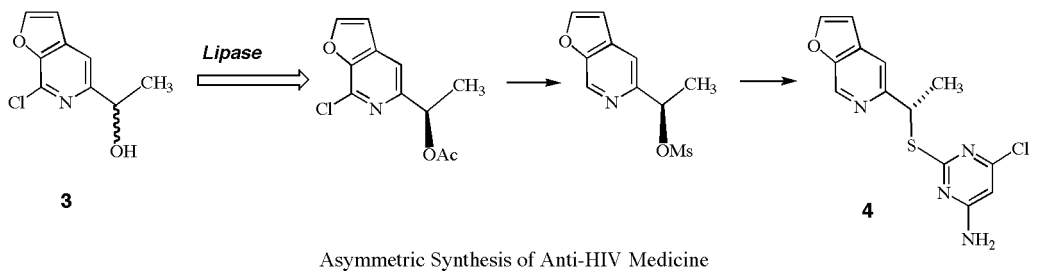
The most common method to alter protein functions is indirect, relying on mutations in the genes encoding proteins of interest, but chemical activation offers a complementary and direct approach [4]. This requires the equivalency of small receptor molecules and mutants in their ability to activate specific proteins. As illustrated in Chart 1, small receptor molecules apparently bind to the target protein ("Type A"), thereby influencing its function, but there are many possibilities that the protein functions may be altered by other means ("Type B", "Type C" and "Type D"). The steroid hormones are typical examples of activating small molecules, which activate transcription following their binding to steroid hormone receptors.

Crown ethers and other macrocyclic receptors can form supramolecular complexes with proteins [2], though only a limited number of macrocycles have been examined. Some of them were demonstrated to modify the physical properties and to enhance the chemical reactivity of the biological protein upon crown ether complexation [10,11]. Hamilton *et al.* developed a calixarene derivative **2** with four peptide loops having a large surface area that allowed strong binding to a complementary surface on α-chymotrypsin [13]. Such an antibody - like complexation gave slow binding kinetics in an analogous manner to those of biological proteinase inhibitors. There are a vast number of crown ether-type receptors reported in the literature which interact with a variety of species common in protein processes (see Chart 1). Since these allow us to construct a library effective for chemical activation, we can choose a suitable crown ether from this library and use it in the chemical activation of a specific protein.

Chemical Activation of Lipase Functions with Small Molecules.

Lipases have been established as valuable catalysts in not only biological reactions but also organic synthesis. They have performed regio- and stereo-selective transformations in laboratory scale up to industry scale, and provided facile accesses to chiral building blocks and selectively protected intermediates [3,5]. They typically catalyze the hydrolysis of carboxylic esters and acyl transfer onto hydroxy and amino groups with the formation of carboxylic esters and amides. The lipases accept a broad range of synthetic substrates, but they cannot always transform them in an optimal manner. Scheme 1 illustrates asymmetric synthesis of HIV reverse transcriptase inhibitor **4**, PNU-142721 [14]. The resolution of racemic pyridylethanol derivative **3** was successfully carried out by lipase-catalyzed asymmetric acetylation with vinyl

Scheme 1



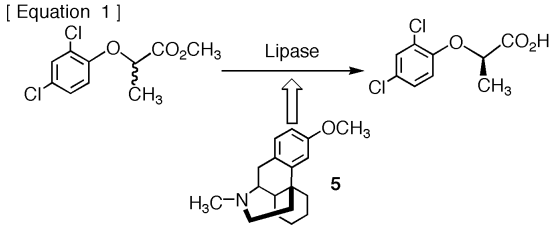
3 **4**

Asymmetric Synthesis of Anti-HIV Medicine

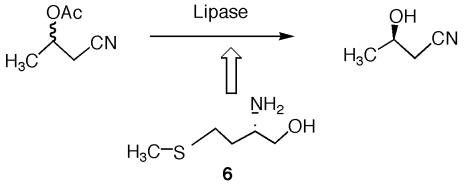
acetate. *Candida antarctica* lipase, CAL, provided perfect optical resolution of this substrate. Acetylation automatically stopped at 50 % conversion and gave an optically pure product (> 98% ee). Since the subsequent substitution in an S_N2 reaction proceeded with inversion of the chiral carbon center, anti-HIV medicine **4** was readily prepared in an asymmetric fashion.

