Reduction of Methemoglobin via Electron Transfer across the Bilayer Membrane of Hb Vesicles

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The hemoglobin vesicle (HbV) has a cellular structure which encapsulates concentrated Hb in the inner aqueous phase of a phospholipid bilayer vesicle. Hb is gradually autoxidized to methemoglobin (metHb), which can not bind oxygen during oxygen transport under physiological conditions. In order to reduce metHb in HbV, we evaluated the reduction of metHb by electron transfer across the bilayer membrane of HbV from a reductant added to the outer aqueous phase. Water-soluble methylene blue (MB) and hydrophobic ubiquinone 10 (UQ) were selected as electron mediators. Under a nitrogen atmosphere, the addition of the reduced form nicotinamide-adenine dinucleotide (NADH) to the outer aqueous phase of UQ-incorporated HbV showed only a slow reduction rate for metHb. On the other hand, when MB and NADH were added under a nitrogen atmosphere to HbV containing 40% metHb, a rapid decrease in the metHb percentage was observed. The entire reaction was controlled by a reaction with NADH and MB in the outer aqueous phase. Under aerobic conditions, the decrease in the efficiency of the metHb reduction and rapid oxidation after reaching the minimal metHb percentage were observed. This was confirmed to be due to the influence of hydrogen peroxide; the decrease was prevented by the co-encapsulation of catalase.

The hemoglobin (Hb) vesicle (HbV) encapsulates a concentrated Hb solution with a phospholipid bilayer membrane, which has been paid much attention concerning use as a red-cell substitute.[1] Recently, an oxygen-transporting ability comparable to that of a red blood cell (RBC) was confirmed in 90% exchanged transfusion tests using rats.[2] However, one of the remaining issues for clinical application is a relatively rapid oxidation of the hemes of Hb: the formation of methemoglobin (metHb), which can not bind oxygen. In order to solve this problem, we studied the co-encapsulation of reductants and enzymes, such as thiols, catalase, and superoxide dismutase in the inner aqueous phase of the vesicles.[3] As a result, the rate of metHb formation during blood circulation was reduced to one-third compared with HbV having no reductant. Because the amount of metHb to be reduced by the reductants is low during the initial stage, the reductant reacts with oxygen to lose its activity for metHb reduction, and produces active oxygen species. Therefore, the durability of the reductant should be short, considering the blood circulation time. The reductant has to be introduced or activated when the metHb percentage becomes high. The problem is whether or not the reduction of metHb that exists in the inner aqueous phase of HbV can be achieved by the reductant added to the outer aqueous phase, because there is a bilayer membrane which behaves like a barrier for the reductant.

Such electron-transfer reactions across the bilayer membrane take place in natural systems, for instance, the photosynthetic system in plants, the respiratory chain in mitochondria, ion-channels and so on.[4] In addition, model studies of such systems have been carried out by using ubiquinones,[5] cytochrome,[6] dyes,[7] and synthetic lipids.[8] The experiments are usually carried out under anaerobic conditions so as to prevent the autoxidation of reductants or electron mediators, and the active oxygen species generated through the autoxidation and participating in the metHb formation. The autoxidation, metHb formation and reduction depend upon the properties of the electron mediators and reductants, especially, their location: the outer or inner aqueous phase, the surface or the hydrophobic phases of the membrane. Because HbV acts as a red-cell substitute, which carries oxygen under physiological conditions, oxygen should exist in the system. Therefore, it is necessary to achieve a reduction of metHb via an electron-transfer reaction across the bilayer membrane under aerobic conditions.

Both the reductant and electron-mediator should be safe for intravenous administration and use in the medical field. Based on these points, methylene blue (MB) and ubiquinone 10 (UQ) were selected as mediators. MB is commercially available and has been used in the therapy of methemoglobinemia for a long time.[9] Because it is soluble in both organic solvents and water, it can be added to the outer aqueous phase to attain electron transfer across a bilayer membrane.[10] In contrast, UQ is a well-known electron mediator, and since it is insoluble in water, it can be incorporated into the hydrophobic region of the membrane. On the other hand, NADH was employed as a reductant because of its excellent biocompatibility and appropriate redox potential (−0.32 V vs. NHE).

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In this study, metHb in HbV was reduced by the co-addition of NADH and MB to the outer aqueous phase; the permeation of MBH resulted from the reduction of MB with NADH through the bilayer membrane. In addition, the reduction of metHb under aerobic conditions was studied based on the kinetics of these reactions.

Experimental

Materials. The lipid bilayer comprised Presome PPG-I (a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG) with a 5/5/1 molar ratio, Nippon Fine Chem. Co., Ltd.). MB and UQ were purchased form Kanto Chem. Co., Inc. and were recrystallized twice from water/ethanol. Nicotinamide-adenine dinucleotide (reduced form) (NADH) was purchased from Yeast Co., Ltd., and was used as received. A 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered solution (5×10^{-3} M, 300mOsm, adjusted with NaCl at pH 7.4, 37 °C) was used as the standard buffer solution (1 M = 1 mol dm^{-3}).

