Suppression of Methemoglobin Formation by Glutathione in a Concentrated Hemoglobin Solution and in a Hemoglobin-Vesicle

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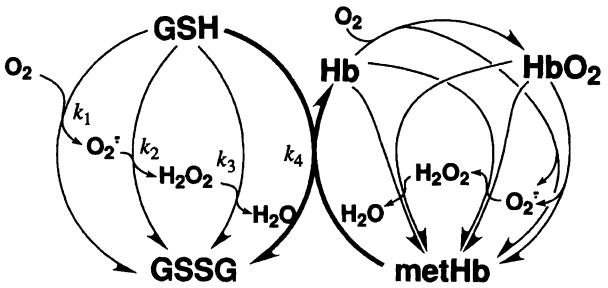
The suppression of methemoglobin (metHb) formation by glutathione (GSH) is difficult because GSH is oxidized not only by the reduction of metHb, but also by oxygen to generate active oxygen species, such as superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2), which contribute to metHb formation. An effective nonenzymatic reduction of metHb was achieved at a high concentration of Hb (40 wt%, 2.48×10^{-2} M subunits (1 M=1 mol dm^{-3})) because the reduction of metHb was accelerated, and at a low partial pressure of oxygen (pO_2=40 Torr (1 Torr=133.322 Pa)) because the oxidation of GSH was effectively suppressed. Hb-vesicles, which encapsulate concentrated Hb (40 wt%, 2.48×10^{-2} M subunits), transport oxygen in the blood stream at relatively low pO_2 (110 Torr in artery and 40 Torr in venous, 58 Torr in average). The rate of metHb formation in Hb-vesicles was effectively decreased from 3.8×10^{-7} to 1.6×10^{-7} M s^{-1} by coencapsulating GSH at 58 Torr.

Blood substitutes, which meet the various clinical requirements, e.g., blood type-free and virus-free as well as chemical and structural stability for long-term storage, have been extensively developed because of fear of infectious diseases and side reactions, demand for emergency transfusions, difficulty in long-term storage, a lack of blood donation anticipated in aging societies, and so on.[1,2] One category of blood substitutes is the utilization of purified hemoglobin (Hb) from outdated human red cells, bovine Hb, recombinant Hb, human Hb from transgenic pigs, etc. These Hbs are chemically modified[3] or encapsulated within phospholipid vesicles (Hb-vesicles).[4,5] We have been developing the Hb-vesicles (ϕ: 200 nm) from the standpoint of macromolecular or molecular assembling.[6] The Hb-vesicles have many advantages, which are related to the significance of red cells, as follows. No modification of Hb is necessary because allosteric effectors to regulate the oxygen affinity are coencapsulated within the vesicles.[6] Damage to tissue due to Hb and active oxygen species from Hb are suppressed owing to the bilayer membrane, which works as a barrier.[2]

One of the problems of Hb-based blood substitutes is methemoglobin (metHb) formation during storage or circulation in the blood stream. Iron(II) oxyhemoglobin (HbO_2) autoxidizes to iron(III) metHb and loses its oxygen binding ability (Scheme 1). This mechanism has been discussed by many researchers.[7—11] It is well-known that HbO_2 dissociates into metHb and $O_2^{-\cdot}$.[7] DeoxyHb, to which H_2O is weakly coordinated, reacts with oxygen to form metHb more rapidly than does HbO_2.[10,11] The generated $O_2^{-\cdot}$ and H_2O_2 rapidly oxidizes Hb to generate metHb.[8] The reaction of H_2O_2 with deoxyHb is 100-times faster than that with HbO_2.[7,12]

In the case of modified Hbs, they sometimes encounter a problem, such that they autoxidize faster than does the native Hb.[13] The active oxygen species, metHb itself or other by-products generated during the course of the autoxidation of Hb, would be factors causing tissue damage.[2] On the other hand, the autoxidation of Hb in the Hb-vesicles is serious, especially for vesicles composed of unsaturated phospholipids.[14] This can be reduced by using α-tocopherol as an antioxidant or saturated phospholipids as membrane components.[14] The water-soluble reductant can also be coencapsulated into the Hb-vesicles.



