CHEMISTRY LETTERS, pp. 1219-1222, 1994. © 1994 The Chemical Society of Japan

Enhanced Peroxidase Activity of Cytochrome c by Phosphate Bilayer Membrane[1)]

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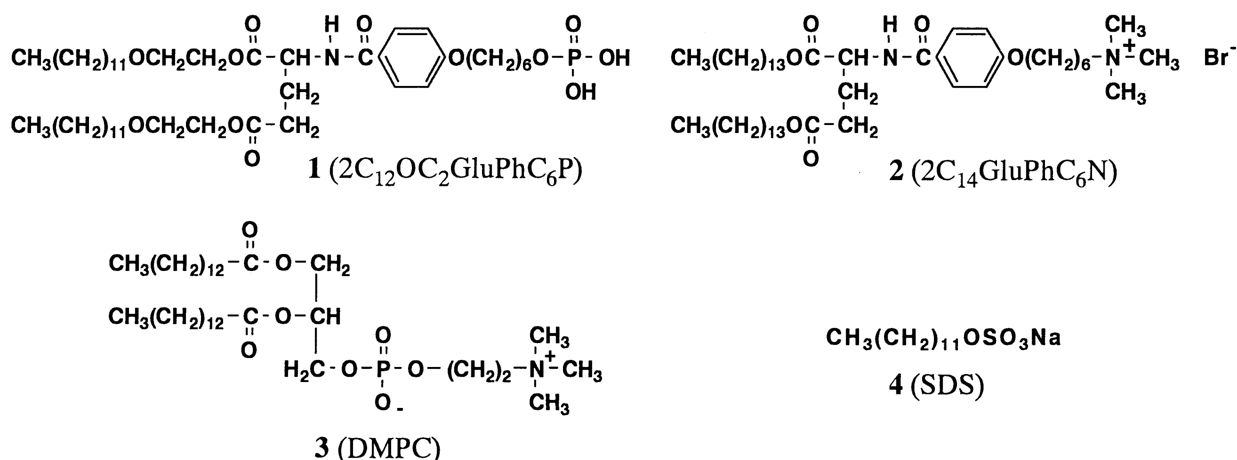
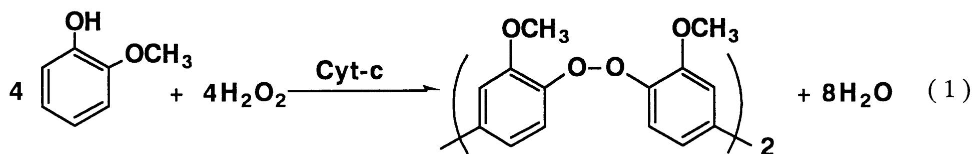
Peroxidase activity of cytochrome c (cyt-c) as measured by oxidation of o-methoxyphenol with H_2O_2, was enhanced due to binding of cyt-c to a phosphate bilayer membrane. The rate enhancement and the membrane binding were not observed with ammonium and zwitterionic bilayer membranes.

Functional modulation and conversion of naturally occurring proteins and enzymes by synthetic materials are one of the promising methodologies in protein-based bioengineering.[2)] We recently reported that functional conversion of myoglobin from a dioxygen storage protein to redox or monooxygenase-like enzymes was successfully induced by its anisotropic binding to synthetic bilayer membranes.[3,4)] Thus, lipid bilayer membranes can act as a functional modulator, rather than a simple matrix for membrane-bound proteins. In order to generalize such a novel function of lipid bilayers, we are currently extending this methodology to other proteins. Here we describe much enhanced peroxidase-activity of cytochrome c which is tightly bound to a phosphate bilayer membrane.



Native hemoproteins conduct important biochemical functions such as electron transport, dioxygen transport and storage, and dioxygen-related chemical transformations (oxygenase, peroxidase and catalase). Cytochrome c (cyt-c) is one of the electron transport hemoproteins located on the biomembrane surface.[5)] With the aim of its functional conversion to an oxidation catalyst, we examined the peroxidase activity of cyt-c in the presence of various bilayer membranes. Commercially available cyt-c (from horse heart, Sigma Chemical Co.,)

was purified through gel chromatography (Sephadex G-25). Powdery amphiphiles (**1**-**4**) were sonicated in 10 mM phosphate buffer solution (pH 7.0). The resultant aqueous dispersion was mixed with o-methoxyphenol (o-MP) and cyt-c. The reaction was initiated by addition of hydrogen peroxide solution.



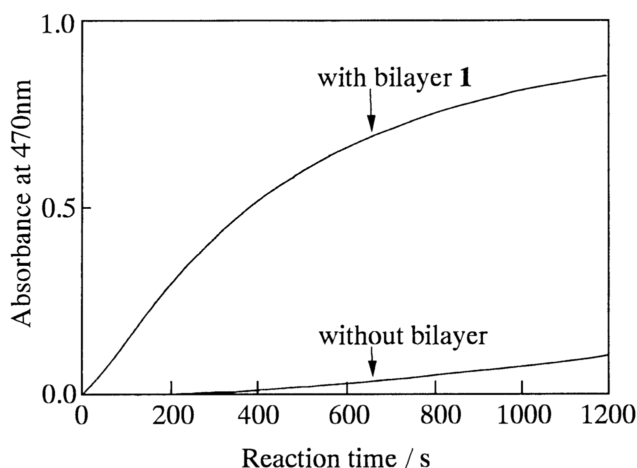


Fig. 1. Time course of tetraguaiacol formation catalyzed by cytochrome c in the absence and presence of membrane **1**. Cyt-c 5 μM, o-MP 10 mM, H_2O_2 0.5 mM, membrane **1** 0.1 mM, 10 mM phosphate buffer, pH 7.0, 30 ℃.

