Functional Conversion of Myoglobin Bound to Synthetic Bilayer Membranes: From Dioxygen Storage Protein to Redox Enzyme

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Abstract: Myoglobin (Mb), a water-soluble hemoprotein, is effectively organized together with NADH and FMN coenzymes on the surface of aqueous synthetic bilayer membranes. The redox activity and the binding mode of membrane-bound Mb molecules were studied by UV-visible and ESR spectroscopies and the ultrafiltration binding assay. Mb molecules bound onto a mixed bilayer of ammonium (**1**) and phosphate (**2**) amphiphiles can efficiently accept an electron from NADH via FMN and subsequently release its electron catalytically to dioxygen and 1,2-naphthoquinone-4-sulfonate. Thus, Mb is converted from an oxygen storage protein to a redox enzyme. ESR examination of a cast film of the aqueous mixture indicates that Mb is bound to the mixed bilayer in a precision comparable to conventional membrane-bound enzymes. Implications of the present methodology were discussed in terms of functional conversion of enzymes and design of novel multienzyme systems.

Introduction

We recently found that myoglobin (Mb), a water-soluble protein was immobilized without denaturation in a cast film of a phosphate-bearing, synthetic bilayer.[1] Myoglobin molecules are incorporated in fixed orientations due to electrostatic interactions with phosphate head groups of the regular multibilayer film. This active role of the matrix membrane could undoubtedly be involved in natural membrane-bound enzyme systems. However, the natural lipid bilayer has been regarded largely as simple vehicles to provide hydrophobic environments in the past reconstitution experiment.[2] This situation was the same when artificial (synthetic) bilayer membranes were used as vehicles. For example, Ringsdorf and co-workers[3] reconstituted ATPase in a polymerized bilayer membrane of a diacetylenic amphiphile, and partially polymerized vesicles of a dienoyl phosphatidylcholine were used by O'Brien et al.[4] to incorporate rhodopsin without denaturation.

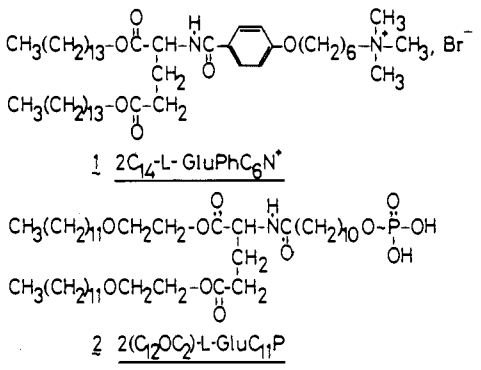
We hypothesized on the basis of our previous work that synthetic bilayer matrices would display more positive roles in the functioning of bound proteins rather than being passive binding sites. This hypothesis led to the present finding that myoglobin could act as a key enzyme in an electron transport system that was constituted on a synthetic bilayer.

Results

Design of Electron Transport System. In order for a myoglobin molecule to play a key role in an artificial electron transport system, reduction and oxidation steps have to be coupled with the myoglobin action. It is reported that metmyoglobin (met-Mb, Fe(III) form) can be reduced by NADPH under a catalytic action of cytochrome P-450 reductase.[5] Therefore, we planned reduction of myoglobin with a nicotinamide coenzyme, NADH, via flavin mononucleotide (FMN), a common prosthetic group of reductases. At the same time, molecular oxygen and naphthoquinone were adopted as electron acceptors from reduced Mb.

The orientation of myoglobin molecules is effectively regulated by a double-chain phosphate bilayer membrane.[1] The electrostatic interaction between positively charged residues on the myoglobin surface and the anionic phosphate bilayer appears to exert a predominant influence on the orientational control. In contrast, NADH and FMN are negatively charged and would be bound effectively only onto cationic bilayer surfaces. Therefore, a bilayer aggregate that includes both positive and negative surfaces appears more suitable as a site for the electron-transfer chain. We thus used a mixed bilayer membrane of **1** and **2** in addition to single-component (either ammonium or phosphate) bilayers. Both of these bilayer components are glutamate-based double-chain amphiphiles. They produce well-organized bilayer membranes, in the form of aqueous dispersions[6] and as cast films.[7]

Chart I



Electron Uptake by Mb. In the presence of a 9:1 mixed bilayer membrane of cationic and anionic amphiphiles, reduction of met-Mb (Fe(III)) to deoxy-Mb ((Fe(II)) upon injection of aqueous NADH is clearly recognized by changes in the UV-visible absorption spectrum, as shown in Figure 1. The absorption maximum at 409 nm (Soret band of met-Mb) diminished with time, and a new λ_{max} of deoxy-Mb appeared at 434 nm with three isosbestic points at 419, 464, and 510 nm.[8] The inset of Figure 1 shows the time course of the absorbance increase at 434 nm. Under the present conditions, deoxy-Mb increases linearly with time after a few minutes of an induction period, and the conversion is completed in 15 min. The reduction was not observed in the absence of FMN under otherwise the same conditions.