Despite the widespread use of lipases in asymmetric reactions, more or less empirical strategies were required to improve reactivity and selectivity. Theil summarized several examples of lipase activation [5]. Two classes of small molecules have proved to enhance the activity of lipase-catalyzed reactions. One is a family of amine derivatives, and the other is a family of macrocyclic receptors. Sih *et al.* reported that chiral amine **5** worked as an enantioselective inhibitor in lipase-catalyzed hydrolysis of (±)-arylpropionic ester and (±)-aryloxypropionic ester (see Equation 1) [15]. Itoh *et al.* found that (*S*)-2-amino-4-methylthio-1-butanol **6** enhanced the enantioselectivity in lipase-catalyzed hydrolysis (see Equation 2) [16]. Their kinetic analyses revealed that these small molecules increased the enantioselectivity by inhibiting the hydrolysis of the slow-reacting enantiomers. Triethylamine and D-sorbitol similarly increased the enantioselectivity [17,18], while lipids and sodium deoxychlorate improved the reactivity [19,20].

[Equation 1]



[Equation 2]



Chemical Activation of Lipase Functions by Crown Ethers.

The crown ether-activated reactions allowed us to develop a smart asymmetric synthesis, though the number of successful applications still remains limited. Several crown ethers and related macrocycles have been reported to activate the lipase functions. Reinhoudt *et al.* reported that crown ethers effectively enhanced the enzymatic activity in non-aqueous media [9]. Addition of 18-crown-6 to the reaction solvent resulted in up to 30 times enhancement of the α-chymotrypsin-catalyzed trans-esterification of amino acid esters. Tyrosinase-catalyzed oxidation reaction was similarly accelerated in the presence of crown ethers. Because the employed enzymes have different mechanistic and structural properties, the crown ether activation of enzymes has a general applicability in organic solvents [21]. We demonstrated that thiacrown ethers promoted both the activity and the enantioselectivity of lipase-catalyzed hydrolytic and trans-esterification reactions as described below [7].

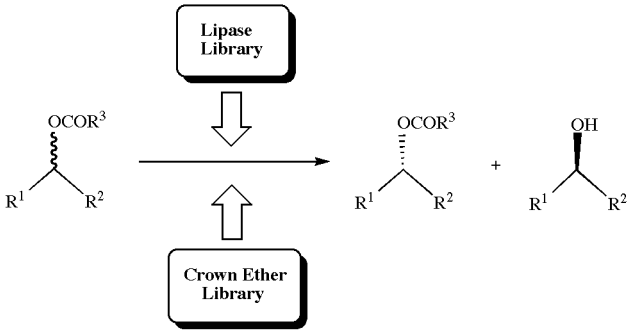


Figure 1. Crown Ether Strategy Toward Lipase Activation

The crown ether strategy toward lipase activation involves (i) search of effective receptors from a large collection, (ii) selection of a proper lipase, and (iii) optimization of a crown ether and protein combination (Figure 1). The hydrolysis of 2-cyano-1-methylethyl acetate **7** was typically chosen to identify the effective crown ether derivative and its lipase partner [22]. Figure 2 indicates a collection of crown ethers and their derivatives, most of which are commercially available. Since more than 30 kinds of lipases are also available, we can easily select a proper pair of lipase and crown ether from more than 1000 combinations. We have no detailed information about the lipase structure and its catalytic mechanism. Thus, such a combinatorial approach is an effective method to select a proper pair of crown ether and lipase.

When *Pseudomonas capacia* lipase, PCL, was employed, 1,4,8,11-tetrathiacyclotetradecane **8** enhanced both enantioselectivity and efficiency more effectively than other crown ether derivatives (see Figure 2). The addition of 5 mol% of thiacrown ether **8** to ester substrate **7** was enough to activate the lipase, which corresponded to 250 times thiacrown ether based on the lipase molecule. It is quite important that the hydrolysis proceeded even at 0 °C, though most of enzymatic reactions did not occur at such low temperature [23].