Scheme 1. Elemental reactions in the GSH/Hb system.

The reduction of metHb in red cells is performed by NADH-cytochrome b_5 reducing systems,[15] direct reduction by glutathione (GSH) and ascorbic acid. The oxidized dehydroascorbic acid and GS· are reduced again by corresponding reductases. Moreover, superoxide dismutase or catalase diminish the active oxygen species. The percent of metHb in red cells is maintained at less than 0.5% of the total Hb.[16] Since these chemicals and enzymes are completely removed in the purified Hb solution,[17] studies about the nonenzymatic reduction of metHb by reductants are requested. The reductants such as ascorbic acid, flavin or 5-hydroxyanthranilic acid show very rapid reduction. However, they easily decompose in air during preparation. For example, ascorbic acid added to a solution of Hb is easily oxidized and generates a "green pigment" as the denatured form of metHb.[18]

GSH is present in a human red cell at a concentration of 1.5×10^{-3} M (1 M=1 mol dm^{-3}), and the reduction of metHb by GSH is known to share 12% of the total reduction.[16] However, it was reported that GSH accel-

erated rather than inhibited metHb formation in vitro without the appropriate enzymes.[12,19—21] The effective reduction of metHb by only GSH has not yet been reported. It was explained in terms of oxidation by active oxygen species generated during GSH oxidation. The process of metHb reduction in a mixture of GSH and Hb is divided into three categories of reactions. The first is the reduction of metHb by GSH. The second is negative: autoxidation of Hb without GSH. The third is also negative: The sum of the rates of metHb formation by the reaction with active oxygen species generated at the oxidation of GSH.

The purpose of this study was to nonenzymatically inhibit the metHb formation by suppressing the oxidation of GSH, itself, and accelerating the reduction of metHb by GSH based on analyses of the redox behavior of the Hb/GSH systems. Moreover, the successful conditions for suppression of metHb in Hb-vesicles are reported.

Experimental

Materials. An Hb solution was purified from outdated human red cells.[17] An outdated red cell solution from the Hokkaido Red Cross Blood Center was diluted with an equivalent volume of saline; the solution was then shaken in a CO atmosphere to convert HbO_2 to HbCO in the red cells. After washing the red cells with saline by centrifugation, the red cell solution ([Hb]=25 wt%) was mixed with a 0.2-fold volume of dichloromethane to lyse the red cells. Centrifugation (1900 g, 15 min) of the solution separated the stroma components. After evaporating the residual solvent under a reduced pressure (20 Torr (1 Torr=133.322 Pa)) at 40 °C, the solution was heated at 60 °C in the dark under a CO atmosphere. The denatured proteins other than Hb precipitated and were removed by centrifugation (1900 g, 20 min). The removal of the stroma components and concomitant proteins in the obtained Hb solution (≥25 wt%, 1.55×10^{-2} M subunits) was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and high-performance liquid chromatography (HPLC). No denaturation of Hb was confirmed by isoelectric focusing, oxygen dissociation equilibrium measurements, visible spectrometry, and HPLC.[17] The resulting Hb solution was dialyzed against pure water and the solution conditions were regulated ([Hb]=10 wt% (6.2×10^{-3} M subunits), pH 7.0).

Chemicals added to Hb solution, GSH (Merck & Co., Inc., purity: >99.8%), pyridoxal 5′-phosphate (PLP, Merck & Co., Inc., >98.5%), inositol hexaphosphate dodecasodium salt (IHP, Sigma Chem. Co., 98%), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Kanto Chem. Co., Inc., >98.0%) were used without further purification.

As for the membrane components of vesicles, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, NOF Co.) was purified by reprecipitation in acetone. Cholesterol and palmitic acid were purchased from Kanto Co., and purified by recrystallization from methanol and hexane, respectively. The purity of these amphiphiles was confirmed by thin-layer chromatography (silica gel plates (Merck & Co., Inc.) eluent: $CHCl_3/CH_3OH/H_2O$=65/25/4 by volume).