It is thus clear that electron transport proceeds from NADH to met-Mb via FMN. The zeroth-order kinetics are observed for appearance of deoxy-Mb. Therefore, the rate-limiting step is not the electron transfer from reduced FMN(FMNH) to met-Mb, since this step should obey the first-order or second-order rate law. Instead, rate-limiting electron flow from excess NADH to a catalytic amount of FMN can satisfy the observed zeroth order kinetics. It is conceivable that negatively charged NADH and

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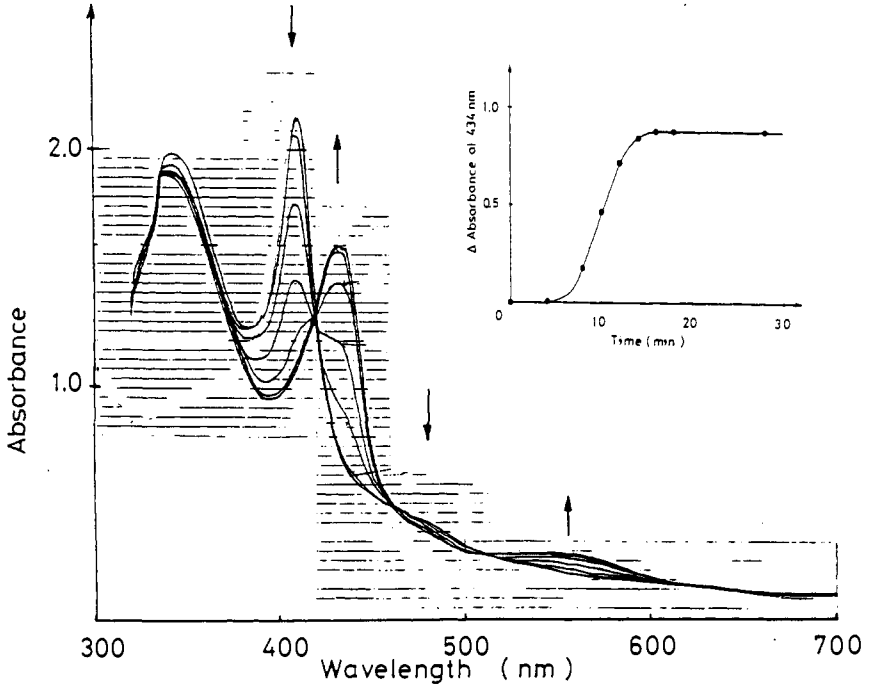

Figure 1. UV-visible spectral changes during the reduction of membrane-bound met-Mb (0.018 μmol) by NADH (0.25 μmol) in the presence of FMN (0.035 μmol) in water. Mixed bilayer dispersion, **1/2** = 9/1 (mol/mol), **1** + **2** = 2.5 μmol, pH 7.5, 30 °C, Ar atmosphere. Measurements were made every 2 min after the addition of NADH. The arrows indicate directions of the intensity change. Inset: time course of the reaction which was monitored by the absorbance increase at 434 nm.

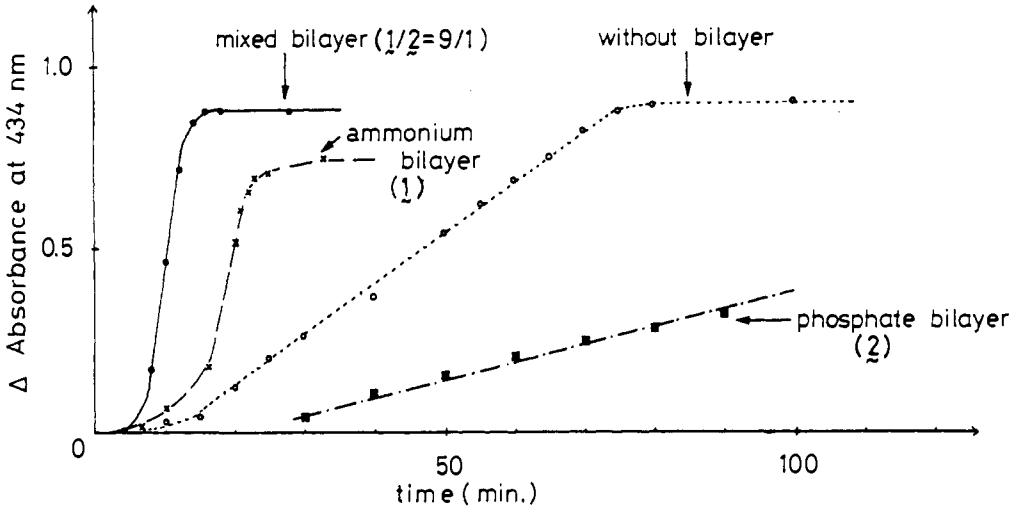

Figure 2. Time course of the met-Mb reduction by NADH and FMN on aqueous dispersions of various matrix membranes. The reaction was monitored by the absorbance at 434 nm. The reaction conditions are identical with those of Figure 1.

FMN are efficiently bound to the cationic domain of the bilayer. These coenzymes display much enhanced electron-transfer reactivities, when bound to cationic polyelectrolytes or to cationic bilayers.[9,10] NAD^+ that is formed by the electron transfer would be bound to the cationic domain less strongly, due to formation of the cationic pyridinium unit.