CarbonylHb (HbCO, 6.2 mM) was prepared according to a previous paper,[11] and pyridoxal 5′-phosphate (PLP, 18.6 mM, Merck) was added to the solution as an allosteric effector. Lastly, 0.3 M Na_2CO_3 was then added to adjust the pH value to 7.4 at 37 °C.

Preparation of HbV.[12] The thin lipid film of PPG-I was cast on the wall of a round-bottom flask from a chloroform solution. After completely removing the solvent in vacuo, a HbCO solution was added to it. The dispersion obtained by rotating it for 3 h was applied to an Extruder (Lipex Bio. Inc.),[13] and then permeated stepwise through membrane filters having a pore size from 3.0 to 0.22 μmϕ (FM type microfilters, Fuji Film Co., Ltd.) using nitrogen gas pressure (10 kgf cm^{-2}). After three permeations through the 0.22 μm pore size filter, the vesicle size was measured using a dynamic light-scattering method (Coulter N4SD) and clarified to 0.27±0.05 μm diameter. Untrapped Hb was removed by ultracentrifugation (50000×g, 30 min, Beckmann Co., Ltd.) as a supernatant. A reddish precipitate was redispersed in the buffered solution. Such a washing procedure was repeated three times to yield HbV ([Hb]/[Lipid] = 1.6 by weight).

The conversion of HbCO to oxyHb was performed by visible-light irradiation (400 W, halogen lamp) under an oxygen flow for 5 min to the HbV ([Hb] = 0.5 g dL^{-1}) suspension rotated using a rotary evaporator. Complete exchange was confirmed by the disappearance of the shoulder of the Soret band at 419 nm and Q band at 540 nm under a nitrogen atmosphere.

HbV ([Hb] = 10 g dL^{-1}) was incubated at 37 °C for 48 h to make HbV with a 40% metHb level. After this was diluted by a buffer solution, a reduction experiment was carried out. Deoxygenation was performed by nitrogen bubbling (50 mL min^{-1}, 5 min), and the remaining oxygen could be estimated at 4.0×10^{-6} M from the triplet lifetime of 5,10,15,20-tetrakis(1-methyl-4-pyridinio)-porphyrinatozinc tetrachloride (10^{-6} M, 500 μs).[14]

Determination of the Concentrations of Hb and Phospholipid. The concentrations of phospholipid and Hb were determined by Allen's method[15] and a cyanometHb method, respectively. In the case of HbV, because the membrane of the vesicle could not be completely destroyed by Triton® X-100 used as a membrane-destroying agent for an Hb concentration measurement, the Hb concentration was determined after heating and agitating at 60 °C for 15 min. All of the UV-visible spectra were recorded using spectrophotometer (MPS-2000, Shimadzu Co., Ltd.).

Determination of the MetHb Percentage of HbV. The metHb percentage of HbV could not be determined by a conventional cyanometHb method, because the vesicle structure could not be completely destroyed by Triton® X-100. Subsequent heating for complete destruction denatures Hb and changes the metHb percentage. Therefore, the absorbance ratio of the Soret bands of metHb (405 nm) and deoxyHb (430 nm) of HbV was used for the measurement.

HbV with 100% metHb was prepared by the following procedure: Through an HbV suspension was bubbled nitrogen for 20 min to remove all oxygen. Subsequently, an excess amount of nitrogen monoxide was added to this suspension to convert deoxyHb to HbNO in the vesicle. After a 20 min incubation for complete conversion, excess nitrogen monoxide was removed by a nitrogen flow for more than 30 min. Oxygen was purged into the suspension, and HbNO was converted to metHb in the vesicle after 12 h. HbV with 100% deoxyHb was prepared by the addition of sodium dithionate under a nitrogen atmosphere. Sodium dithionate can penetrate through the bilayer membrane of HbV and completely reduce metHb.

Mixtures of HbV with 100% metHb and HbV with 100% deoxyHb were used to determine the calibration constants of the metHb percentage. The concentrations of metHb and deoxyHb were obtained using Eqs. 1 and 2, and the metHb percentage was determined using Eq. 3,[16]

$$[\text{metHb}] = \frac{\varepsilon^{\text{d}}_{430}A_{405} - \varepsilon^{\text{d}}_{405}A_{430}}{\varepsilon^{\text{d}}_{430}\varepsilon^{\text{m}}_{405} - \varepsilon^{\text{m}}_{430}\varepsilon^{\text{d}}_{405}} \quad (1)$$

$$[\text{deoxyHb}] = \frac{\varepsilon^{\text{m}}_{430}A_{405} - \varepsilon^{\text{m}}_{405}A_{430}}{\varepsilon^{\text{d}}_{430}\varepsilon^{\text{m}}_{405} - \varepsilon^{\text{m}}_{430}\varepsilon^{\text{d}}_{405}} \quad (2)$$

$$\text{MetHb percentage (\%)} = \frac{[\text{metHb}]}{([\text{metHb}] + [\text{deoxyHb}])} = \frac{AX - B}{CX - D}, \quad (3)$$