As shown in Eq.1, o-MP is oxidized to its tetramer (so-called tetraguaiacol) in a typical peroxidation reaction.[6] The reaction can be followed spectrophotometrically by monitoring the absorbance of product tetramer at 470 nm. Figure 1 shows time courses of the absorbance change in the presence and absence of phosphate bilayer membrane **1**. It is clear that the reaction is much accelerated by the phosphate bilayer. The overall peroxidation reaction is divided into generation of an active species of hemoprotein and the subsequent oxidation of substrates.[7] The initial rate does not increase with the o-MP concentration, and substrate inhibition is observed instead, as shown in Fig. 2a. In contrast, the rate increases with increasing H_2O_2 concentrations (Fig. 2b). These results suggest that the rate-determining step of the overall reaction is formation of an activated intermediate from H_2O_2 and cyt-c rather than

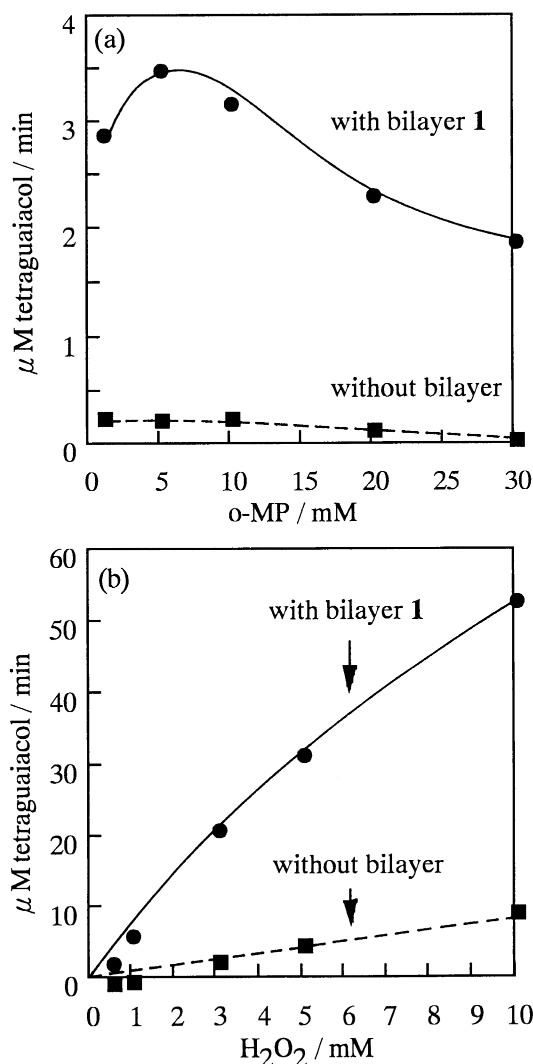


Fig. 2. (a) Dependence of the initial rate of tetraguaiacol formation on substrate concentration. Experimantal conditions are identical with those given in Fig. 1 except for o-MP concentration. (b) Dependence of the initial rate of tetraguaiacol formation on H_2O_2 concentration.

the direct oxidation step of o-MP. Thus, it is clear that the enhanced peroxidase activity of cyt-c in the presence of phosphate bilayer membrane **1**, is mainly attributed to increased reactivity of cyt-c toward H_2O_2.

Table 1 summarizes initial rates of the peroxidation reaction in the presence of various amphiphiles. Negatively-charged phosphate bilayer **1** enhances the peroxidase activity by a factor of 10, relative to that of native cyt-c. On the other hands, positively-charged ammonium bilayer **2** does not affect the rate and zwitterionic dimyristoylphosphatidylcholine **3** (DMPC) rather suppresses the reaction. The suppession may be caused by reaction of DMPC with the precursor of tetraguaiacol. Negatively-charged, micelle-forming amphiphile **4** (sodium dodecyl sulfate, SDS) does not influence the cyt-c activity. Apparently, the bilayer structure is required for the rate enhancement in addition to the negative surface charge.

Table 1. Initial rates of tetraguaiacol formation in various matrix membranes[a)]

Matrix membrane	Initial rate / μM min^{-1}	Relative activity
1	3.47	10.9
2	0.33	1.1
3	0.12	0.4
4	0.47	1.5
none	0.32	1.0

a) Experimental conditions are identical with those given in Fig. 1.