The reduction of met-Mb was also conducted without bilayer or in the presence of single component matrices of the ammonium or phosphate bilayers. Their time courses are summarized in Figure 2. The reduction rate in the ammonium bilayer is as fast as that in the mixed-bilayer matrix. The reduction of FMN by NADH does not proceed detectably in the absence of Mb and/or the bilayer. In contrast, the rate of the electron transport via FMN is diminished only by 90% in the absence of the bilayer relative to that in the presence of the bilayer. NADH and FMN may form aggregates with met-Mb by the electrostatic effect, with the consequent promotion of the electron flow even without the bilayer. The single-component phosphate bilayer suppresses the reaction even more. Although Mb molecules are bound to the phosphate bilayer, as inferred from the binding experiment (see below), negatively charged NADH and FMN should not be bound strongly. The absence of the simultaneous binding would explain the smaller rate observed with the phosphate bilayer.

On the basis of these results we summarize the advantages of the mixed bilayer as follows: (i) NADH and FMN are preferentially bound to the cationic domain of the mixed bilayer, and their reactivities are enhanced. Electron transfer from NADH to FMN is thus facilitated. (ii) Mb molecules are probably bound to the phosphate domain of the bilayer, where their denaturation may be avoided as was the case in the cast film.[1] (iii) Since all the reacting species are concentrated on the bilayer, FMNH formed can efficiently reduce met-Mb.

Electron Release from Mb. When oxygen gas is introduced to aqueous deoxy-Mb, oxygen-bound Mb is formed instantaneously. The oxy-Mb thus formed of course remains unchanged for many hours. The situation was quite different when Mb was bound to the mixed bilayer. As shown in Figure 3, when oxygen gas was introduced to a solution of bilayer-bound deoxy-Mb, a new ab-

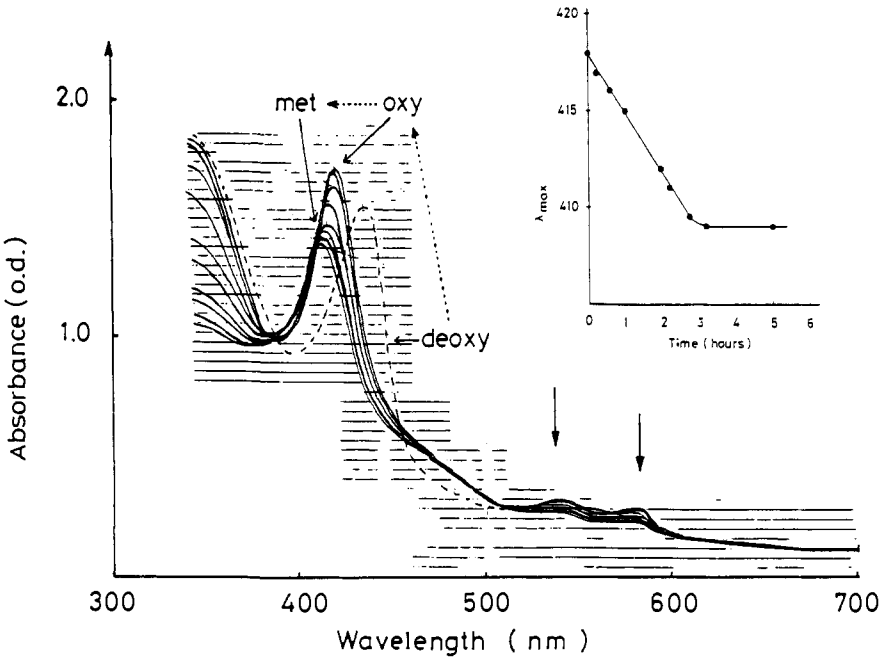

Figure 3. UV-visible spectral changes due to O_2 binding to and reoxidation of myoglobin. O_2 gas was bubbled for 30 s into aqueous deoxy-Mb that was prepared as described in Figure 1. The spectral scan was repeated every 19 min after the introduction of O_2 gas. Inset: time course of the reaction which was monitored by the shift of λ_{max}.

sorption peak of the dioxygen complex (oxy-Mb) appeared immediately (Soret band, λ_{max} 418 nm),[8] in concert with disappearance of the deoxy-Mb peak at 434 nm. Surprisingly, newly formed oxy-Mb was gradually converted to the oxidized Mb (met-Mb). This process can be followed by a λ_{max} change from 418 to 409 nm. The inset of Figure 3 displays the time course of the λ_{max} shift. The conversion was complete in 3 h. It is clear that an electron which is accepted by Mb from NADH is subsequently transferred to molecular oxygen. This electron transport process was possible only when Mb was bound to the synthetic bilayer membrane.

Extra NADH remaining in the reaction mixture is apparently consumed by $HO_2\cdot$ radical or H_2O_2 which is formed from FMNH and O_2. This is supported by depression of the 350-nm peak.