Here, $\varepsilon^{\text{d}}_{430}$, $\varepsilon^{\text{m}}_{430}$, $\varepsilon^{\text{d}}_{405}$ and $\varepsilon^{\text{m}}_{405}$ are the extinction coefficients of deoxyHb and metHb at 430 and 405 nm. X is A_{405}/A_{430}. A, B, C, and D are different constants in HbV, and were determined from the calibration; for instance, in the case of the HbV prepared by the above conditions, they are 0.55, 9.8, 0.51, and −0.49, respectively.[17]

In the case of an air-saturated solution, the same method could be employed as a determining method of the metHb percentage using ε at the λ_{max} of oxyHb at 415 nm instead of those of deoxyHb in Eqs. 1 and 2.

Fluorescence Polarization Measurement. The fluorescence polarization of MB was measured with a spectrofluorometer (JASCO FP-770) equipped with polarizers at excitation and emission. The polarity (P) and the fluorescent intensity were measured at an emission maximum of 680 nm by irradiating with excitation light of 600 nm.[18]

Stopped-Flow Measurement.[19] Stopped-flow measurements for the reduction of metHb by MBH were carried out using an automatic mixer equipped with a Xe lamp and a photomultiplier connected with optical fibers (Unisoku). The obtained data were averaged from 5 runs. The reaction mixtures from the nitrogen-bubbled reserver cells was rapidly mixed with a high-pressured nitrogen gas (4 kgf cm^{-2}).

The reduced form MB (MBH) was prepared by the reduction of MB with equimolar NADH under a nitrogen atmosphere, being irradiated by visible light from a halogen lamp. The reduction rate of metHb was obtained by a change in the absorbance at 405 and 430 nm, and was calculated using Eq. 1. The oxidation rate of MBH was obtained by the change in the absorbance at 664 nm (ε=78000).

Result and Discussion

Comparison between UQ and MB as Electron Mediators. Figure 1 shows the changes in the metHb percentage after NADH was added to the outer aqueous phase of HbV having 40% metHb with or without electron mediators at 37 °C under a nitrogen atmosphere. MetHb formation does not occur under a nitrogen atmosphere. No leakage of Hb from HbV during the experiment was confirmed by gel-filtration chromatography (Sepharose CL4B). Without any electron mediators, there is no change in the metHb percentage even after 30 min (square plots). From the fact that NADH can reduce metHb in a homogeneous solution state, it was clarified that NADH was not able to permeate through the bilayer membrane of HbV to react with metHb. Furthermore, there was no component having the ability to transport electrons in the HbV membrane.

Next, in order to transport electrons through the bilayer membrane, we studied the incorporation of UQ into the membrane of the HbV. When NADH was added to the outer aqueous phase of UQ-incorporated HbV, a low rate of metHb reduction ($<1\times10^{-9}$ M min^{-1}) was observed, and the metHb percentage decreased by only 5% from the initial value (40%) after 30 min (triangle plots). On the other hand, the co-addition of MB and NADH showed that the rate of metHb reduction was one-hundred times (1×10^{-7} M min^{-1}) as fast as that of the UQ-incorporated HbV. The spectral change shown in Fig. 2 indicates that the absorbance of the Soret band of metHb at 405 nm decreased, and that of the deoxyHb at 430 nm increased with isosbestic points at 417 and 448 nm. In addition, the metHb percentage reached less than 5% within 20 min (circle plots). From these results, the reduction in metHb of the HbV was possible with the addition of water-soluble reductants from outside via electron transfer across the bilayer membrane using an appropriate electron mediator, such as MB.

The large difference in the rates of metHb reduction between MB and UQ would be explained from the solubility and location of the electron mediators. Because UQ is completely hydrophobic, it should locate in the center of the hydrophobic part (far from the water–lipid interface).[5a] Furthermore, because the viscosity of the concentrated Hb solution (36 g dL^{-1}) in the inner aqueous phase was high (ca. 8 cP),[12c] the slow diffusion of metHb would lead to the small frequency of the interfacial reaction with the UQ. Therefore, it is easy to understand that UQ is not suitable as an electron mediator for the reduction of metHb in HbV.

In contrast, it is well known that MB has high solubilities in both water and organic solvents, and binding properties on a bilayer membrane,[20] polyelectrolyte,[21] polynucleotide,[22] zeolite,[23] etc. This implies that MB can react with both metHb and a reductant with a high probability because of the wide distribution of MB in both aqueous phases and a membrane phase. However, it is difficult to know the location and orientation of MB, because the adsorption or partition was affected by the ionic strength, the concentration in the bulk phase, the surface charge of the substrate and so on. In the case of HbV, DPPG having an anionic headgroup is contained in the lipid component, so that the negatively charged surface of HbV electrostatically interacts with MB having a positive charge. MB tends to adsorb on the negatively charged membrane or polyanion as associated (dimeric or polymeric) forms.[20–23] This phenomenon was analyzed based on visible spectral changes, namely a decrease in the absorbance at 664 nm, ascribed to the MB monomer, and to an increase in the absorbance at 600 nm due to association. However, no spectral change of MB was observed after the

