CHEMISTRY LETTERS, pp. 1175-1178, 1993.

Lipid Anchored Myoglobin 2. Effect of the Anchor Structure on Membrane Binding [1)]

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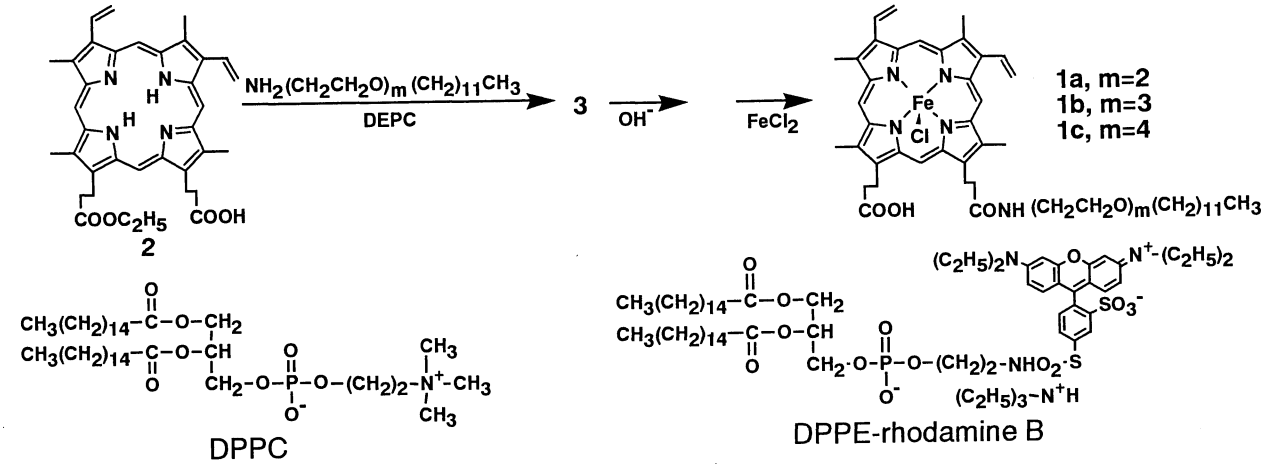
Protoheme derivatives with different anchor structures were successfull incorporated into apomyoglobin. A longer oxyethylene unit in the anchor was required for efficient, anisotropic binding of the reconstituted myoglobin onto the DPPC bilayer membrane.

We recently reported that a reconstituted myoglobin with a long alkyl chain anchor (lipid-anchored Mb) was anisotropically incorporated into a phospholipid bilayer membrane.[2)] The presence of the alkyl chain as anchor was crucial not only for enhanced membrane affinity of the protein but also for controlled orientation on the bilayer membrane. This finding suggests that a water-soluble protein can be successfully converted to a membrane-bound one by a properly designed alkyl chain anchor. In order to establish a general strategy for design and synthesis of membrane-bound proteins, we started a detailed study on the role of the anchor. We describe herein a correlation between the length of the oxyethylene unit in the anchor and the membrane affinity of lipid-anchored myoglobins.



Scheme 1.

Protoporphyrin derivatives (**1a-c**) with different oxyethylene lengths were synthesized as shown in Scheme 1. Protoporphyrin monoethyl ester **2** was condensed with appropriate amines[3] in the presence of diethyl cyanophosphate. The monoester monoamide **3** thus obtained was hydrolyzed and subjected to complexation of Fe(III) and purification with silica-gel chromatography ($CHCl_3$/MeOH = 4/6, V/V) to yield **1a-c.**[4] Apomyoglobin (apo-Mb) was prepared by extraction of protoheme from met-myoglobin (horse heart from Sigma Chem. Co.,) with 2-butanone, according to the literature.[5] To an aqueous solution of apo-Mb (0.1 mM(mmol·dm^{-3}), 8 mL) was added dropwise 1.2 equivalent of hemes (**1a-c**) dissolved in 10 % aqueous pyridine (10 mM of heme, 0.1 mL) at 0 °C. After the mixture was incubated at 4 °C for 12 h, the reconstituted Mb (**Mb(1a-c)**) was purified by centrifugation (10 000 rpm x 15 min, 4 °C), dialysis and gel filtration chromatography.