After the first cycle is over, reduction and oxidation of Mb can be repeated by adding NADH under the anaerobic conditions.

o-Naphthoquinone can oxidize deoxy-Mb.[11] Similarly, the bilayer-bound deoxy-Mb was oxidized to met-Mb within a few seconds upon injection of aqueous 1,2-naphthoquinone-4-sulfonate (NQ). Figure 4 shows the repeated redox cycle of Mb, as followed by the met-Mb absorption at 409 nm. Met-Mb was first converted to deoxy-Mb by excess NADH (0.12 mM). When the quinone was added by 1/3 of the initial NADH concentration, ca. 1/3 of met-Mb was regenerated immediately, but they were reduced again to deoxy-Mb by the remaining NADH in about 20 min. The regeneration of met-Mb would not be observed if the direct reaction occurred between the *o*-naphthoquinone and NADH.

Interestingly, regeneration of met-Mb by *o*-naphthoquinone was not quantitative, although the oxidant was added in excess and the oxidation is much faster than reduction of met-Mb by NADH (via FMN). As mentioned above, formation of FMNH is rate-limiting in the reduction process. The regenerated met-Mb may be rapidly reduced by the surrounding FMNH which has been formed by reaction with NADH. Therefore, the descending arm of the 409-nm absorbance should be composed of slow reduction of met-Mb (production of FMNH being rate-limiting) and rapid reoxidation of deoxy-Mb by NQ. The Mb molecule thus plays a catalytic role in the electron flow from NADH to NQ. This process lasts until the naphthoquinone is consumed completely.

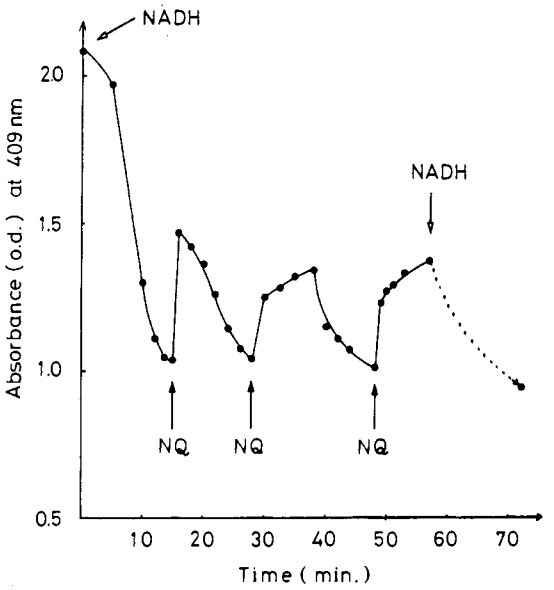

Figure 4. Repeated redox cycles of membrane-bound Mb in water, as followed by the absorbance at 409 nm. The reaction conditions of the initial reduction of met-Mb are the same as those described in Figure 1. Aqueous NADH (0.25 μmol) and aqueous sodium 1,2-naphthoquinone-4-sulfonate (0.09 μmol) were injected into the reaction mixture, as indicated by the arrows.

Deoxy-Mb thus obtained is reoxidized to met-Mb by adding another 0.04 mM of the quinone. This cycle is repeated three times, until NADH is completely consumed by the quinone. The reduction of met-Mb could be induced again by additional injection of NADH.

Binding of Mb to the Aqueous Bilayer Dispersion. The above-mentioned favorable effects of bilayer membranes can be related to binding of water-soluble Mb onto the membrane surface. The protein-membrane association was in fact supported by the ultrafiltration experiment. Mb molecules (MW 18 000) should readily pass an ultrafiltration membrane of cut-off molecular weight of 100 000. The bilayer aggregates possess molecular weights not smaller than ca. 10^6. Therefore, the bilayer and bilayer-bound Mb cannot pass the filter. Under the conditions

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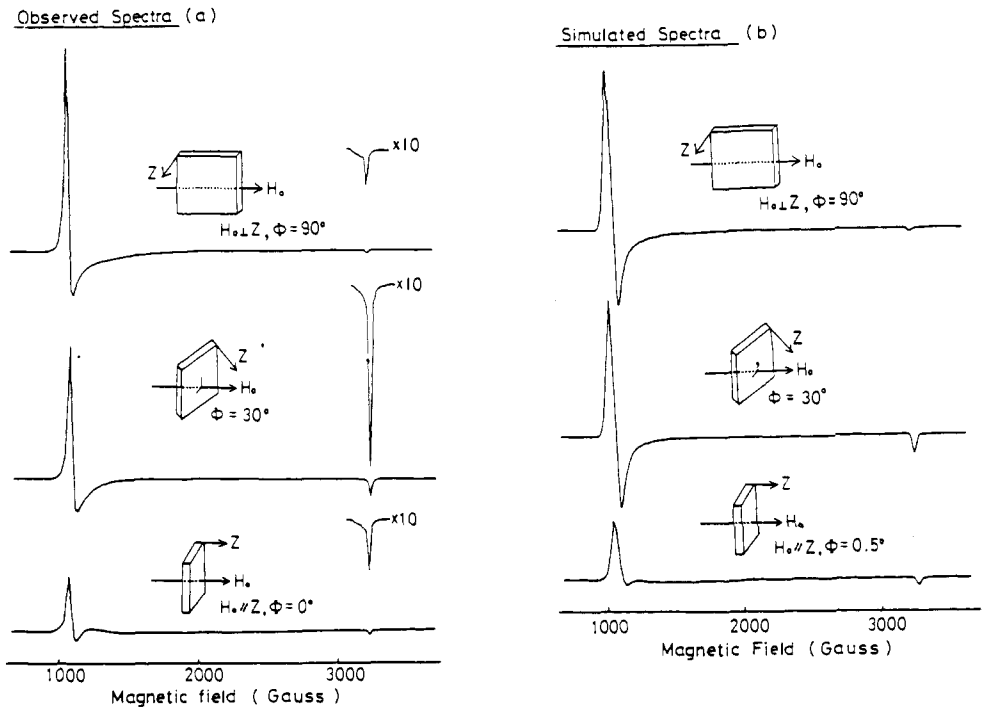