This chemical activation method was applied in the regio-selective hydrolysis of 2-acetoxy-2-methylbut-2-enyl acetate **9** (Equation 4) [24]. Although this diacetate has two reactive sites for lipase-catalyzed hydrolysis, the

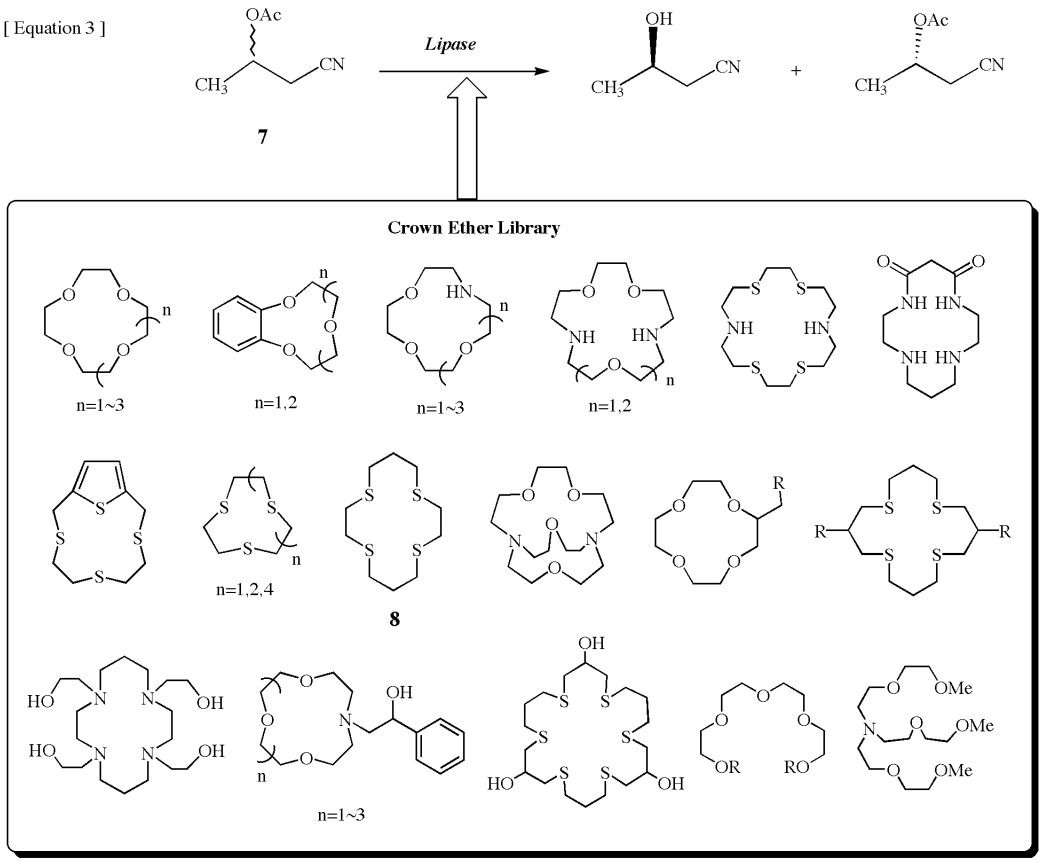
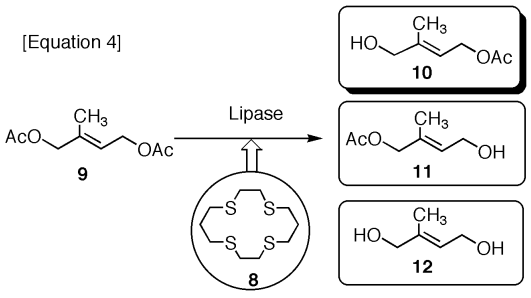


Figure 2. Lipase-catalyzed hydrolysis and crown ether library.

addition of thiacrown ether **8** strongly enhanced regioselectivity and efficiency to give monoacetate **10**. Table 2 compares rate-enhancements by thiacrown ether **8** in the five lipase systems. *Candida rugosa* and *Pseudomonas cepacia* lipases, CRL and PCL, exhibited improved reactivity, though *Procine pancrease* and other lipases, PPL, AL and F, slightly influenced the reaction rate and enantioselectivity. Since *trans*-esterification of racemic alcohols was also improved with the addition of crown ethers [25], a variety of lipase-catalyzed reactions can be chemically modified by crown ethers. The chemical activation of the lipases with crown ethers did not change the original stereochemistry of the product, but the enhanced reactivity was obtained at a practical level.