These reconstituted myoglobins (**Mb(1a)-Mb(1c)**) gave the same Soret band (408 nm) and Q-bands (503, 630 nm) as those of native myoglobin[6] in absorption spectra. The shift of these bands upon exchange of the sixth ligand from H_2O was also close to that of native Mb. : 418(418), 542(540), 572(572) nm in the azide form, and 406(404), 495(488), 606(604) nm in the fluoride form (the values in parentheses are λ_{max} of the corresponding native Mb).[6] It is clear that the lipid-anchored Mb's were successfully reconstituted from heme **1a-1c.**

Interaction of anchored Mb's with a phospholipid bilayer membrane (dipalmitoylphosphatidylcholine: DPPC) was examined by ultrafiltration binding assay and fluorescence quenching experiments. Mb molecules pass through a filter membrane of cut off molecular weight of 100 000, because of their lower molecular weights (about 18 000). Since the aggregate weight of DPPC bilayers is larger than 100 000, Mb's bound to aqueous bilayer dispersions cannot pass the filter.[7] In Fig. 1, the fraction of the bilayer-bound Mb was 53, 28, 15 and 14% for **Mb(1c)**, **Mb(1b)**, **Mb(1a)** and native Mb, respectively. It is clear that the bilayer affinity of anchored Mb's increases with the length of the oxyethylene unit.

As shown in Fig. 2, fluorescence emission from a chromophore (DPPE-rhodamine B, Molecular Probes INC.) bound to the DPPC bilayer is effectively quenched by introduction of lipid-anchored Mb.[8] Figure 3 compares the quenching efficiency of anchored Mb's and native Mb in the form of the Stern-Volmer plot. The efficiency is lessened gradually in order of **Mb(1c)**, **Mb(1b)**, **Mb(1a)** and then native Mb, that of **Mb(1c)** being 10 times greater than that of native Mb. Biphasic quenching behaviors were observed in the case of anchored Mb's. It is probable that the fast process was mainly due to quenching by membrane-bound Mb and the slow process was due to that by free Mb (See Scheme 2). These results are consistent with the

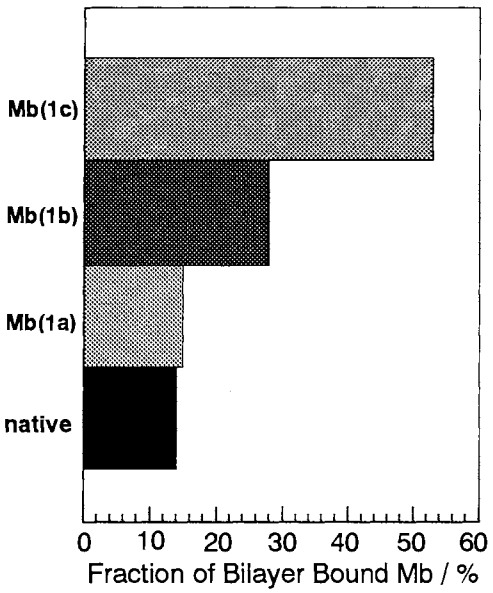


Fig.1. Ultrafiltration binding assay of anchored Mb's in aqueous DPPC dispersion. Mb 9.0 μM, DPPC 1.0 mM in phosphate buffer(pH 6.0). Flter : MOLCUT II, cut off molecular weight 100 000.

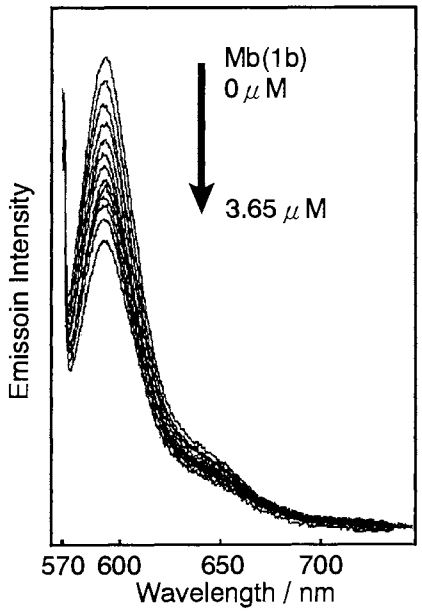


Fig.2. Quenching of emission from DPPE-rhodamine B by **Mb(1b)**. DPPC 0.1 mM, DPPE-rhodamine B 0.1 μM, **Mb(1b)** 0—3.65 μM, 10 mM phosphate buffer pH 6.0 at 20 ℃.

