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Chemical Activation of Cytochrome *c* Proteins via Crown Ether Complexation: Cold-Active Synzymes for Enantiomer-Selective Sulfoxide Oxidation in Methanol

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There are several chemical approaches to activate proteins and modify their functions, which include introduction of metal coordinating sites, organization of substrate recognition pockets, use of functionalized prosthetic groups, and attachment of cofactors.[1] In addition to various covalent modifications of proteins, noncovalent complexation has recently been recognized as a useful alternative to functionalize the proteins.[2] This requires relatively small molecules which interact with specific proteins to alter their biological structures and add nonbiological functions. Typically, several crown ethers bind $-NH_3^+$, $-CO_2^-M^+$, or other functional moieties exposed on the protein surface and form *n*:1 (crown ether:protein) types of supramolecular complexes. Reinhoudt et al. and our group have demonstrated that crown ether complexation enhanced reactivity and enantiomer-selectivity in the hydrolytic enzymatic reactions.[3] Here, we report that supramolecular complexation with 18-crown-6 can convert electron-transfer cytochromes *c* to "cold-active" synzymes.[4]

Cytochrome *c* proteins mediate electron-transfer processes in mitochondrial respiratory chains.[5] They have two coordinative residues (His 18 and Met 80) to form six-coordinate low-spin heme complexes, although most of the catalytically active heme enzymes have highly reactive five-coordinate hemes with open sites. Some mutants and heme peptide fragments have been derived from the cytochromes *c*, which have lost the methionine ligands and had five-coordinate hemes. Although they were used as oxidation catalysts,[6] the degradation of their hemes readily occurred in the presence of H_2O_2 and other oxidants. Effective receptors for cytochrome *c* functionalization have been developed. Hamilton et al. and Goto et al. designed calixarenes having polycarboxylic acids to bind the positively charged lysine cluster on the horse heart cytochrome *c* surface.[7] Odell and Earlam reported earlier that crown ethers solubilized the cytochrome *c* into organic solvents. On the basis of ESI-MS studies, Julian and Beauchamp found that four 18-crown-6 molecules strongly bound the exposed lysine moieties on the cytochrome *c* surface.[8] These cytochrome *c* crown ether complexes have particularly interesting features as asymmetric cold-active synzymes effective in organic media: (1) The biologically inactive heme structure can be converted to the catalytically active form;[9] (2) the protein matrix provides an asymmetric environment around the heme center; (3) high solubility in organic media allows a low-temperature reaction so that the oxidative degradation of heme is suppressed and enantiomer-selective reaction is promoted; (4) a different variety of substrates and conditions are available from those in aqueous systems; and (5) their preparation requires no laborious procedures such as derivatization/purification, dialysis, or lyophilization.

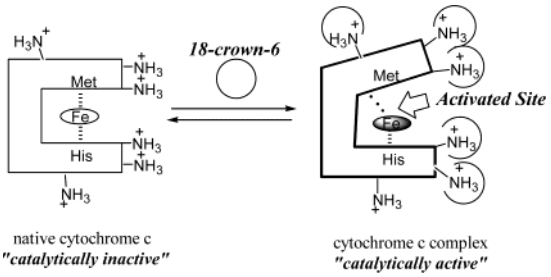

Figure 1. Activation of cytochrome *c* via crown ether complexation.

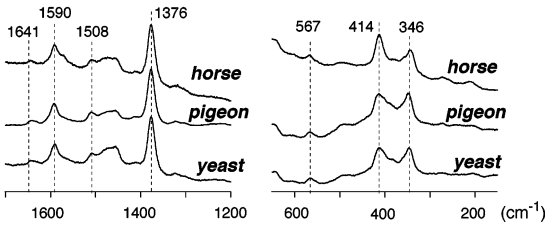

Figure 2. Resonance Raman spectra of three cytochrome *c*−crown ether complexes. Conditions: cytochrome *c*, 5.00×10^{-5} mol/L; 18-crown-6, 1.00×10^{-2} mol/L; in methanol; $\lambda_{ex} = 406.7$ nm.

We demonstrate below that supramolecular complexation with 18-crown-6 converted three kinds of cytochrome *c* proteins to cold-active synzymes, which effectively promote the asymmetric oxidation of sulfoxides in methanol. 18-Crown-6 well solubilized horse heart, pigeon breast, and yeast cytochrome *c* proteins[10] in methanol when their powders (6.0 mg) were stirred mechanically with a methanol solution of 18-crown-6 (25.4 mg/1.2 mL) for 1.5 h. Although these cytochromes *c* are insoluble in methanol, their supramolecular complexes gave homogeneous solutions and rarely precipitated even at −75 °C after a few days (see picture in Table of Contents). Polyethylene-glycolated horse heart cytochrome *c* was reported to be soluble in several organic media.[11] This formed an insoluble gel material in methanol at <0 °C and is inapplicable at low temperatures. Spectroscopic characterizations indicated that each cytochrome *c* complex has a nonbiological six-coordinate heme in methanol (Figure 1). The LMCT bands due to methionine coordination disappeared in the electronic spectra around 695 nm,[12] suggesting that the coordinative methionine was displaced from the sixth coordination position of the heme. In the resonance Raman spectra (Figure 2),[13] these cytochrome *c* complexes exhibited characteristic bands of six-coordinate low-spin iron(III) complexes at 1376, 1508, 1590, and 1641 cm^{-1}, suggesting that the neighboring amino acid residue occupied the sixth coordination position. Because the enhanced circular dichroism bands were observed around 209 and 420 nm, the three kinds of supramolecular complexes have artificially activated hemes in the asymmetrically structured protein domains.