Table 2
Regio-Selective Hydrolysis of Diacetate **9**
Catalyzed by Five Kinds of Lipases

Lipase	Thiacrown	Relative Rate	Regioselectivity (**10**:**11**)
CRL	0	4	74:26
	5 mol %	54	87:13
PCL	0	37	96:14
	5 mol %	87	98: 2
PPL	0	0.4	94:6
	5 mol %	0.1	94: 6
AL	0	2	88:12
	5 mol %	5	94: 6
F	0	0.02	81:19
	5 mol %	2	86:14



The mechanism of the lipase activation with crown ether involves several possibilities as illustrated in Chart 1. Complexation of crown ether with ionic residues on the surface or around the active center of the lipase should be considered a key factor to alter the structure and function of the protein ("Type A"). Another possibility is that crown ethers bind water molecules or active species prior to substrate binding ("Type B"). They may also transport substrate or other components between the active site and bulk

medium ("Type C" and "Type D"). Since the chemical equilibrium of the reaction is modified to inhibit the reverse reaction, both rate and enantioselectivity can be enhanced in these cases. Thiacrown ether **8** was demonstrated to bind several alcohols in the hydrophobic media, suggesting that the mechanism *via "*Type C or D" would be involved in the above-described reactions. Nakamura *et al.* [26] and Easton *et al.* [27] reported that the addition of cyclodextrin increased the efficiency and selectivity in the enzymatic reactions. Since the cyclodextrin traps the substrates (see "Type C" in Chart 1), this can be used to manipulate the substrate concentration, reduce the substrate inhibition and alter reaction selectivity. Thus, chemical activation with synthetic receptors is widely applicable in the protein systems.

Chemical Activation of Cytochrome c Functions with Small Molecules.

The physical properties and the chemical reactivities of biological metallo-proteins can be controlled upon non-covalent interaction with small molecules. Cytochrome c is a representative of water-soluble heme proteins and mediates electron transfer in the mitochondrial respiratory chain [28]. This is positively charged at neutral pH and functions as an electron-transporting protein to cytochrome c peroxidase and oxidase. Its crystal and solution structures indicated that several charged amino acids were distributed around the heme edge and interacted with the negatively charged residues of its reaction partners [29]. Hildebrandt *et al.* observed the modified redox properties of cytochrome c upon non-covalent interaction with negatively charged heteropolytungstate, phospholipid vesicles and electrodes [30]. Hamachi *et al*. [31,32] and Hirota *et al.* [33] employed negatively charged small molecules such as surfactants, Ru complexes and peptides to modulate the functions of cytochrome c.

The peroxide activity of myoglobin was enhanced by chemical activation. This heme-protein is used for oxygen storage with a single non-covalently bound iron proto porphyrin in a hydrophobic pocket of protein matrix. Hayashi *et al.* employed heme derivative having polycarboxylate anion groups to increase the accessibility of neutral aromatic substrates to the heme active site, resulting in higher peroxidase activity [34]. Therefore, the chemical activation with small molecules can be applied in various metallo-protein systems.

Chemical Activation of Cytochrome c Functions by Crown Ethers.

Cytochrome c is not an enzyme in the biological processes but a promising candidate for an effective biocatalyst having great advantage over common heme enzymes: covalently protein-bound heme group and stable protein backbone [35]. Since there are a number of lysine residues (-NH_3^+) and other functional groups on the surface, crown ethers can form supramolecular complexes