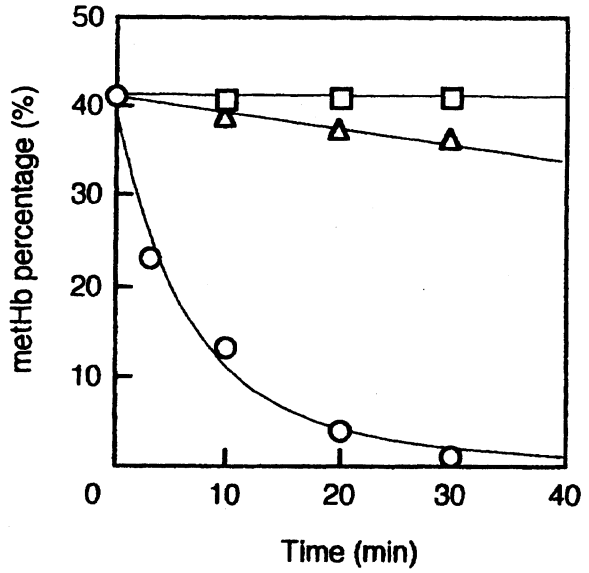

Fig. 1. Changes of metHb percentages after the addition of MB/NADH to the HbV or NADH to the UQ incorporated HbV under a nitrogen atmosphere at 37 °C. ○: MB/NADH=9/100, △:NADH=500 (to UQ 6 μM incorporated HbV), □: NADH=200 μM.

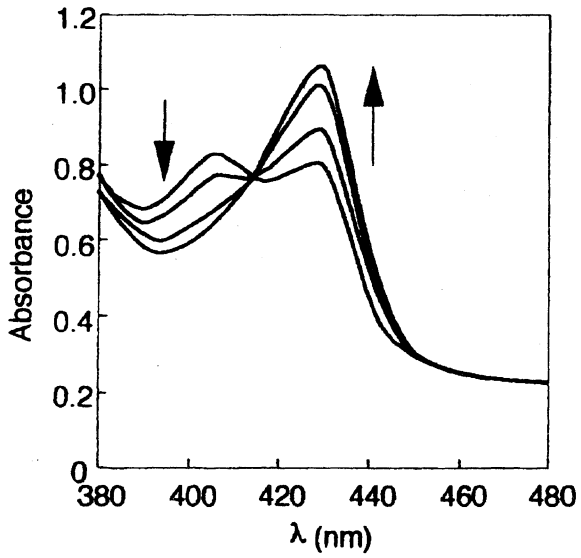

Fig. 2. Spectral change of Soret band of metHb (405 nm) and deoxyHb (430 nm) when MB/NADH (6/200 μM) was added to the Hb vesicles including 40% metHb. From below line: 0, 3, 10, 20 min.

addition of vesicles having the same membrane composition as HbV in the lipid concentration range of 1—5×10^{-4} M. This would have been due to the high ionic strength of the bulk saline solution and/or the low vesicle concentration, which should weaken the interaction between MB and the vesicular surface. The association of MB molecules at the surface of the vesicles can be confirmed by a fluorescence quenching method.

The fluorescent intensity of MB with various concentrations was measured at 680 nm before and after mixing with the vesicles; the results are summarized in Table 1. The maximum wavelengths of the emission and excitation of MB did not shift after mixing with vesicles for all concentrations of MB. For a low concentration (3×10^{-6} M) of MB, no fluorescent quenching of MB was observed after the addition of vesicles, indicating that the association of MB on the surface of the vesicles did not occur. When the MB concentration was 6×10^{-6} M, the fluorescent intensity of MB became lower with increasing vesicle concentration. This was caused by the increased amount of MB adsorbed on the surface, followed by the association of MB. At a concentration higher than 9×10^{-6} M, the fluorescence of MB even in a homogeneous buffered solution was self-quenched.

On the other hand, the fluorescence polarity (P) (ca. 0.3) in a homogeneous solution showed no dependence on the MB concentration, whereas the addition of vesicles increased P to ca. 0.4—0.5 (Table 1). This means that the molecular rotation of MB was restricted by adsorption on the vesicular surface, or incorporation into the bilayer membrane. In addition, P was lowered with increasing concentration of MB. There are at least two components of MB, namely MB dissolved in the bulk solution and MB fixed on the vesicle surface. The increase in P was due to the MB fixed on the surface of the vesicles, and the ratio of dissolved MB having a low P was decreased with increasing MB concentration. Therefore, at a high MB concentration, MB adsorbed on the surface of the vesicles should be saturated.

