Enhanced *N*-Demethylase Activity of Cytochrome *c* Bound to a Phosphate-Bearing Synthetic Bilayer Membrane[1]

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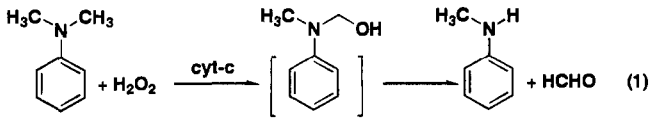
Lipid–protein interactions are probably the single most crucial factor that determines the action of protein molecules in biological membranes.[2] It has been commonly regarded that lipid bilayer membranes are passive matrices only to preserve the three-dimensional structure of membrane-bound proteins both in vivo and in reconstituted systems.[3] We recently found, however, that lipid bilayer membranes played more positive roles in the function of myoglobin molecules.[4] It is important to expand this new concept by elucidating the nature of lipid–protein interactions. We describe herein enhanced *N*-demethylase activity of cytochrome-*c* (Cyt-*c*) that is tightly bound to a phosphate-bearing synthetic bilayer membrane. Cyt-*c*, a peripheral, electron transport protein in its native form, has been recently reported to display weak peroxidase and/or P-450-like activities.[5] *N*-Demethylation is one of the typical reactions that have been examined with these biocatalysts.



(1)

The *N*-demethylase activity of Cyt-*c* (from horse heart) can be determined by monitoring the generation of the formaldehyde product according to the standard method.[6] The formation of *N*-methylaniline was also confirmed by HPLC. The reaction was initiated by addition of hydrogen peroxide to aqueous mixtures of *N*,*N*-dimethylaniline (substrate), Cyt-*c* (catalyst), and a sonicated dispersion of bilayer membrane (**1**, **2**, **3**, or **4**; Chart 1).[7]

As shown in Figure 1, the initial rate, v, of demethylation in the presence of phosphate bilayer **1** (v = 13.8 nmol/mL min) was accelerated by a factor of 10.6, relative to that in the absence of bilayers. Ammonium-bearing bilayer **2** (v = 2.0 nmol/mL min) and zwitterionic phosphatidylcholine bilayer **3** (v = 0.8 nmol/mL min) were not effective. Addition of anionic micelle-forming surfactant, sodium dodecyl sulfate (SDS), had no influence on the demethylation activity (v = 1.8 nmol/mL min). This implies that an anionic regular bilayer structure is essential for the rate enhancement.

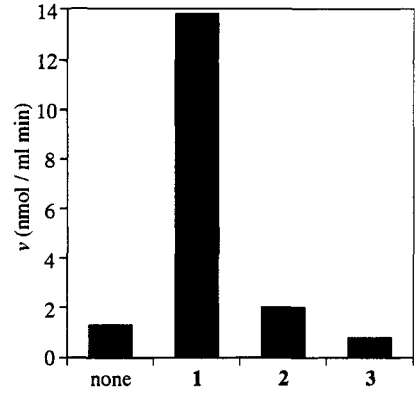
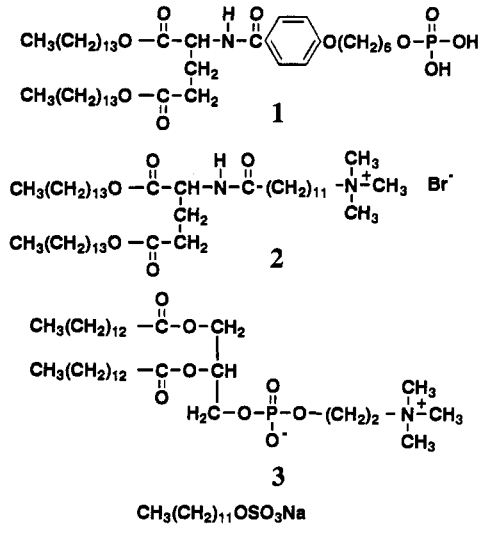


Figure 1. Dependence of the initial rate of *N*-demethylation on matrix membranes: 5 μM Cyt-*c*, 2 mM *N*,*N*-dimethylaniline, 1 mM H_2O_2, and 250 μM matrix membranes (**1**, **2**, or **3**) in 25 mM phosphate buffer (pH = 7.0) at 30 °C. The reaction was terminated after 5 min by addition of 30% trichloroacetic acid, and the amount of the formed formaldehyde was determined according to the Nash method.[6,7] The amount of product formaldehyde increased linearly with time.