Figure 5. Observed (a) and simulated (b) ESR spectra of met-Mb immobilized in a cast film of the mixed bilayer membrane (**1/2** = 9/1, (mol/mol)). Detailed conditions are described in the Experimetnal Section.

similar to the redox experiments (Mb 8.7 μM, bilayer 1.20 mM, 5 mM TrisHCl, pH 7.5), the fraction of the bilayer-bound Mb was 59, 69, and 54% with bilayers **1**, **2**, and a 9:1 (**1/2**) mixed bilayer, respectively. A control experiment without the bilayer showed 20% retention of Mb, which is conceivably caused by adsorption. Therefore, appreciable amounts of Mb must be bound to bilayers under the reaction conditions used.

Mode of Binding of Mb onto Bilayer Matrix. Mb molecules bound onto synthetic bilayers assume certain fixed orientations, as confirmed by ESR experiments of the cast film. This cast film technique that has been used also by others for examining orientation of cytochrome oxidase, etc.[12] was applied to the present system.

Figure 5 compares observed and simulated ESR spectra of met-Mb immobilized in a cast film of the mixed bilayer. The observed g values ($g_\perp$ = 5.9, $g_\parallel$ = 2.0) at 4 K reveal that the heme unit of met-Mb maintains the ferric high-spin state even in the membrane matrix (Figure 5a).[13] Denaturation of met-Mb is not detected in the spectra, as is also the case with its absorption spectra. There is observed a macroscopic anisotropy that depends on the angle (ϕ) between the applied magnetic field and the normal of the film plane. When the film plane is parallel to the magnetic field (ϕ = 90°), a strong signal of the $g_\perp$ component appears at 1000 G, but the $g_\parallel$ signal at 3300 G is weak. As angle ϕ decreases from 90° to 0°, the $g_\parallel$ component is intensified and reaches a maximum at 30–40°. The $g_\perp$ component is drastically weakened as angle ϕ decreases from 45° to 0°. The observed angular dependence clearly indicates that the heme plane of met-Mb is oriented with high regularity at a tilt angle of 30–40° against the membrane surface. The simulated spectra are in essential agreement with the observed spectra at a tilt angle of 35° and a standard deviation of 25° (Figure 5b). The orientational specificity in the mixed-bilayer membrane is comparable to those of native membrane-bound proteins. In contrast, ESR spectra of Mb in ammonium bilayer **1** indicate a lesser extent of Mb ordering, and peaks due to denaturation are observed at g = 6.5, 4, and 3. The single component ammonium membrane is not suitable as a matrix of specific Mb binding.

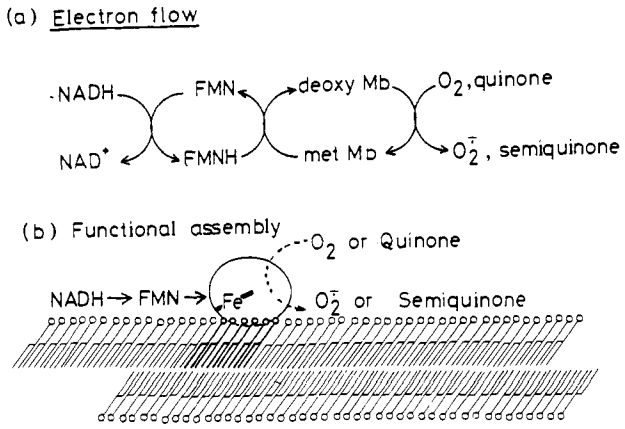

Figure 6. Electron transport chain and a schematic illustration of the functional assembly on the bilayer membrane.

Discussion

Figure 6 summarizes the electron flow that has been accomplished in this study. It proceeds from NADH to O_2 or *o*-naphthoquinone with intervening FMN and Mb. Mb molecule plays a key role as a redox enzyme in this electron transport chain. Most important findings obtained herein are as follows: (i) Oxy-Mb is readily converted to met-Mb when bound onto a synthetic bilayer membrane. The functional conversion from a dioxygen storage protein to a redox enzyme may be attributed to changes in the relative redox potential of oxy-Mb and met-Mb or to a microenvironmental effect of the synthetic bilayer which stabilizes the transition state of the electron transfer. It has been reported that the redox potential of cytochrome *c* shifts by −60 mV upon binding to the mitochondrial membrane.[14] The controlled orientation of Mb at the bilayer surface would have important bearings on the reactivity change of Mb. (ii) Mb, NADH, and FMN molecules are efficiently concentrated on the mixed bilayer membrane. This effect must have led to formation of the

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effective electron transport chain. FMN acts as an efficient converter between the one-electron and two-electron transfer systems.

