# Aniline-Hydroxylase Activity of Myoglobin by Coupling with a Membrane-Bound Electron Transport System[1]

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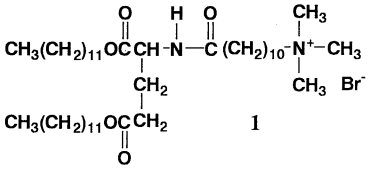
Aniline-hydroxylase activity of myoglobin (Mb) can be induced by a rationally assembled semi-artificial multienzyme system of NADH - FMN - Mb on a synthetic bilayer membrane

We describe herein that mono-oxygenase activity of myoglobin (Mb) is enhanced upon its coupling with an efficient electron transport system assembled on synthetic bilayer membranes.

Oxygenase enzymes such as cytochrome P-450 can oxidize diverse substrates by molecular oxygen under ambient conditions.[2] This activity is attractive particularly as an oxidation catalyst, and is an important target in the field of protein engineering. Recently, we reported functional conversion of Mb bound to synthetic bilayer membranes from dioxygen storage protein to redox enzyme.[3] Electron injection from NADH to Mb facilitated by the bilayer membrane, was conceivably a crucial factor for generating activated dioxygen as oxidant. This conclusion suggests an interesting possibility to employ Mb as mono-oxygenase. Thus, we examined aniline hydroxylase activity as a representative case.



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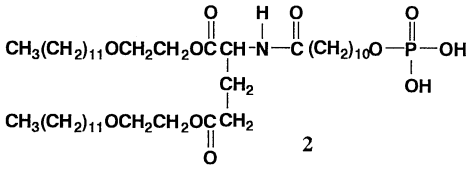

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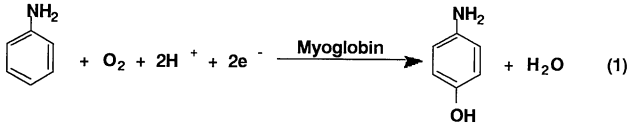
Hemoproteins require an efficient electron transport system, in order to function as mono-oxygenase enzymes.[4] Therefore, we used dihydronicotinamide adeninedinucleotide (NADH) as an electron donor and flavin mononucleotide (FMN) as an electron mediator. Matrix membranes were prepared from ammonium amphiphile **1**, phosphate amphiphile **2**, and phospholipid DMPC. The hydroxylation reaction was initiated by addition of NADH to mixed solutions of Mb (from horse heart, Sigma), FMN, aniline and a bilayer membrane. Formation of the oxidation product, p-aminophenol, was monitored spectroscopically after it was converted to indophenol according to the phenol-indophenol method.[5] During the reaction, Mb molecules gradually turned from its met-form (oxidized Mb, (FeIII)) to the oxy-form (dioxygen complex), as confirmed by UV-visible spectra.[6] Denaturation and/or degradation of Mb was not detected in absorption spectroscopy.



(1)

**Table 1.** Depencence of initial rates of the hydroxylation reaction on reaction components[a]

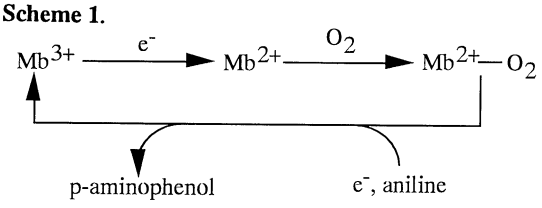
| system | relative activity (%)[b] | p-aminophenol ($\mu$M / h) |
|---|---|---|
| complete (with membrane **1**) | 100 | 27.3 |
| without membrane | 27 | 7.3 |
| without Mb | 0 | 0 |
| without FMN | 3 | 0.8 |
| without NADH | 0 | 0 |
| without $O_2$ | 0 | 0 |
| complete (with membrane **2**) | 0 | 0 |
| complete (with DMPC) | 19 | 5.3 |

[a]Standard assay mixture: 5 $\mu$M Mb, 5 $\mu$M FMN, 1mM membrane, 1mM NADH, in 25 mM phosphate buffer (pH = 7.0) at 38 °C. Reactions were initiated by addition of NADH, and terminated by trichloroacetic acid. [b]Relative activities are based on the initial rate of the complete system of membrane **1**.

Table I compares relative activities of the hydroxylation reaction for various reactant combinations. The hydroxylase activity of Mb is enhanced 4 times in the presence of ammonium bilayer **1**. In the present conditions, the turnover number of Mb was about 5.5 $h^{-1}$ based on the amount of p-aminophenol formed. The reaction does not proceed under anaerobic conditions, indicating that dioxygen is the oxygen source of p-aminophenol. It does not occur also in the absence of Mb and/or NADH (electron source). When FMN that acts electron mediator is omitted, the activity is suppressed to 3 % of that of the complete system. In the past examples of the mono-oxygenase activity of hemoproteins, flavoproteins such as cyt P-450 reductase has been used as electron mediator.[7] The present system shows that the cofactor itself (without apoprotein) act as an efficient electron mediator from NADH to Mb. This is probably caused by efficient binding of negatively-charged FMN onto the positively-charged surface of the ammonium bilayer membrane.