Chart 1



The hemoprotein-catalyzed *N*-demethylation reaction has been reported to proceed via a ping-pong mechanism or a sequential mechanism,[8] where the hemoprotein reacts with H_2O_2 to give an activated intermediate (in some cases, hypervalent iron–oxo species), followed by oxidation of substrates. The initial rate of demethylation was rather insensitive to the concentration of *N*,*N*-dimethylaniline (inset of Figure 2). Under these conditions, the rate-determining step of the overall reaction is the formation of the active intermediate from H_2O_2 and Cyt-*c*. Double reciprocal plots against the H_2O_2 concentration gave satisfactory linear relationships (Figure 2). The kinetic parameters in the Michaelis–Menten scheme were obtained as follows: k_{cat} = 9 ± 4 min^{-1}, K_m

(1) Functional Conversion of Hemoproteins by Synthetic Bilayer Membranes. 4. Contribution No. 998 from the Department of Chemical Science and Technology.

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(7) Cytochrome *c* (from horse heart; Sigma Chemical Co., Type VI) was purified by Sephadex G-25 column chromatography before use. Powdery amphiphiles **1**-**4** were sonicated in 25 mM phosphate buffer solution (pH = 7.0). The aqueous dispersion was mixed with *N*,*N*-dimethylaniline and Cyt-*c*. The reaction was initiated by addition of H_2O_2 solution and terminated by addition of trichloroacetic acid. The resultant precipitates were filtered off, and the amount of formaldehyde formed in the filtrate was determined spectrophotometrically by a modified Nash method.[6] However, the accurate determination of formaldehyde by the Nash method was difficult at high H_2O_2 concentrations (e.g., 50 mM) because excess H_2O_2 caused suppression of discoloration. This suppression was not significant up to about 10 mM of H_2O_2.

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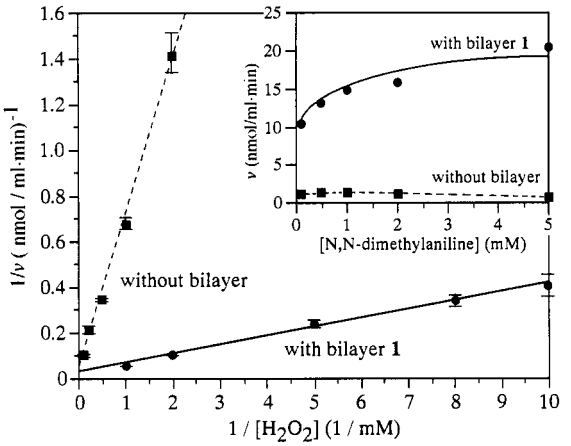

Figure 2. Double-reciprocal (Lineweaver–Burk) plots of the initial rate of *N*-demethylation reaction against H_2O_2 concentration in the absence and presence of bilayer membrane **1** (1mM): 5 μM Cyt-*c*, 2 mM *N*,*N*-dimethylaniline, 0.1–10 mM H_2O_2 in 25 mM phosphate buffer (pH = 7.0) at 30 °C. Inset: dependence of the initial rates on *N*,*N*-dimethylaniline concentration (0.1–5 mM).

= 2 ± 1 mM in the presence of **1** and k_{cat} = 12 ± 6 min^{-1}, K_m = 40 ± 20 mM in the absence of bilayers. Since the k_{cat} values are virtually identical, phosphate bilayer **1** accelerates demethylation by enhancing the affinity of H_2O_2 toward Cyt-*c*.