The Rate of metHb Formation. Decarbonylation of HbCO was performed by irradiating visible light from a halogen lamp under an O_2 flow at 4 °C by using a rotary evaporator.[22] The ratio of HbCO after decarbonylation was less than 5%.[23] The Hb solution was concentrated to 40 wt% (2.48×10^{-2} M) by ultrafiltration, and PLP ([PLP]/[Hb]=0.75) or IHP ([IHP]/[Hb]=0.125) and GSH (0—3.0×10^{-2} M) were added to the solution. After deoxygenation of the solution by stirring the solution in an atmosphere of N_2, each 10 cm^3 of the deoxyHb solution was put into a glass tube (100 cm^3). The gas phase was then regulated by gas flowing at pO_2=36, 72, 142 or 149 Torr. The measurement of the time course of metHb formation using a cyanometHb method[23] was started with incubation of the solution at 37 °C.

The rate of metHb formation: $\left(\frac{d[metHb]}{dt}\right)_{t=0}$ ($M\,s^{-1}$) was calculated from the initial slope of the time course of [metHb]. The increasing rate of metHb percent is represented as follows:

$$\text{Rate of metHb\%} = \left(\frac{d[metHb]}{dt}\right)_{t=0} \cdot \frac{100}{[Hb]} (\%s^{-1}). \quad (1)$$

The Rate of GSH Oxidation. A GSH solution (pH 7.3) was freeze-dried, and the resulting powder was dissolved in D_2O at a concentration of 3.0×10^{-2} M. The solution was put into a glass tube and stirred under regulated pO_2 at 37 °C. The sample solution (200 mm^3) was pipette out and ^1H NMR was measured under an atmosphere of N_2. The oxidation rate was calculated based on the decrease in the integral of CH_2 in $-CH_2SH$ (δ=2.73, d).[19]

The Rate of metHb Reduction by GSH. A metHb solution was prepared from HbO_2 by incubation with $K_3[Fe(CN)_6]$ and subsequent purification by column separation with Sephadex-G25 (Pharmacia). To a GSH solution (5.0×10^{-2} M HEPES, pH 7.0 at 37 °C) was added the metHb solution in an atmosphere of N_2 at [GSH]=2.2×10^{-2} or 4.1×10^{-2} M and [metHb]=6.2×10^{-6} M. From the decrease in the absorbance at 405 nm, attributed to λ_{max} of the Soret band of metHb, the rate of metHb reduction was calculated.

Preparation of Hb-vesicles and Measurement of metHb Formation. A chloroform solution of 0.5 g of lipid mixture (DPPC/cholesterol/palmitic acid/α−tocopherol= 7/7/2/0.4 by mol) was evaporated in a 1000 cm^3 flask. To the resulting thin film was added a concentrated Hb solution (2.48×10^{-2} M, 10 cm^3) containing GSH (1.5×10^{-2} M) and PLP ([PLP]=1.86×10^{-3} M). After dispersion of the mixed lipids in the solution, this was passed through polycarbonate membrane filters (final pore size: 0.2 μm) using an Extruder™ (Lipex Biomembrance Inc., Canada).[6] After removing Hb in an exogenous phase by gel filtration with Sepharose CL-4B (Pharmacia, eluent: 2.0×10^{-2} M HEPES, pH 7.0) and subsequent decarbonylation, the Hb-vesicles suspension (ϕ, ca. 200 nm) was put into a glass tube and stirred at pO_2=58 and 149 Torr. The rate of metHb formation was measured in the same manner as previously mentioned.