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Table 1. Degradation of Cytochrome *c*−Crown Ether Complexes with H_2O_2 and Their Activities in Sulfoxide Oxidation

cytochrome *c*	temp (°C)	degradation[a] half-life (min)	oxidation[b] conversion (preferred isomer[c])	oxidation[b] ee %[d]
horse	+20	0.3	10% (*S*)	18
	0	6	39% (*S*)	32
	−20	52	68% (*S*)	41
	−40	150	73% (*S*)	47
	−60	>2500	39% (*S*)	43
pigeon	−40	700	79% (*S*)	49
yeast	−40	2400	31% (*S*)	31

[a] Cytochrome *c*, 3.30×10^{-5} mol/L; 18-crown-6, 6.66×10^{-3} mol/L; H_2O_2, 2.00×10^{-3} mol/L, in methanol. Monitored at 407 nm. [b] Cytochrome *c*, 2.00×10^{-4} mol/L; naphthyl methyl sulfoxide, 4.00×10^{-4} mol/L; 18-crown-6, 4.00×10^{-2} mol/L; H_2O_2, 6.00×10^{-3} mol/L, for 15 h in methanol. [c] (*S*)-Isomer was more rapidly oxidized. [d] Calculated at 80% conversion.[16]

The cytochrome *c* proteins are known to display minor peroxidase activities in aqueous solution,[14] but the degradation of their hemes with H_2O_2 has frequently been reported, accompanied by bleaching and catalyst destruction.[15] The supramolecular complexes exhibited higher reactivities toward H_2O_2 degradation because of the activated heme structures (Table 1). The half-life of horse heart cytochrome *c* at 20 °C was estimated to be 0.3 min in methanol and 20 min in water, but lowering the temperature greatly depressed the degradation in methanol: half-life = 6 min (0 °C); 52 min (−20 °C); 150 min (−40 °C); >2500 min (−60 °C). Therefore, the supramolecular complexes have uncommon heme structures with long-term activities at low temperature.

These cytochrome *c*−crown ether complexes promoted oxidation of several organic sulfoxides, sulfides, and anthracene in methanol, although styrene, ethyl benzene, and benzyl alcohol rarely reacted. When racemic naphthyl methyl sulfoxide was oxidized with H_2O_2, the reaction mixture was typically analyzed by the chiral HPLC method (DAICEL, Chiracel OB, hexane/ethanol = 7/3) after centrifugal filtration (Millipore Corp. Microcon YM-3). We observed only three HPLC peaks for the reaction mixture, indicating that oxidation was very clean: two peaks due to the sulfoxide enantiomers and one peak due to the sulfone. Blank experiments in the absence of either cytochrome *c* complex or H_2O_2 confirmed that the sulfoxide was slightly adsorbed on the filtration membrane (<5%) but was rarely oxidized (<1%). Table 1 also summarizes asymmetric oxidation profiles with three cytochrome *c* complexes, in which (*S*)-naphthyl methyl sulfoxide was more rapidly oxidized than the (*R*)-isomer. Their reaction behaviors were quantitatively analyzed in terms of the enantiomeric ratio as done in many enzymatic reactions,[16] and the ee % values at 80% conversion were calculated. The horse heart cytochrome *c* complex remarkably exhibited the highest reactivity and enantiomer-selectivity at −40 °C: for conversion after 15 h, 10% (+20 °C) < 39% (0 °C) < 68% (−20 °C) < 73% (−40 °C) > 39% (−60 °C); for ee % at 80% conversion, 18% (+20 °C) < 32% (0 °C) < 41% (−20 °C) < 47% (−40 °C) ≥ 43% (−60 °C). Methyl tolyl sulfoxide, isopropyl phenyl sulfoxide, benzyl methyl sulfoxide, and 4-methylsulfenyl acetophenone were also oxidized in an (*S*)-selective fashion: conversion after 15 h, 15−93%; ee % at 80% conversion, 8−47%. Pigeon breast cytochrome *c* complex exhibited comparable activities to those of horse heart cytochrome *c*, while the yeast complex modestly promoted the oxidation. Their conversion after 15 h/ee % values at 80% conversion for naphthyl methyl sulfoxide at −40 °C were estimated as 79%/43% for pigeon and 31%/31% for yeast. Because our Raman spectroscopic results indicated that the three cytochrome *c* complexes have similarly activated heme centers, the natures of their proteins may cause these reactivity differences. Probably, the yeast cytochrome *c* has the protein matrix which may impede the access of sulfoxides to the heme even in the absence of methionine ligand.[17] When $H_2{}^{18}O_2$ was employed,[18] >92% of the oxygen atom of the formed sulfone was labeled. Furthermore, the degradation with H_2O_2 was largely suppressed by the addition of 4 equiv of naphthyl methyl sulfoxide. Its half-life increased from 150 to 1060 min at −40 °C, although the addition of this substrate induced no UV or CD spectral change of the complex. Although the active intermediate could not be detected, these observations support a mechanism in which the oxygen atom of the reactive heme is directly added to the sulfoxide substrate as proposed in microperoxidase-catalyzed oxidations.[19] In the heme enzymes, protein compositions, external circumstances, and other factors are finely tuned to generate the sophisticated biological functions. The crown ether worked as an artificial factor activating the cytochrome *c* proteins to act as cold-active synzymes in asymmetric oxidation.

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