In order to confirm the interaction of MB with the bilayer membrane as a Langmuir adsorption, and not the partition of MB between the membrane and the aqueous phase, 1 to 9×10^{-6} M MB was added to the outer aqueous phase of the vesicles which encapsulates NADH (3×10^{-7} M). If MB was parted in both the membrane and the aqueous phases with a partition coefficient, a part of MB that existed in the inner aqueous phase could be reduced by NADH. In contrast, if the adsorption of MB onto the outer surface of the vesicles was the main process, a small amount of MB could be reduced. Indeed, when MB was added to a suspension of the vesicles encapsulating NADH under a nitrogen atmosphere, no reduction of MB was confirmed, even after 6 h at 37 °C. Therefore, it was clarified that MB added to the outer aqueous phase existed only in the outer aqueous phase or at the outer side of the membrane.

Table 1. Fluorescence Intensity and Polarization of MB with or without Vesicles

[MB]/M $\times10^6$	[Lipid]/M $\times10^4$	I_{680}	P
3	0	0.027	0.28
3	1	0.027	0.54
6	0	0.058	0.33
6	1	0.050	0.43
6	2	0.044	0.46
6	3	0.040	0.49
9	0	0.029	0.33
9	1	0.029	0.38

When a small amount of MBH (2×10^{-7} M), obtained by the reduction of MB with the equimolar NADH, was added to this system, a high reduction rate of 6×10^{-8} M min^{-1} was observed, which was in good accordance with that in a homogeneous solution (3×10^{-8} M min^{-1}). This result means that MBH can be dissolved in the bilayer membrane and freely diffuse to the inner aqueous phase, and plays the role as an electron mediator from the inside NADH to MB adsorbed on the membrane. Therefore, the electron transfer across the membrane of HbV was carried out by MBH. MB was not able to act as an electron mediator through the bilayer membrane if not reduced by NADH. This was supported by the results that no significant change in the reduction rate was observed when MB was added to the HbV suspension before and after the addition of NADH.

In order to determine the amount of adsorbed MB and the partition coefficient (Φ) of MBH, ultracentrifugation (300000×g, 30 min) was employed to separate vesicles from the MB solution without changing the MB concentration in the solution. Vesicles ([Lipid] = 10^{-4} M) were added to the buffered solution of various concentrations of MB, and incubated for 20 min at room temperature under a nitrogen atmosphere. The amount of MB included in the precipitate was calculated from the concentration of MB in the supernatant. The amount of MB in the precipitate for all samples was plotted versus the concentration of MB before separation and shown in Fig. 3a. This diagram could be analyzed using a Langmuir adsorption isotherm and fitted to

$$w = \frac{w_{st}b[\mathrm{MB}]}{1+b[\mathrm{MB}]}. \tag{4}$$

The saturated adsorption value (w_{st}) and adsorption constant (b) were determined to 1.7×10^{-6} mol and 4.0×10^5, respectively, with a good correlation.

On the other hand, Fig. 3b shows the amount of MBH included in the precipitate, plotted versus the concentration of MBH before separation. After measuring the concentration of MBH in the supernatant, MBH was oxidized by bubbling with oxygen for 30 min. The amount of MBH in the precipitate showed a linear relationship versus the MBH concentration. This suggests that MBH would not be adsorbed on the surface of the vesicle, but would be situated between the buffer solution and bilayer membrane. Φ can be calculated using

$$\Phi = \frac{m_b/V_b}{m_{aq}/(V_w - V_b)}. \tag{5}$$

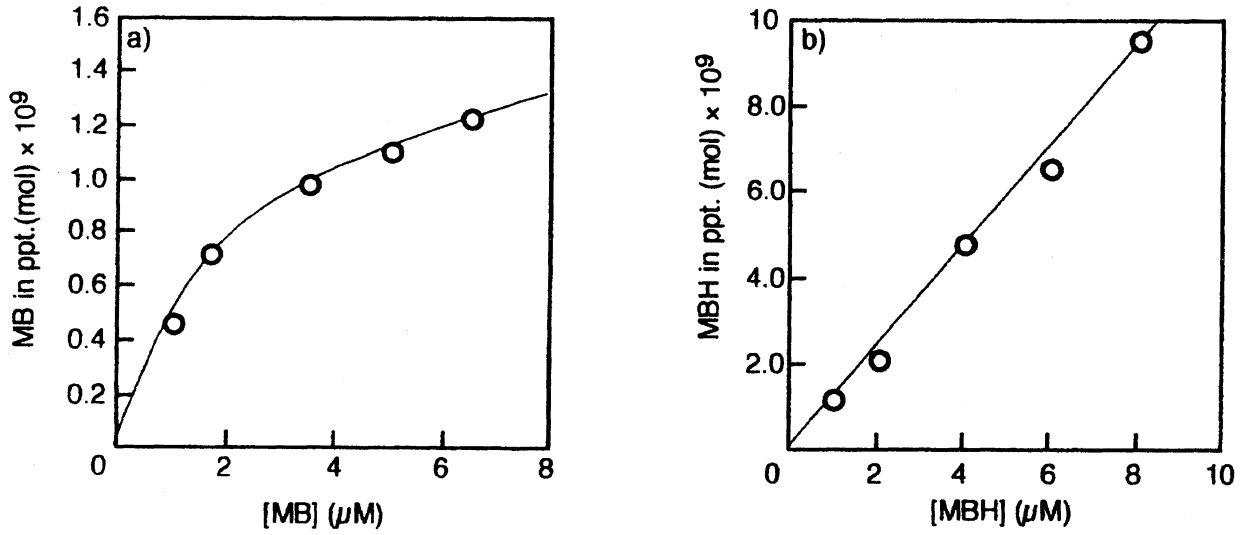

Fig. 3. Relationship between the concentration and the amount of (a) MB and (b) MBH contained in the vesicles as precipitates after ultracentrifugation.