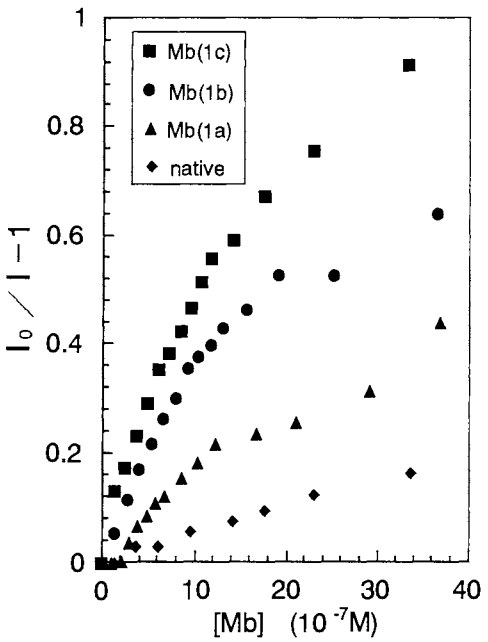
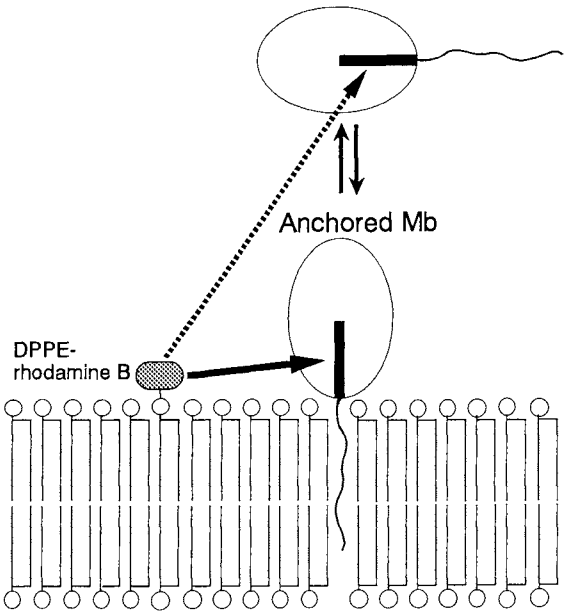


Fig.3. Stern-Volmer plots of emission quenching by anchored Mb's. I_0: emission intensity without Mb's, I: emission in the presence of Mb's.



Scheme 2. Schematic illustration of emission quenching by anchored Mb.

results of the ultrafiltration binding assay. The lipid-anchored Mb with the tetra-oxyethylene unit has the highest affinity for the DPPC bilayer membrane and quenches emission of the membrane-bound chromophore most efficiently.

Macroscopic anisotropy was not found in electron spin resonance (ESR) spectra of **Mb(1a)** and **Mb(1b)** imbedded in cast films of the DPPC bilayer (data not shown), in contrast to a strong anisotropy of the film-bound **Mb(1c)**.[2] This indicates that effective binding observed with **Mb(1c)** is related to anisotropic insertion of the anchor into the bilayer. Shorter oxyethylene units as in **Mb(1a)** and **Mb(1b)** are not sufficent for effective anchor insertion. The tetra-oxyethylene unit appears to be required to keep intact the hydration layer of Mb that are tightly bound to the DPPC bilayer.

The lipid anchor method can be applied to other cofactor-bearing enzymes and the present finding is useful to design the artificial lipid-anchor protein in general.

References

1) Contribution No. 977 from Department of Chemical Science & Technology.
2) I. Hamachi, K. Nakamura, A. Fujita, and T. Kunitake, J. Am. Chem. Soc., in press.
3) These amines (clear oil) were synthesized from the corresponding alcohols via tosyl esters by the Gabriel method.
4) A detailed synthetic procedure for **1c** was described in ref. 2. Other derivatives were prepared in similar manners, **1a**: Anal. Found: C, 63.71; H, 7.18; N, 7.33%. Calcd for $C_{50}H_{65}N_5O_5FeCl\ 2H_2O$: C, 63.67; H, 7.37; N, 7.42%. **1b**: Anal. Found: C, 65.52; H, 7.19; N, 7.13%. Calcd for $C_{52}H_{69}N_5O_6FeCl$: C, 65.64; H, 7.31; N, 7.36%.
5) F. W. Teale, Biochim. Biophy. Acta **35**, 543 (1959).
6) T. Asakura, and T. Yonetani, J. Biol. Chem., **262**, 6725 (1969).
7) Aqueous mixture of Mb and DPPC bilayer membrane were filtered by using the MOLCUT II system. The total and bilayer-bound Mb's were monitored by absorption spectra of the solution before and after filtration, respectively. I. Hamachi, S. Noda, and T. Kunitake, J. Am. Chem. Soc., **113**, 9625 (1991).
8) A DPPC bilayer dispersion containing DPPE-rhodamine B was prepared by sonication. Fluorescence quenching was conducted by addition of an appropriate amount of Mb's to the DPPC solution (excitation at 560 nm).

(Received April 9, 1993)