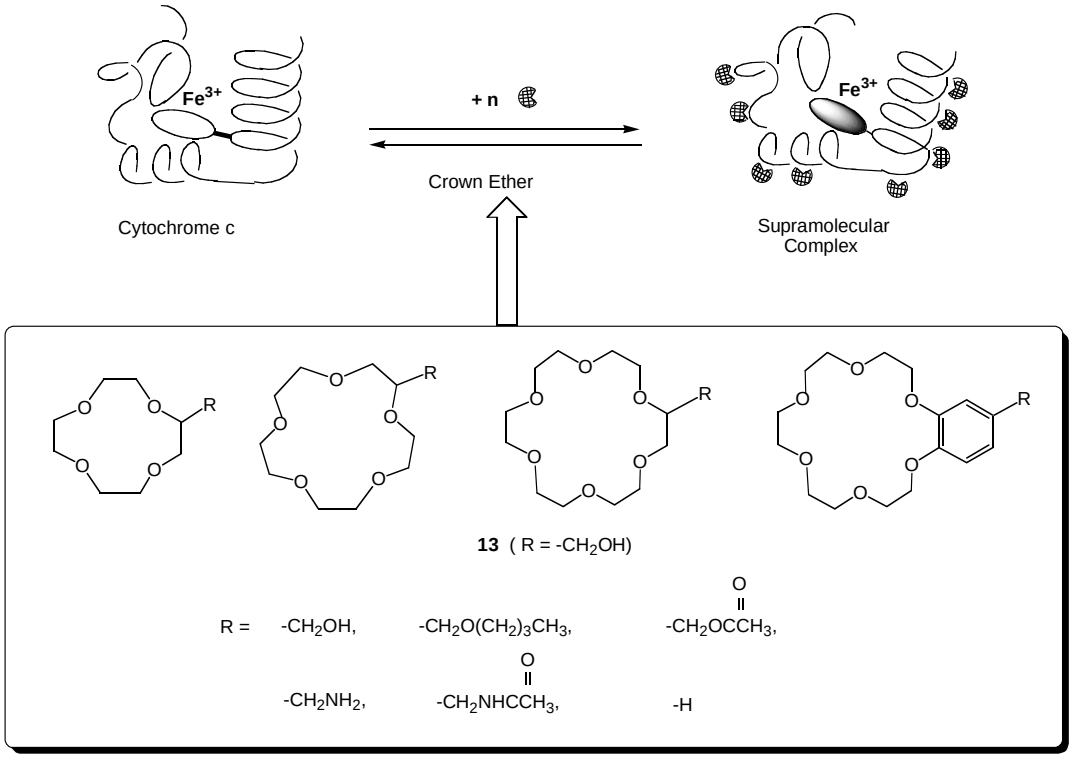


Figure 3. Crown ether library for supramolecular complexation with cytochrome c.

with cytochrome c. Typically, cytochrome c from horse heart (Mw=12,500) having 19 Lys, 9 Glu, 3 Asp and other ionic amino acids was complexed with crown ethers and solubilized into methanol [10]. Calixarene with four peptide loops **2** was also reported to bind cytochrome c and inhibit electron transfer process [36].

A series of crown ethers having alcohol, ether, ester, amine and amide moieties on their sidearms are potential candidates for small molecules to interact with cytochrome c (Figure 3), because the parent crown rings and sidearm functionalities can bind metal ions, organic ammonium groups, water, and other common species in biological systems [8]. Among the employed crown ethers, alcohol-armed crown ethers interacted with cytochrome c and solubilized it in methanol most effectively. When 90 equivalent of alcohol-armed 18-crown-6 **13** completely solubilized cytochrome c powder in CD_3OD, the obtained solution gave a broad, singlet NMR signal for crown ring protons of -CH_2- even at -80 °C. In contrast, the supramolecular complex with unsubstituted 18-crown-6 provided one set of the NMR signals for the complexed and the free 18-crown-6 at < -32 °C. Its shape analysis suggested that about 30 molecules of 18-crown-6 bound to one cytochrome c molecule. Thus, alcohol-armed crown ether **13** bound to cytochrome c more dynamically than parent crown ether. Spectroscopic characterizations revealed that the cytochrome c - crown ether complexes had structurally ordered protein structures in methanol, where their heme coordination was modified: displacement of methionine-80 by lysine-79 at an axial ligand of the heme; unnatural 6-coordination low-spin heme state; and enhanced α–helix contents in the protein backbone. Table 3 lists the reduction potentials of several cytochrome c derivatives, indicating that cytochrome c exhibited more negative reduction potentials in methanol than those in aqueous solutions. This suggests that the cytochrome c have several different conformations and functions depending on microenvironments in both aqueous and methanolic media. Methoxypolyethylene glycol, polyethylene glycol mono-*p*-isooctylphenyl ether and tris[2-(2-methoxyethoxy)-ethyl]amine were examined for comparison, but exhibited lower solubilization ability than crown ethers.