These membrane effects on reactivity show good correspondence to membrane affinities of Cyt-*c*. The fraction of the bilayer-bound Cyt-*c* was estimated by ultrafiltration assay[9] to be 96, 6, and 10% for bilayer membranes **1**, **2**, and **3**, respectively. Because of its high isoelectric point (pI = 10.0),[12] Cyt-*c* is positively charged under the experimental conditions and is strongly bound to an anionic bilayer membrane.

A structural change of Cyt-*c* induced by **1** was detected by UV–visible absorption and electron paramagnetic resonance (EPR) spectroscopies. The Soret band of Cyt-*c* in the oxidized form shifted from 408 (native)[13] to 406 nm by addition of **1**. A weak charge-transfer band due to bonding of sulfur of 80-Met with heme iron(III) is observed at 695 nm in native Cyt-*c*.[14] This band completely disappeared in membrane (**1**)-bound Cyt-*c*. In the reduced form of the membrane (**1**)-bound Cyt-*c*, the Soret band showed a red shift (426 from 416 nm), with a lessened molecular extinction coefficient (ε = 85 mM^{-1} cm^{-1}),[15] and the shape of the Q-band changed from characteristic peaks at 550 and 520 nm (native) to a broad one at 550 nm. The altered spectra show resemblance to those of myoglobin, in which histidine and water coordinate to the heme iron as axial ligands. The EPR pattern of membrane (**1**)-bound Cyt-*c* is different from that of low-spin native Cyt-*c* (*g* = 3.06, 2.24, and 1.24).[16] Instead, it is characteristic of high-spin iron(III) (*g* = 6.0, 2.0).[17] Absorption spectra and EPR signals of Cyt-*c* in the presence of ammonium bilayer **2** and zwitterionic bilayer **3** were almost identical with those of native Cyt-*c*. It is clear from the binding assay and the spectral measurements that membranes **2** and **3** do not specifically interact with Cyt-*c*.

The preceding kinetic and structural studies suggest that binding of Cyt-*c* to phosphate bilayer **1** induces dissociation of the axal ligand (80-Met),[18] accompanied by the spin-state change of the heme. The vacant axial site apparently facilitates coordination of H_2O_2 and its subsequent reaction with the heme center,[19] giving rise to functional conversion of bilayer-bound Cyt-*c* from an electron transport protein to demethylase. The bilayer membrane acts as an active effector to regulate the function of membrane-bound Cyt-*c*. The large variety of hemoprotein functions such as dioxygen transport and storage (hemoglobin, myoglobin), electron transport (cytochromes), and substrate oxidation (oxygenase, peroxidase and catalase) are exactly differentiated in natural systems, even though the prosthetic group is identical. Although the nature of the heme–apoprotein interaction is regarded as a major controlling factor of these functions, molecular mechanisms of such functional differentiation have not been clearly presented. The present finding suggests that specific microenviroments provided by lipid bilayer matrices facilitate the interconversion of diverse functions of hemoproteins. This aspect has not been explicitly considered in the mechanistic analysis of membrane-bound proteins.

(9) Aqueous mixtures of Cyt-*c* (10 μM) and bilayer membrane (**1**, **2**, or **3**, 0.25 mM) were filtered by using the MOLCUT II system (filter of cutoff molecular weight 100 000; Millipore Co.). Experimental details were described elsewhere.[4a,10,11]

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(15) A molecular extinction coefficient of native cytochrome *c* in the reduced form (from horse heart) was reported to be 129.1 mM^{-1} cm^{-1} (λ_{max} = 416 nm).[13]

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(17) Aqueous mixtures containing Cyt-*c* (250 nmol) and bilayer membrane (**1**, **2**, or **3**, 50 μmol) were lyophilized, and resulting powders were used for EPR measurement (JEOL JES-RE3X X-band ESR spectrometer with a liquid helium cryostat, microwave power 5 mW, frequency 9.08 GHz at 4 K).

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