Table I also includes distinct effects of the matrix membranes on the hydroxylase activity. In contrast with the re-

**Scheme 1.**



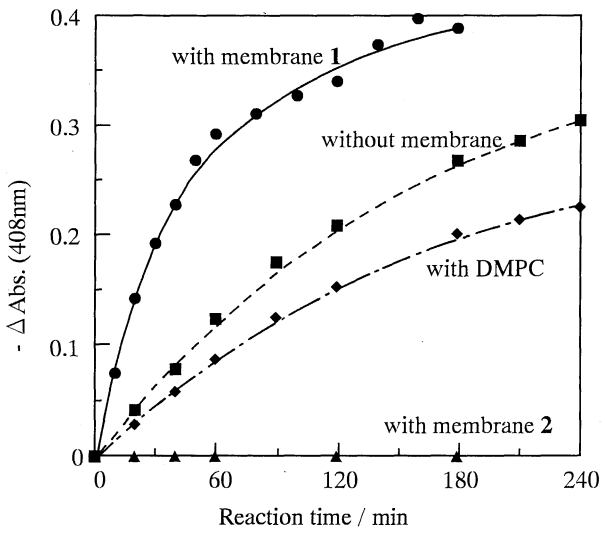



**Figure 1.** Electron injection rates from NADH to met-Mb via FMN in the presence of various matrix membranes. 5 $\mu$ M Mb, 5 $\mu$ M FMN, 1mM membrane, 250 $\mu$ M NADH, in 10 mM phosphate buffer (pH = 7.0) at 38 ℃. Reaction was monitored by the disapparence of 408-nm band.

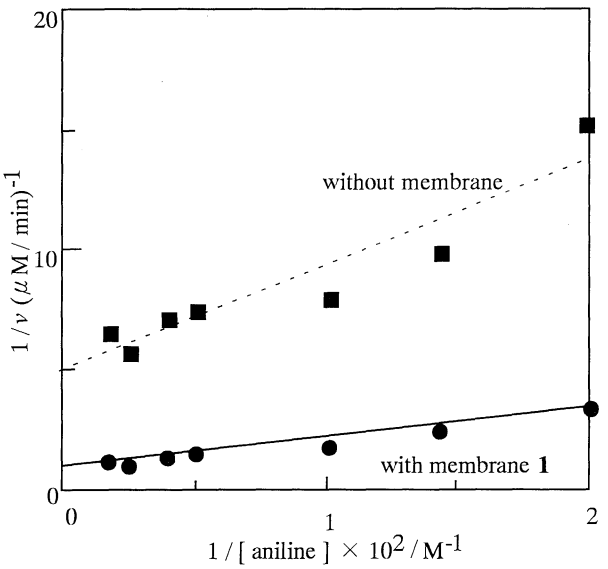



**Figure 2.** Double-reciprocal (Lineweaver - Burk) plots of the initial rates of hydroxylation reaction against aniline concentration in the absence and presence of bilayer membrane **1**. Experimental conditions are identical with those described in Table 1.

markable enhancement by ammonium bilayer **1**, a zwitterionic lipid, DMPC, partially depressed the activity and the reaction was completely suppressed by phosphate bilayer **2**. Such membrane effects show good correspondence with the electron injection rate from NADH to met-Mb in the mono-oxygenase action of Mb proposed by Mieyal (Scheme 1).[7] As shown in Figure 1, the rate is accelerated by a factor of 3.6 in the presence of membrane **1** relative to that of the homogeneous system.[8] The reduction rate slightly decreased in the presence of DMPC. No electron transfer occurred in the presence of **2**, probably due to its interaction with Mb but not with FMN and NADH, thus an efficient electron transport system not being formed on the bilayer surface. These results demonstrate that facilitated electron transport from NADH to Mb via FMN as assembled on the bilayer surface is essential for the enhanced aniline-hydroxylase activity.

The aniline hydroxylation obeys saturation kinetics with respect to the bilayer concentration. The rate saturation was also observed with respect to aniline at a constant bilayer concentration (**1**, 1 mM). Figure 2 shows double-reciprocal plots of the rates against aniline concentration (the Lineweaver-Burk plot) in the absence and presence of the ammonium bilayer membrane. Linear relationships in both cases yielded the values of apparent $k_{cat}$ and $K_m$ as follows: $k_{cat}$ (without bilayer) = 0.03 $min^{-1}$, $K_m$ (without bilayer) = 8 mM; $k_{cat}$ (with bilayer **1**) = 0.14 $min^{-1}$, $K_m$ (with bilayer **1**) = 8.4 mM.[9] The binding affinity of aniline to Mb ($1/K_m$) does not significantly change by addition of the bilayer membrane **1**. On the other hand, $k_{cat}$ is enhanced about four times with bilayer membrane **1**. It is apparent that the enhanced hydroxylase activity of the membrane-bound Mb is mainly ascribed to the incresed catalytic efficiency compared to that of native Mb. In aniline hydroxylation by microsomal cyt P-450, $k_{cat}$ values are reported to be in the range of 0.22 to 0.65 $min^{-1}$.[4,5] The activity of the present membrane-bound Mb is comparable to that of P-450.

In conclusion, NADH and FMN are successfully assembled on the surface of the cationic bilayer membrane, and act as an efficient pseudo-reductase system. The mono-oxygenase activity of Mb is efficiently enhanced. Very recently, we found enhanced peroxidase and N-demethylase activities of membrane-bound cytochrome c, where the synthetic bilayer membrane directly operates on cyt-c as an active effector.[10] Functional conversion of proteins induced by non-covalent interaction with the synthetic bilayer membrane may provide a novel approach toward protein engineering.

## References and Notes

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8 The rate in the presence of membrane **1** does not obey a simple exponential curve at the end of the reaction. This must be attributed to decreased lifetime of oxy-Mb by the interaction with membrane **1**, and/or to decomposition of oxy-Mb by active oxygen species generated by NADH and FMN.
9 This $k_{cat}$ value with bilayer **1** corresponds to a turnover number of 8.4 $h^{-1}$, which is in fair agreement with 5.5 $h^{-1}$ (= 27.3 / 5) of Table 1.
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