Figure 3 shows ultrafiltration binding assay of cyt-c in aqueous bilayer dispersions. When a filter membrane of cut off molecular weight of 100 000 is used, individual cyt-c molecules (M.W. 12 000) can pass through the filter but the bilayer-bound cyt-c can not.[8)] The fraction of the bilayer-bound cyt-c was 90, 10, and 13% for bilayer **1**, **2**, and **3**, respectively. Because of its high isoelectric point (pI=10.0),[10)] cyt-c is positively charged in the neutral aqueous solution. It is reasonable that the cationic cyt-c most strongly interacts with the anionic surface of bilayer membrane **1**. Undoubtedly, the membrane affinity of cyt-c is related to its enhanced reactivity.

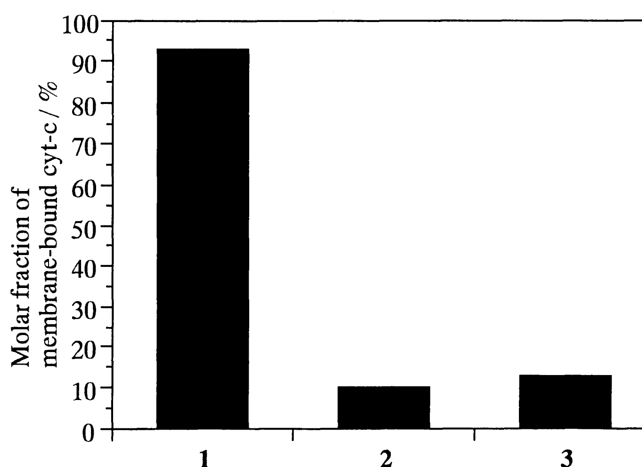


Fig. 3. Ultrafiltration binding assay of cytochrome c in various membrane dispersions. Cyt-c 5 μM, membrane 0.1 mM, 10 mM phosphate buffer, pH 7.0. Filter, MOLCUT II, cut off molecular weight 100 000.

In conclusion, the peroxidase activity of cyt-c which is low in its native form, is considerably enhanced by its binding to a phosphate bilayer membrane (Fig. 4). The non-covalent interaction provided by synthetic bilayer membranes is clearly useful for the functional conversion of originally water-soluble hemoproteins. It is known that cyt-c is bound to a negatively-charged domain of a lipid bilayer surface or to a membrane-bound protein in the natural system.[5)] The present finding implies that cyt-c, when membrane-bound, displays varied biological functions in addition to the conventional electron transport role. The metabolism of H_2O_2 as the inevitable by-product of mitochondrial respiration[11)] can

be a candidate of the hidden role of cyt-c.

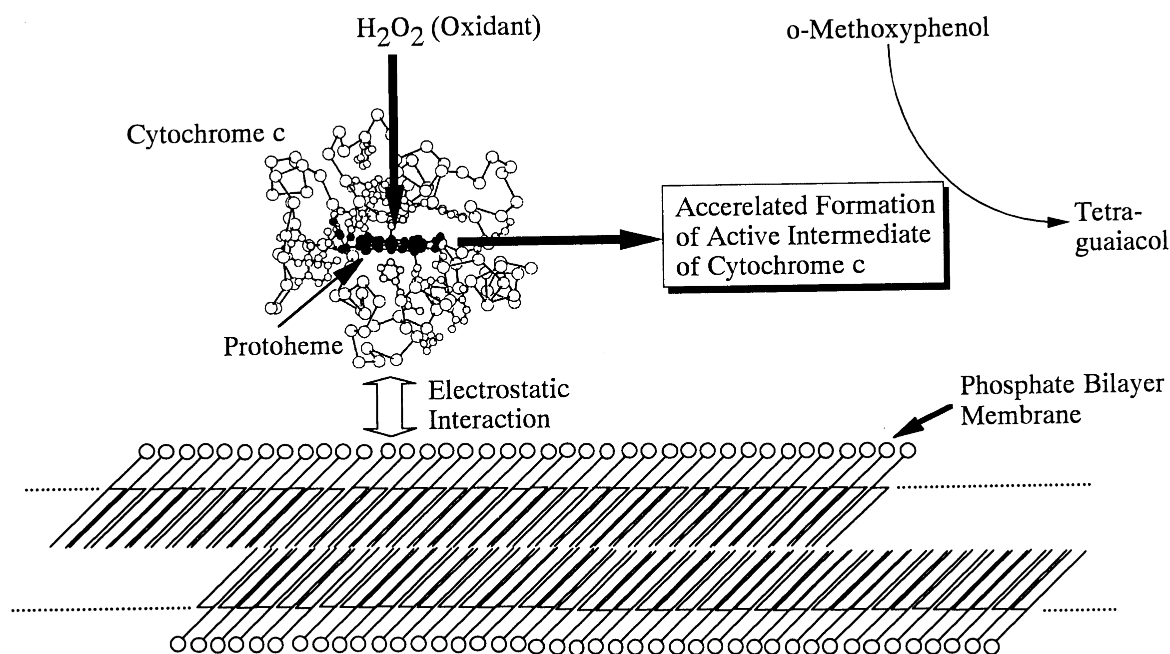


Fig. 4. Schematic illustration of cytochrome c bound on a phosphate bilayer membrane.[12)]

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(Received March 28, 1994)