The volume of the membrane phase (V_b) and the inner aqueous phase (V_{in}) were calculated using the following equations:[12a)]

$$V_b = V_w - V_{in}, \tag{6}$$

$$V_{in} = \frac{4}{3}\pi\{r - n(d + d_w) + d_w\}^3, \tag{7}$$

where, r, d, d_w, and n mean the average radius of the vesicle (135 nm), the thickness of the bilayer membrane (0.43 nm), the distance between the bilayer membrane (0.13 nm), and the average number of bilayer membranes (2.3), respectively. From above, Φ was determined to be 5.8—6.7×10^{14}, which was a very high value. Under these conditions, no decrease in the dispersibility of vesicles was observed using a dynamic light-scattering method and turbidimetry.

Consequently, the membrane phase can dissolve a very large amount of MBH while keeping its structure. MBH generated in the outer aqueous phase by the reduction of MB with NADH immediately moved into the membrane phase, and reduced metHb in the inner aqueous phase of the vesicle. The MB converted from MBH at the inner surface of the membrane during the reduction of metHb should move to the inner aqueous phase of the vesicle, and act as an electron mediator from the membrane to metHb. Finally, the electron pathway from the outer aqueous phase to the inner aqueous phased should be completed; such an MBH property leads to the high efficiency of the metHb reduction.

The Kinetics of metHb Reduction. Figures 4(a) and 4(b) show the kinetics of the metHb reduction when NADH and MB were added to the outer aqueous phase under a nitrogen atmosphere. The rate of metHb reduction shows a linear relationship versus the concentrations of MB and NADH. This means that reduction could be analyzed for a pseudo-first-order reaction. The rate-determining step of the metHb reduction in Fig. 5 was expected to be the reduction of MB with NADH in the outer aqueous phase. In HbV, the membrane permeability or the amount of adsorbed MB, however, should affect the entire reaction because metHb and NADH are separated by the bilayer membrane. In order to obtain the basic data about the electron transfer across the bilayer membrane, the reaction in a homogeneous solution was first studied. When MB and NADH were mixed with metHb in a buffered solution, the metHb reduction rate obeyed the pseudo-first-order reaction versus the concentrations of MB and NADH as well as that in the case of HbV. Therefore, the entire reaction rate can be described by the second-order reaction, as shown by

$$-\frac{\mathrm{d[metHb]}}{\mathrm{d}t} = k\,[\mathrm{MB}][\mathrm{NADH}], \tag{8}$$

where k was calculated to be 3.7×10^2 M^{-1} min^{-1}. This value was almost the same as the rate constant of the MB reduction with NADH in a homogeneous solution without metHb (k_1=4.3×10^2 M^{-1} min^{-1}). In addition, the rate constant of metHb reduction with MBH obtained from a stopped-flow measurement was four-orders higher (k_2=1.8×10^6 M^{-1} min^{-1}) than that of the reduction of MB with NADH. From these results, it is clear that the rate-determining step of metHb reduction is the MB reduction with NADH.

When the results in the case of HbV (Fig. 4) were analyzed using Eq. 8, k was determined to be 3.5×10^2 M^{-1} min^{-1}. This value was in good accordance with a system without a membrane. Indeed, the rate constant of the metHb reduction in HbV with MBH was determined to be 1.2×10^6 M^{-1} min^{-1}. Therefore, the separation between MBH and metHb through a membrane shows no influence on the rate of metHb reduction; also, permeation rate (P_d) of MBH could be estimated to be more than 10^9 cm s^{-1}. Although this value seems to be too large, when the determined above Φ and the diffusion coefficient (D=10^5 cm^2 s^{-1}) obtained from the literature[24)] were used with Overton's rule, as shown in the following equation, it could be understood that the P_d of MBH, as estimated, was appropriate:

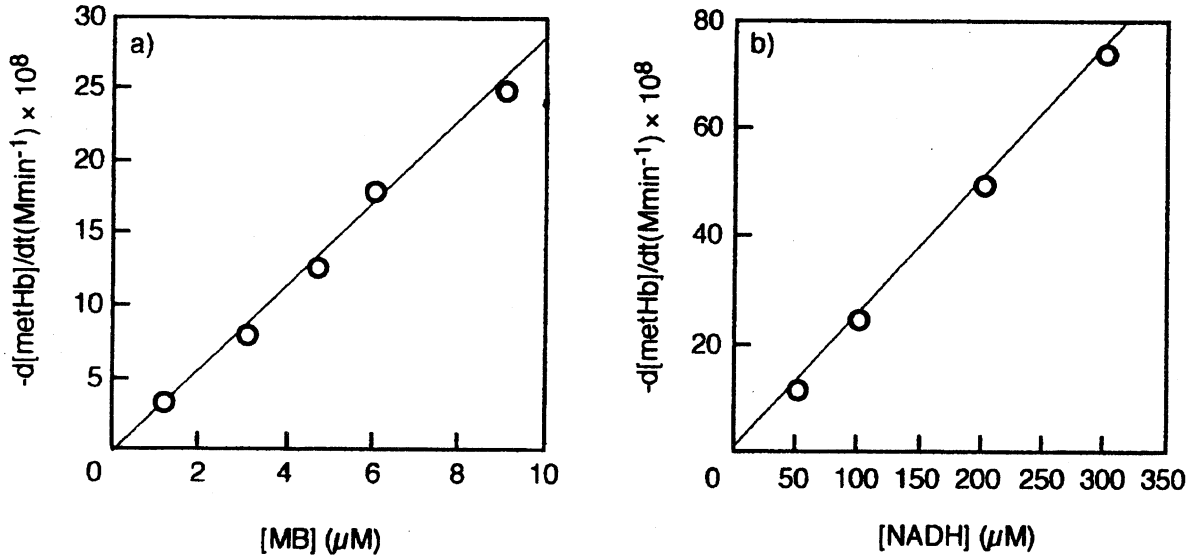

Fig. 4. Kinetics of metHb reduction in HbV by the addition of MB/NADH under a nitrogen atmosphere.

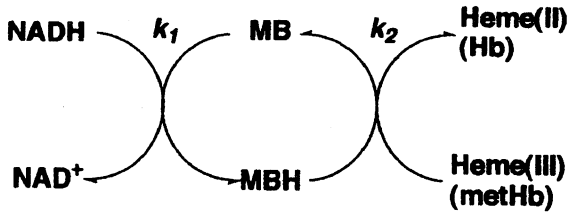

Fig. 5. The reaction scheme of metHb reduction.

$$P_d = P_0 D \cdot \Phi / l, \tag{9}$$

where P_0 was the constant and l represents the thickness of the membrane. Theses elemental reaction rates of Fig. 5 are summarized in Table 2.

Reduction of MetHb in HbV under Aerobic Conditions. The same experiment as shown in Fig. 1 were performed in an air-saturated solution (P_{O_2}=149 Torr, 1 Torr=133.322 Pa); the data are given in Fig. 6. When a reductant was added to the outer aqueous phase of UQ-incorporated HbV, no reduction of metHb was observed, and Hb was gradually oxidized. Because the oxidation rate was much faster than the intrinsic oxidation rate without reductants, it was suggested that the incorporated UQ should promote metHb formation. Namely, active oxygen species would be generated by the autoxidation of UQ to oxidize Hb to metHb. Therefore, the incorporation of UQ into HbV does not work under aerobic conditions.

On the other hand, the co-addition of MB and the reductant was also influenced by the presence of oxygen. When MB and NADH were added at the same concentration as in Fig. 1, the metHb percentage was definitely decreased within 20 min; however, subsequently, the percentage abruptly increased. The minimal value of the percentage did not reach less than 10%. These unfavorable phenomena were considered to be due to active oxygen species. In the case of MBH oxidation, the main product of the active oxygen species is hydrogen peroxide,[25] not the superoxide radical. According to the result obtained under a nitrogen atmosphere, the reduction rate of metHb by the co-addition of MB and NADH is controllable by changing their concentrations. The kinetics was obtained with increasing NADH concentration until 5×10^{-4} M, as shown in Fig. 6 (triangle plots). The increase in the NADH concentration made the metHb percentage less than 10% within 20 min.

In order to analyze the redox behavior in Fig. 6, the rate of metHb reduction was expressed as

Table 2. Reaction Rate Constant of the Elemental Reactions of the metHb Reduction via Electron Transfer across the Bilayer Membrane under Nitrogen Atmosphere

Reaction	Rate constant $M^{-1}min^{-1}$
metHb reduction by MB/NADH	3.7×10^2
metMb reduction by MB/NADH in HbV	3.5×10^2
MBH+metHb in solution	1.8×10^6
MBH+metHb in HbV	1.2×10^6
MB+NADH→ MBH+NADH	4.3×10^2

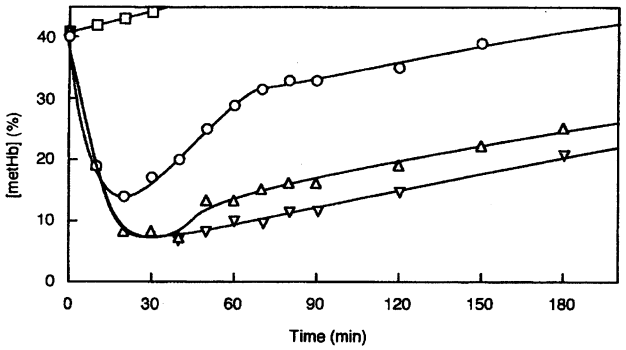

Fig. 6. Change in metHb percentage by the addition of MB/NADH under air at 37 °C. ○: MB/NADH=9/100, △:MB/NADH=9/300, ▽:MB/NADH=9/300 (to HbV having 10^5 U/L catalase), □: NADH=500 μM (to UQ (6 μM) incorporated HbV).

$$-\frac{d[metHb]}{dt} = k_2[metHb][MBH] - k_3[H_2O_2][Hb] - k_4[O_2][MBH]. \quad (10)$$

In this equation, the autoxidation of NADH (4×10^{-2} M^{-1} min^{-1}) could be neglected.