Recently, functional conversion of proteins has been actively studied. For example, Sligar and co-workers altered the heme axial ligand of cytochrome b_5 from histidine to methionine by site-directed mutagenesis, with the consequent functional change from a redox enzyme to demethylase of amine.[15] Gray et al. displaced the axial ligand of cytochrome *c* from methionine to histidine by a semisynthetic method and produced a remarkable shift of the redox potential.[16] It is reported by Mieyal and co-workers that hemoglobin showed a monooxygenase activity by combination with cytochrome P-450 reductase.[17] Hayaishi and co-workers also found that indoledioxygenase act as a monooxygenase in the presence of P-450 reductase.[18] Kaiser et al. prepared various semisynthetic enzymes in which designed cofactors were covalently bound to specific sites and displayed novel functions (flavopapain, flavohemoglobin).[19]

These methodologies depend on modifications of the protein primary structure, on combinations of biologically irrelevant enzymes, or on introduction of nonnatural functional units into natural proteins. The present approach is unique in that noncovalent association is used. The synthetic bilayer can provide highly anisotropic binding sites with controlled microenvironments. It is important that the noncovalent association affect the reactivity of a protein that is not membrane-bound in the biological system. We can envisage novel semiartificial systems which are composed of natural or modified water-soluble proteins and synthetic bilayer membranes. As for the natural membrane-bound proteins, the relevance of the biological membrane on the protein action has not been discussed in molecular terms. Examination of the protein action in the synthetic bilayer system may help define the microenvironmental role of the biological membrane. On the practical side, designed construction of multienzymatic systems will lead to sophisticated bioreactors.

Experimental Section

Materials. Metmyoglobin from horse heart was purchased from Sigma Chemical Co. and used without further purification. Dihydronicotinamide adenine dinucleotide (NADH) and flavin mononucleotide (FMN) were purchased from Wako Pure Chem. Amphiphile **1**, mp 60–210 °C (liquid crystal), was prepared according to the procedure of Y. Ishikawa et al.[20]

Bis(3-oxapentadecyl)-L-glutamate Hydrochloride. 3-Oxapentadecan-1-ol (Nikko Chemicals, 13.1 g, 0.057 mol), L-glutamic acid (3.8 g, 0.026 mol), and *p*-toluenesulfonic acid monohydrate (9.5 g, 0.05 mol) were suspended in 350 mL of toluene and refluxed for 7 h with a Dean-Stark trap. After evaporating toluene at room temperature, the pale yellow residual oil was dissolved in 300 mL of chloroform and washed with aqueous K_2CO_3 (pH 11, 200 mL × 2) and with saturated aqueous NaCl (100 mL × 1). The chloroform layer was dried over anhydrous $MgSO_4$, and solvent was removed. Colorless precipitates were formed from the colorless oily residue dissolved in 200 mL of acetone by addition of 35% hydrochloric acid (2.7 mL) on ice cooling. Recrystallization from acetone gave colorless crystals, mp 84–85 °C, yield 5.6 g (36%): IR (KBr, cm^{-1}) $\nu_{C=O}$ 1760, ν_{C-O} 1130.

***O,O*-Bis(3-oxapentadecyl)-*N*-(11-hydroxyundecanoyl)-L-glutamate.** The *O,O*-disubstituted glutamate hydrochloride (3.5 g, 5.8 mmol) and triethylamine (0.7 g, 6.9 mmol) were dissolved in 50 mL of dry (refluxed over NaH) THF and stirred at room temperature for 20 min. The precipitates of triethylamine hydrochloride were removed, and 11-hydroxyundecanoic acid (1.3 g, 6.3 mmol) and diethyl phosphocyanide (Aldrich 90%, 1.5 g, 9.0 mmol) were added with ice cooling. After stirring for 72 h at room temperature, the solvent was removed. Recrystallization of the residual solid from acetone gave colorless powder in 48% (2.1 g) yield: mp 66.5–67 °C, TLC (silica gel, $CHCl_3$) R_f 0.15; IR (KBr, cm^{-1}) ν_{O-H} 3560, ν_{N-H} 3320, $\nu_{C=O}$ 1655, ν_{C-O} 1130; ^1H NMR (60 MHz, $CDCl_3$) δ 0.88 (t, 6 H, CH_3), 1.27 (m, 58 H, CCH_2C), 2.31 (m, 4 H, $COCH_2$), 3.49 (m, 10 H, CH_2O–), 4.25 (m, 4 H, CH_2OCO), 4.85 (m, 1 H, NCHCO), 6.48 (d, 1 H, NH).