Table 3

Reduction Potentials of Cytochrome c Derivatives

Cytochrome c	$E^{o'}$(V *vs* SHE)	Medium
Cytochrome c	0.260	H_2O (pH 6.0)
Cytochrome c	0.060	H_2O (pH 9.0)
Cytochrome c - **13**	-0.360	MeOH (0.05M Et_4NClO_4)
Cytochrome c - 18-Crown-6	-0.393	MeOH (0.05M Et_4NClO_4)
Polyethylene Glycolated Cytochrome c	-0.439	MeOH (0.05M Et_4NClO_4)

Polyethylene glycolation is widely applied in the use of biological proteins, but the protein derivatization usually includes a series of laborious experimental procedures [37]. Typically, the total preparation of polyethylene glycolated cytochrome c requires more than 3 days: activation of polyethylene glycol (1 day); reaction with polyethylene glycol and cychrome c (3 hours); and dialysis (1 day) and lyophilization (1 day). Since 2 hours are enough for the crown ether complexation, the chemical activation of biological protein remarkably shortened experimental periods [38-41] and realized satisfactorily high functionality in non-biological systems. The polyethylene glycolated cytochrome c catalyzed the oxidation of aromatic hydrocarbons and organosulfides in aqueous methanol solution, but did not work well in non-aqueous methanol [35]. In contrast, the cytochrome c - crown ether complexes promoted the oxidation of pinacyanol chloride in non-aqueous methanol [42]. The cytochrome c itself was suspended as powder in the non-aqueous methanol and did not catalyze the oxidation. Since the crown ethers complexed it and solubilized it in the methanol, the resulting supramolecular complexes of cytochrome c with crown ethers acted as homogeneous catalysts in such non-biological oxidation.

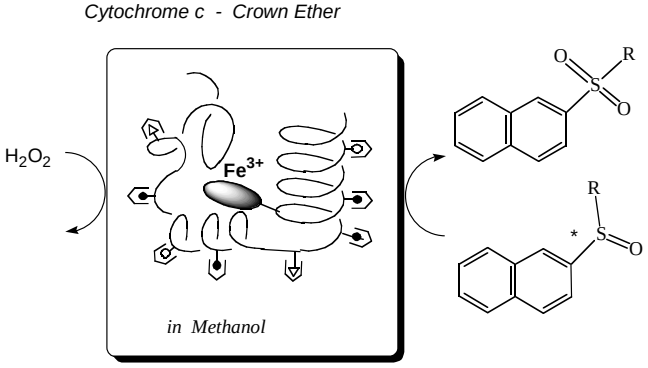


Figure 4. Asymmetric oxidation by cytochrome c - crown ether complex.

As observed with many metallo-enzymes, cytochrome c is composed of chiral amino acid sequences and provides the integrated chiral environments around the heme redox center. Since it has highly ordered structure not only in the aqueous solution but also in the organic media, its crown ether complexes effectively promoted the oxidation of racemic sulfoxide with hydrogen peroxide in methanol (Figure 4). Most biological proteins are known to work only around ambient temperature, but the present cytochrome c complexes exhibited both the highest efficiency and enantioselectivity at -40 °C. Some lipases were exceptionally reported to work on heating at 100 °C or lowering the temperature to -40 °C [43,44], but the cytochrome c complexes oxidized (*R*)-sulfoxide three times faster than (*S*)-sulfoxide in methanol at -40 °C. Therefore, crown ether complexation gave uncommon reactivity, enantiomer selectivity and substrate specificity in the cytochrome c reaction systems.

Concluding Remarks.

We are still in the early stage of chemical activation of biological proteins based on crown ether chemistry. The functions of protein and related components can be controlled by non-covalent interaction with small molecules. Since crown ether derivatives provide precise molecular recognition depending on structural characteristics, they can be widely used to activate biological proteins. This review demonstrated that several types of crown ethers successfully activated protein functions, as was readily discovered from a library containing a series of crown ethers. Since crown ether derivatives have the advantage of facile structural optimization and offer characteristic molecular recognition ability for various biological proteins, they can modify the solubility of the target protein and enhance its reactivity under non-biological conditions. Indeed, enantioselectivity was magnified in lipase catalyzed-reaction and uncommon catalytic activity was generated in the cytochrome c system. The folding at non-permissive temperature, inhibition with small molecules and denaturation in organic media are commonly observed phenomena, but these factors can be used to generate the non-biological functions in the protein processes. Our examples demonstrated that a proper optimization of small molecule, solvent, temperature and other environmental factors effectively enhanced the protein functions. Therefore, the chemical activation based on crown ether chemistry proved a complementary method to biological mutation in regulation of the biological protein functions.

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