First, during the initial stage of less than 3 min, which is denoted as the first stage, the amount of hydrogen peroxide is small. It is reasonably expected that the rate of metHb reduction has a similar linear profile as that under a nitrogen atmosphere, because the second term could be neglected and third term included the MBH concentration. Figure 7 is the relationship between the rate of metHb reduction during the initial stage and the NADH concentration in the presence of MB (9 μM). A linear relationship was obtained, and the rate constant was 35% of that under a nitrogen atmosphere. Considering that the MBH autoxidation rate constant was 4×10^3 M^{-1} min^{-1} and the oxygen concentration (1×10^{-3} M) was much higher than that of metHb (3×10^{-6} M), the rate of MBH autoxidation ($4\times$[MBH] M min^{-1}) was comparable with that of metHb reduction ($3.6\times$[MBH] M min^{-1}). Therefore, the kinetics obtained from Fig. 7 were reasonable.

The oxidation following metHb reduction, as shown in Fig. 6, could be explained by Eq. 10. The second term becomes predominant based on a decrease in the concentration of the reductant, and metHb decreases the first term; also, an increase in hydrogen peroxide, which can oxidize the metHb through the HbV membrane, increases the second term.

In order to suppress the following oxidation, 10^5 unit L^{-1} of catalase was incorporated into the inner aqueous phase of HbV to remove the hydrogen peroxide. As a result, when both MB (9×10^{-6} M) and NADH (5×10^{-4} M) were added, the rate of metHb reduction during the initial stage was almost the same as that without catalase. From this result, it could be confirmed that the influence of hydrogen peroxide was negligible during the initial stage (< 3 min). On the other hand, the oxidation rate during the next stage decreased the rate by one-forth without catalase, as shown in Fig. 6 (reversed triangle plots). The rate of oxidation was effectively suppressed by the addition of catalase, supporting the fact that the metHb formation was mainly caused by hydrogen peroxide.

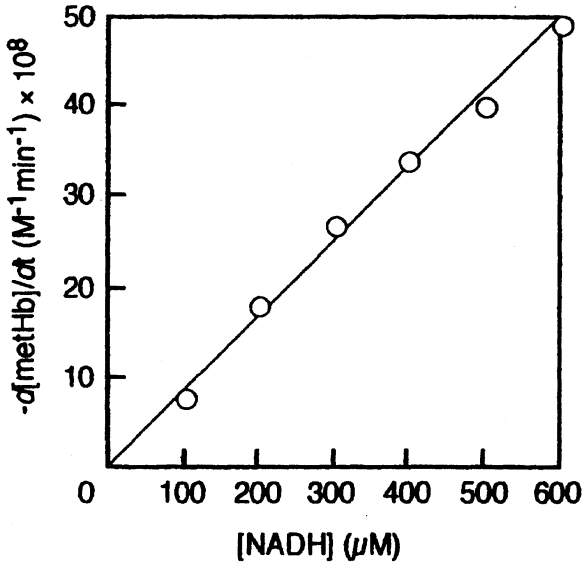

Fig. 7. The rate of metHb reduction by the addition of MB (9 μM)/NADH as related to the concentration of NADH under air.

Finally, the amount of MB for administration and the retention time should be studied in more detail as well as the side effects of this system in medical use. Actually, during the treatment for methemoglobinemia, 100—200 μM MB could be injected each hour.[26]

Conclusions

The metHb reduction in HbV was possible by adding reductants, such as NADH, with electron mediators to the outer aqueous phase. When UQ was used as an electron mediator, a slow reduction rate of metHb was observed under a nitrogen atmosphere. UQ in the bilayer membrane ineffectively reacts with both the reductant and metHb at only the surface of the membrane. In contrast, MB showed a high efficiency as an electron mediator, and the metHb percentage rapidly decreased to less than 10% within 30 min. This was due to the excellent affinity of MBH, which was formed by the reduction with NADH, with both the membrane and hydrophobic phase. Under a nitrogen atmosphere,the kinetics of metHb reduction in metHb/MB/NADH systems were controlled by the reaction of MB with NADH in the outer aqueous phase. On the other hand, under air, the decreased efficiency of the metHb reduction and the following oxidation was observed due to the effect of oxygen and hydrogen peroxide. This oxidation was suppressed by the incorporation of catalase into the inner aqueous phase of HbV.

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