***O,O*-Bis(3-oxapentadecyl)-*N*-(11-phosphoroxyundecanoyl)-L-glutamate.** The preceding product (2.0 g, 2.7 mmol) and triethylamine (0.8 g, 8.1 mmol) were dissolved in 50 mL of dry THF. This solution was added dropwise to 4.1 g (27 mmol) of phosphorus oxychloride (distilled from Na wire) in 10 mL of dry THF in 2.5 h with ice cooling under vigorous stirring. After stirring for additional 3.5 h, precipitates of triethylamine hydrochloride were removed, and 20 mL of distilled water was added with ice cooling. The pH of the solution was about 2. The solution was extracted with 100 mL of chloroform twice and the combined chloroform layer was dried over anhydrous Na_2SO_4. The solvent was evaporated, and the colorless residue was recrystallized from acetone three times to give a colorless powder of **2**: yield 1.2 g (54%); mp 59–59.5 °C; IR (cm^{-1}, KBr) ν_{O-H} 3300, $\nu_{C=O}$ 1740, ν_{C-O} 1655, ν_{N-H} 1540, $\nu_{P=O}$ 1200, ν_{C-O} 1135; ^1H NMR (60 MHz, $CDCl_3$) δ 0.88 (t, 6 H, CH_3), 1.26 (m, 58 H, CCH_2C), 2.31 (m, 4 H, $COCH_2$), 3.49 (m, 8 H, CH_2O–), 4.24 (m, 6 H, CH_2OCO), 4.85 (m, 1 H, NCHCO), 6.48 (d, 1 H, NH). Anal. Calcd for $C_{44}H_{86}NO_{11}P$: C, 63.20; H, 10.36; N, 1.67. Found: C, 63.00; H, 10.31; N, 1.71.

Reactions of Membrane-Bound Myoglobin. Ammonium amphiphile **1** (79 mg, 0.09 mmol) and phosphate amphiphile **2** (8 mg, 0.01 mmol) were dissolved in 50 μL of 0.2 M aqueous Tris, and 4 mL of Tris-HCl buffer (5 mM, pH 7.5) was added. The mixture was sonicated at 50–60 °C for 5 min with a Bransonic Cell disrupter 185 (sonic power 20–30). This stock solution was diluted 20 times with 5 mM Tris-HCl buffer to the final membrane concentration of 1.25 mM. Aqueous FMN (20 μL, 0.035 μmol) and 50 μL of aqueous met-Mb (0.018 μmol) were added to 2.0 mL of the diluted bilayer dispersion in a 10-mm quartz cell equipped with a three-way stopcock. The mixture was carefully deaerated by evacuation and introduction of argon at room temperature. Reduction of met-Mb was started by injection of 0.10 mL of aqueous NADH (0.25 μmol) through a syringe at 30 °C.

Oxidation of the resulting deoxy-Mb was performed by bubbling of oxygen gas into the reaction mixture for 30 s or by injection of aqueous sodium 1,2-naphthoquinone-4-sulfonate (Tokyo Kasei Co., 0.09 μmol) through a syringe. The redox behavior of Mb was followed by repeated scanning of the absorption spectra.

In the case of the single component phosphate bilayer, amphiphile **2** (84 mg, 0.1 mmol) was neutralized with 500 μL of 0.2 M Tris and diluted to 4 mL with Tris-HCl buffer (5 mM, pH 7.5). In the case of the ammonium bilayer, amphiphile **1** (88 mg, 0.1 mmol) was suspended in 4 mL Tris-HCl buffer (5 mM, pH 7.5). The subsequent procedures were the same as those for the mixed bilayer membrane.

Ultrafiltration of Aqueous Mixture of Mb and Bilayer. To 2 mL of an aqueous mixed bilayer (see above, mixed bilayer, 1.25 mM, 5 mM Tris-HCl, pH 7.5) was added 50 μL of aqueous met-Mb (0.018 μmol). The solution (1.0 mL) was filtered using a Milipore system (MOLCUT II, UFP1 THK24, cut-off molecular weight 100000). The concentration of Mb in the filtrate was determined by the absorbance at 409 nm (Soret band of met-Mb, ϵ = 160000).[21] The membrane-bound Mb was estimated by comparing Mb concentrations before and after ultrafiltration.

Preparation of Mb Bound Cast Films. To 2 mL of an aqueous bilayer dispersion (see above) (bilayer, 25 mM, 5 mM Tris-HCl, pH 7.5) was added 5 mg of met-Mb in 0.2 mL of a 5 mM Tris buffer, and the mixture (Mb, 1.4 mM) was gently shaken at room temperature. The resulting solution was spread on flat Teflon sheets (Sumitomo Electric, Fluoropore FP-010, pore size 0.1 μm). Water was allowed to evaporate to dryness at 20–25 °C over a few days. Cast films of less than 0.1-mm thickness were obtained. These films were self-supporting and could be peeled off from the Teflon sheet.

ESR Measurement. ESR spectra of the cast films were measured at 4 K with a JEOL JES-RE2X X-band spectrometer equipped with a liquid helium cryostat with 100-kHz magnetic field modulation, 5 mW microwave power, and 0.79 mT modulation amplitude. A quartz rod (2 mm × 20 mm) with a flattened end (4 mm width × 30 mm length) was used as a sample holder. A cast film was cut into about eight pieces (3 mm × 20 mm), which were stacked and glued to the flat portion of the rod by silicon grease. The ESR sample holder was placed in a quartz tube (5 mm × 270 mm) and sealed with a Sealon film (Fuji Film). The sealed sample was inserted in the cavity of the ESR spectrometer and rotated to observe magnetic anisotropy of the sample. ESR spectral simulation was conducted by using a computer program developed for Cu(II) chelates by Y. Ishikawa which is based on the method of Blum et al.[22] Met-Mb contains a high-spin Fe(III) ion with five unpaired