Results and Discussion

Influence of [Hb] and [GSH]. In Fig. 1 are shown the increasing rates of metHb percent in Hb solutions with different [Hb]s (6.20×10^{-4}—2.48×10^{-2} M (1—40 wt%)) in the presence or absence of GSH at

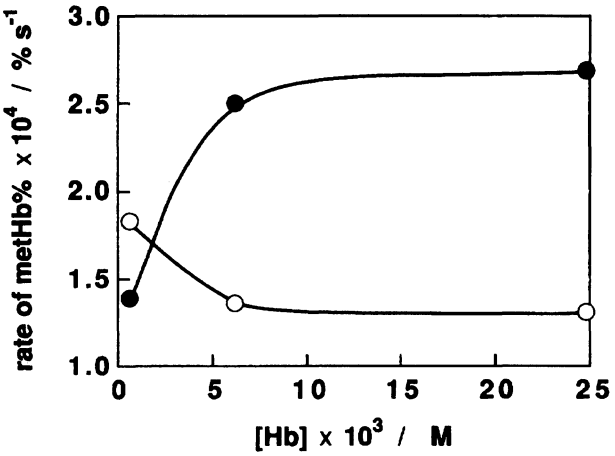


Fig. 1. Influence of the concentration of Hb on the increasing rate of metHb percent in the absence (●) or presence (○) of GSH (1.5×10^{-2} M). $pO_2=142$ Torr.

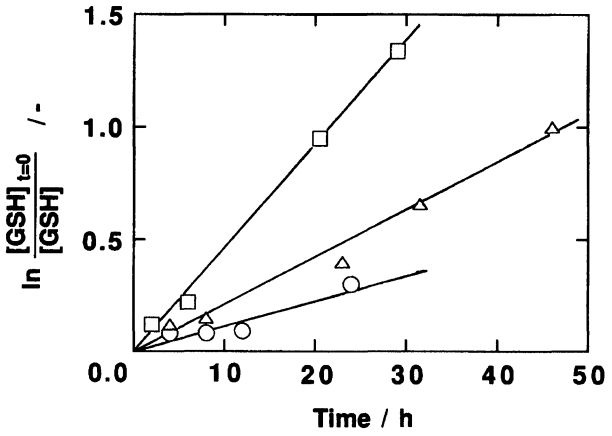


Fig. 2. Time course of the oxidation of GSH (3.0×10^{-2} M) at various pO_2 s measured by ^{1}H NMR (in D_2O, pH 7.3, 37 °C); 36 Torr (○), 72 Torr (△), and 142 Torr (□).

$pO_2=142$ Torr. In the absence of GSH, the rate increase with [Hb], e.g., 1.4×10^{-4}% s^{-1} at 6.2×10^{-4} M and 2.7×10^{-4}% s^{-1} at 2.48×10^{-2} M. MetHb formation occurs by the electron reduction of O_2 of HbO_2 to $O_2^{-\cdot}$. The reaction rate increases proportionally with the concentration of HbO_2. This results in an increase in the concentration of active oxygen species, and then metHb formation is accelerated by these active oxygen species. Therefore, metHb formation exceeds the proportional relation to the [Hb], which explains the rise in the increasing rate of metHb percent.

In the presence of GSH (1.5×10^{-2} M) as a reductant, on the other hand, the rate decreased from 1.8×10^{-4} to 1.3×10^{-4}% s^{-1} when [Hb] increased from 6.20×10^{-4} to 2.48×10^{-2} M. Effective suppression of metHb formation was confirmed at higher Hb concentrations. At lower concentrations, however, metHb formation was accelerated by the addition of GSH. The reduction of metHb by GSH is represented as metHb+GSH→Hb+GS·+H^+. The rate constant of this reaction (k_4) in an atmosphere of N_2 was measured spectroscopically to be 1.1×10^{-3} $M^{-1}s^{-1}$.