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electrons ($S = {}^5/_2$). Only the transition between the lowest energies (M_s $+{}^1/_2 \rightarrow -{}^1/_2$) is measurable by ESR.[23] Since it is assumed that $g_x = g_y = g_\perp$ and $g_z = g_\parallel$, the simulation program is essentially the same as that used for Cu(II) porphyrins.[20] The superhyperfine interaction can be neglected, as the nuclear spin of iron is zero and the superhyperfine splitting with the ligand nitrogen atom is not observed. ϕ represents the tilt angle between the Z axis of the cast film and $g_\parallel$ with a standard deviation of σ. The spectral line shape was assumed to be Gaussian, and the spectral intensity was calculated from 600 to 3600 G at every 5 G.

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Photocyclization of α-(*o*-Tolyl)acetophenones: Triplet and 1,5-Biradical Reactivity

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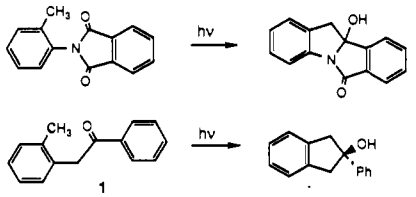
Abstract: Several ring-substituted α-(*o*-tolyl)acetophenones undergo photocyclization to 2-indanol derivatives in high quantum efficiency in solution and in high chemical yield as solids. The mechanism for reaction involves triplet state δ-hydrogen atom abstraction that generates 1,5-biradicals. Quenching studies indicate that the n,π* excited triplets of these ketones react, with rate constants $>10^8$ s^{-1}. Variations in triplet reactivity are ascribed to conformational equilibria that populate reactive and unreactive geometries to different extents. The α-aryl ring eclipses the carbonyl in the lowest energy geometry, from which the most favorable geometry for reaction can be reached by small bond rotations. α-(2,4,6-Triisopropylphenyl)acetophenone forms the relatively long lived enol as well as indanol in solvent-dependent ratios; deuterium labeling indicates that the 1,5-biradical disproportionates to form enol. This does not happen with α-mesitylacetophenone, so its 54% cyclization quantum efficiency is ascribed to an internal triplet quenching that competes with hydrogen abstraction. This internal quenching is presumed to be of the charge-transfer type and does not appear to lead directly to 1,5-biradicals. 1-Methyl-2-phenyl-2-indanol is formed from α-(*o*-ethylphenyl)acetophenone with a Z/E ratio of 20:1 in benzene and 2:1 in methanol. The 1,5-biradical intermediates were characterized by flash spectroscopy; they have lifetimes between 15 and 45 ns, with those derived from α-(*o*-isopropylphenyl) ketones being twice as long-lived as those derived from α-(*o*-methylphenyl) ketones, and show only a small solvent dependence. Biradical lifetimes and the diastereoselectivity of cyclization are interpreted in terms of biradical intersystem crossing occurring preferentially along the reaction coordinate for cyclization, such that the two processes effectively occur concurrently. The applicability of this concept to other biradicals is discussed.

For the past few years we have been conducting a systematic study of δ-hydrogen abstraction by triplet ketones and the competing reactions of the intermediate 1,5-biradicals.[1] Our studies began with a reinvestigation of the photocyclization of *o*-alkoxyphenyl ketones,[2,3] which we recently published in full.[4] A study of *o*-*tert*-butylbenzophenone[5] confirmed hints in the literature[6,7] that its triplet is highly reactive. Kanaoka's report that *N*-(*o*-tolyl)phthalimide undergoes photocyclization[8] prompted us to investigate α-(*o*-tolyl)acetophenone (**1**). We were delighted to discover that it and simple derivatives undergo highly efficient photocyclization to 2-indanols.[9]



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This paper reports in full our studies on the mechanism of this new photocyclization reaction, including flash spectroscopic characterization of the 1,5-biradical intermediates. The following paper[10] presents the much more complicated photochemistry that α-substituted α-(*o*-tolyl)acetophenones undergo.

Results

Product Identification. The ketones listed in Table I were all synthesized by standard procedures, as described in the Experimental Section. They all undergo clean photocyclization to 2-phenyl-2-indanols when irradiated at 313 or 365 nm or with the output of a medium-pressure mercury arc lamp filtered only by Pyrex. In all but one case the chemical yields were within experimental error of 100%. The exception is α-(2,4,6-triisopropylphenyl)acetophenone (**7**), which gives varying amounts of its enol as well. This fact was first established by irradiating a dioxane-d_8 solution of **7** with 350-nm Rayonet lamps and following the reaction by NMR. At high conversion, two separate signals for vinyl protons at 6.10 and 5.99 ppm, in a 4:1 ratio, indicated the formation of Z and E enols as the major products.[11,12] Integration of the vinyl signals and those of the indanol methyls indicated a 15:1 enol/indanol ratio. After one day, the E enol had disappeared; its NMR signals were replaced with those of the starting ketone. The Z enol was quite persistent and required treatment with acid or base to be tautomerized to the starting ketone. A portion of the unacidified solution of Z enol was

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