In order to elucidate the adverse effect of GSH at lower [Hb], we first analyzed the profile of GSH oxidation. The mechanism of GSH oxidation includes oxidation steps by active oxygen species (Scheme 1). Oxygen first oxidizes GSH to produce GS·, $O_2^{-\cdot}$, and H^+; the resulting $O_2^{-\cdot}$ oxidizes GSH again to H_2O_2, which also oxidizes GSH. Furthermore, in the case of a mixture of Hb and GSH, these active oxygen species generated from GSH oxidation contribute to metHb formation. Figure 2 is the oxidation profile of GSH in D_2O measured by ^{1}H NMR spectroscopy. At each constant pO_2, the net reaction (4 GSH+O_2→2 GSSG+2 H_2O) followed the pseudo-first-order rate law. The rate constant of the second-order rate law[24] was therefore calculated as 1.6×10^{-2} $M^{-1}s^{-1}$ from the results. The rate constant (k_1) was estimated to be 1.7×10^{-2} $M^{-1}s^{-1}$ by a computer simulation (Runge–Kutta method) with the rate constants of elemental reactions in Scheme 1; k_2,[25] 6.7×10^5 $M^{-1}s^{-1}$; k_3,[26] 1.2 $M^{-1}s^{-1}$; dismutation[27] of $O_2^{-\cdot}$, 5.0×10^5 $M^{-1}s^{-1}$. From this value, the initial rate of $O_2^{-\cdot}$ generation at $pO_2=142$ Torr was calculated to be 5.5×10^{-8} Ms^{-1}.

As for the Hb solution, the rate of autoxidation at low Hb concentration (6.2×10^{-4} M) without GSH is 8.6×10^{-10} Ms^{-1}. Therefore, the rate of $O_2^{-\cdot}$ generation is less than this value because the autoxidation includes various oxidations besides $O_2^{-\cdot}$ generation. Comparing the rates of $O_2^{-\cdot}$ generation from GSH and Hb, it is obvious that a larger amount of active oxygen species, which is generated from the GSH oxidation, is present when [Hb] is significantly low compared with the added [GSH]. This leads to the acceleration of metHb formation at lower [Hb]. On the other hand, the rate of metHb reduction by GSH simply depends on the concentration of metHb. Therefore, a relatively large amount of active oxygen species and the low rate of metHb reduction at low [Hb] resulted in the acceleration of metHb formation due to the addition of GSH.

The oxidation of Hb by the addition of GSH has already been reported,[12,19] in which the [Hb] was very low ([Hb]=6.0×10^{-5} M, [GSH]=6.0×10^{-4} M) in order to spectroscopically measure the time course of metHb formation in situ. Our results indicate that GSH accelerates the metHb formation at such a low concentration and that GSH works effectively as a reducing agent when a concentrated Hb solution is used.

In Fig. 3 is shown the influence of the GSH concentration on the rate of metHb formation at the high [Hb] (2.48×10^{-2} M). Suppression of metHb formation was observed under the conditions employed. The rate decreased monotonously with [GSH] and shows a minimum rate of 2.2×10^{-8} Ms^{-1} at [GSH]=1.5×10^{-2} M. The addition of more GSH reduced the suppression of metHb formation. In the concentrated Hb solution, the

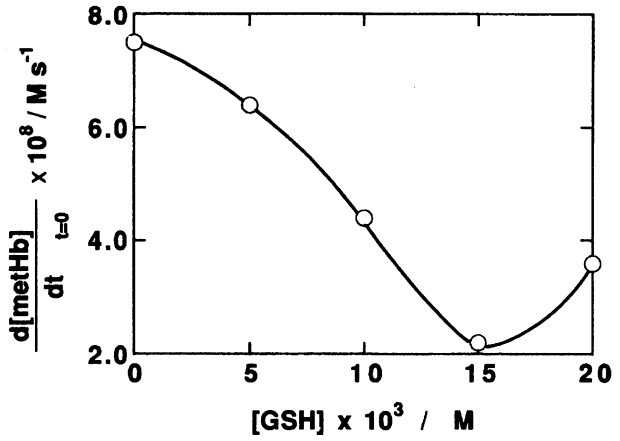


Fig. 3. Influence of the concentration of GSH on the rate of metHb formation ([Hb]=2.48×10^{-2} M subunits).

influence of the active oxygen species generated from GSH oxidation is small and the reductive effect of GSH is prominent. Therefore, the rate of metHb formation decreases with increasing [GSH]. However, the addition of the large amount of GSH induces the adverse effect of GSH There is an optimal amount of GSH to suppress metHb formation.

pO_2 Dependence of the Rate of metHb Formation. The oxygen dissociation curve of an Hb solution (40 wt%, 2.48×10^{-2} M subunits) is shown in Fig. 4(a). The Hill number and p_{50} (partial pressure of oxygen at which the percent of HbO_2 is 50%) were controlled at 2.0 and 43 Torr, respectively, by the addition of PLP (1.86×10^{-2} M). Figure 4(b) is the initial rate of metHb formation calculated from the time course of [metHb] in the Hb solution containing PLP under regulated pO_2. In the absence of GSH, the rate increases with decreasing pO_2 from 149 Torr, and shows a maximum value at around 40 Torr, followed by a subsequent decrease. The pO_2 dependence has already been reported, and the rate of metHb formation is represented by[10]

$$\frac{d[metHb]}{dt} = k_{oxy}[HbO_2] + k_{deoxy}[deoxyHb][O_2]. \quad (2)$$

The rate of metHb formation should be constant when pO_2 is between ca. 120 and 149 Torr because the percent of HbO_2 is 100%. The increase in the rate of metHb formation at a pO_2 below 120 Torr is due to an increase in the percent of reactive deoxyHb. The subsequent decrease in the rate of metHb formation at pO_2 lower than ca. 40 Torr is due to a lower concentration of O_2 and active oxygen species in the Hb solution.

Equation 2 coincides well with the experimental values in Fig. 4(b) when k_{oxy} and k_{deoxy} are ca. 4.4×10^{-6} s^{-1} and 2×10^{-1} $M^{-1}s^{-1}$, respectively, and the maximum is confirmed at 36 Torr. The maximum rate appeared at around 7 Torr in the literature. The difference is explained in terms of the difference in the oxygen affinity (p_{50}) of the Hb solutions (p_{50} in the literature

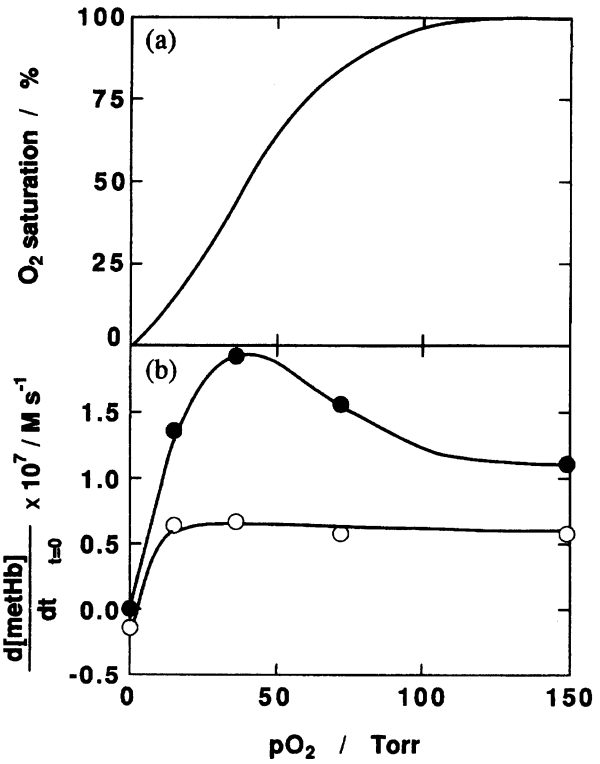


Fig. 4. (a) Oxygen dissociation curve of Hb solution (40 wt% (2.48×10^{-2} M subunits), 1.86×10^{-2} M PLP, pH 7.4, 37 °C), and (b) rates of metHb formation with (○) or without (●) 1.5×10^{-2} M of GSH at various pO_2s.

was 7 Torr). In our system, since p_{50} was controlled at 40 Torr by the addition of PLP, as shown in Fig. 4(a), the oxygen dissociation curve in our system shifted to the right side (upper pO_2 side), as did the maximum rate position of the metHb formation.

The effect of GSH on the rate of metHb formation was obserbed next (Fig. 4(b)). The concentration of GSH was selected to be 1.5×10^{-2} M from the results in Fig. 3. The decrease in the rate of metHb formation by the addition of GSH was confirmed at all pO_2s, indicating that GSH could effectively suppress metHb formation. The rate of metHb formation was about 6.1×10^{-2} $M\,s^{-1}$ between 15 and 149 Torr. The difference in the rate of metHb formation between the solutions with and without GSH increases with decreasing pO_2, indicating that GSH suppressed metHb formation more effectively at lower pO_2s. This can be easily understood from the oxidation of GSH by O_2. From our experimental data, 70% GSH decomposes after 30 h under pO_2 of 142 Torr at 37 °C, and 20% GSH under pO_2 of 36 Torr. The low rate of GSH oxidation at low pO_2 results in a decrease in the rate of metHb formation by oxygen species from GSH. Simultaneously, the reduction of metHb increases due to the high concentration of remaining GSH.

Inhibition of metHb Formation in Hb-vesicles by GSH. A concentrated Hb solution (40 wt%, 2.48×10^{-2} M) was encapsulated in the Hb-vesicles in

order to increase the amount of O_2 transport in the blood stream. The oxygen affinity of Hb vesicles was adjusted to 40 Torr (pH 7.0) by coencapsulation of PLP ([PLP] = 1.86×10^{-2} M) to increase the oxygen transporting efficiency (the percentage of the amount of O_2 released between 40 Torr (mixed venous) and 110 Torr (artery)). The rate of metHb formation in Hb-vesicles was 2.8×10^{-7} M s^{-1} at a pO_2 of 149 Torr (Table 1); this was higher than that in a concentrated Hb solution. It was reported that negatively-charged membrane components electrostatically or hydrophobically interacted with Hb under particular conditions, e.g., at pH 5.6. This results in a deformation of the globin and metHb formation.[14,28,29] There might be some influence of these unfavorable phenomena in spite of the fact that the pH of the solution is 7. Since the pO_2 in the blood stream varies from 40 to 110 Torr, the rate of metHb formation was measured at 58 Torr, which was estimated to be average pO_2 in the blood stream. The rate of metHb formation at 58 Torr was 3.8×10^{-7} M s^{-1}, higher than that at a pO_2 of 149 Torr. This indicates that deoxyHb is more susceptible to oxidation, which is the same tendency observed in the Hb solution in Fig. 4. Coencapsulation of 2.0×10^{-2} M HEPES, which is a known scavenger of hydroxyl radicals,[30] showed the rate of 4.0×10^{-7} M s^{-1} at 58 Torr and no effect on the suppression of metHb formation. The concentration of GSH for encapsulation of Hb solution was selected to be 1.5×10^{-2} M for a 2.48×10^{-2} M (40 wt%) Hb solution from the results in Fig. 3. Coencapsulation of GSH decreased the rate of metHb formation of Hb-vesicles at 58 Torr from 3.8×10^{-7} to 1.6×10^{-7} M s^{-1}.

In conclusion, the partial pressure of oxygen and the relationship between [Hb] and [GSH] are important for the effective reduction of metHb by GSH, and 1.5×10^{-2} M GSH is appropriate in a 40 wt% Hb solution. By coencapsulating GSH into Hb-vesicles, the effective suppression of met Hb formation was confirmed because Hb-vesicles have appropriate conditions to induce the effectiveness of GSH. It has been reported that the reduction of metHb in vesicles was confirmed only when some enzymatic systems are present in the stroma-free Hb. In our purification method of Hb, all proteins other than Hb are completely removed. However, nonenzymatic suppression of metHb formation by GSH was revealed by considering the appropriate conditions to prevent GSH oxidation and to accelerate metHb reduction. Further exploration is necessary to enhance the nonenzymatic suppression of metHb formation by molecular design of the reductant and selecting the appropriate conditions.

Table 1. Rates of metHb Formation in Hb-vesicles under Various Conditions

pO_2 / Torr	HEPES[a]	GSH[b]	$\frac{d[metHb]}{dt}\times10^7$ / M s^{-1}
149	−	−	2.8
58	−	−	3.8
58	−	+	1.6
58	+	−	4.0

a) 2.0×10^{-2} M, b) 1.5×10^{-2} M.

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