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Hidden Antioxidative Functions of NADH Coexisting with Hemoglobin

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ABSTRACT

Ferrous oxyhemoglobin (HbO_2) in red blood cells (RBCs) invariably and slowly autoxidizes to form ferric methemoglobin (metHb). However, the level of metHb is always maintained below 0.5% by intracellular metHb reduction of enzymatic systems with coenzymes such as NADH, and by superoxide dismutase (SOD) and catalase (CAT), which eliminate reactive oxygen species. Unquestionably, NADH cannot reduce metHb without the corresponding enzymatic system. Our study, however, demonstrated that a high concentration of NADH (100-fold of normal level, equimolar to HbO_2) retarded autoxidation of HbO_2 in a highly purified Hb solution with no enzymatic system. Furthermore, an inhibitory effect of NADH on metHb formation was observed with additions of oxidants such as H_2O_2, NO, and $NaNO_2$. Our mechanism assessment elucidated extremely high pseudo-CAT and pseudo-SOD activities of NADH with coexistence of HbO_2, and reactivity of NADH with NO. We prepared a model of RBC (Hb-vesicles, Hb-V) encapsulating purified HbO_2 solution and NADH, but no enzymatic system within liposome. We confirmed the inhibitory effect of NADH on both autoxidation and oxidant-induced metHb formation. In addition, an intravenous administration of these Hb-Vs to rats caused significant retardation of metHb formation by approximately 50% compared to the case without NADH co-encapsulation. Based on these results, we elucidated a new role of NADH, *i.e.*, anti-oxidative effect via interaction with Hb, in addition to its classical role as a coenzyme.

1. INTRODUCTION

Hemoglobin (Hb), the most abundant protein in RBCs, which binds reversibly to oxygen, serves an important role in oxygen transport to peripheral tissues. Ferrous oxyhemoglobin (HbO_2) gradually autoxidizes to form ferric methemoglobin (metHb), which cannot bind to oxygen.[1] During this process, superoxide anion ($O_2^{-}\cdot$) is released ($HbO_2 \rightarrow$ metHb + $O_2^{-}\cdot$). It forms H_2O_2 via a disproportionation reaction. These reactive oxygen species (ROS) enhance HbO_2 and deoxyHb oxidation. Along with ROS, endogenous reactive nitrogen species (RNS) such as NO react with Hb to form metHb. RBCs are always exposed to ROS and RNS, which are produced from white blood cells, macrophages, endothelial cells, and so forth. NO is also released with medications such as nitroglycerin and sildenafil. NO inhalation therapy applied in newborn infants adversely converts Hb to metHb.[2] NO_2^- promotes Hb oxidation. $NaNO_2$ is used as a preservative for meat products.[3] Actually, NO_2^- generated from nitrate in vegetables and drinking water via reduction by oral microbes[4] also induces metHb formation.[5–7]

RBCs have functions to reduce metHb and eliminate ROS to maintain a low metHb level below 0.5%:[8] (i) Ascorbic acid and glutathione directly reduce metHb. Then the resulting oxidized dehydroascorbate and glutathione disulfide are reconverted to the reduced forms by enzymes. (ii) An NADH-dependent enzyme, NADH-cytochrome b_5 reductase, also reduces metHb via cytochrome b_5 as an electron mediator.[9] The resulting oxidized form, NAD^+, is reduced to NADH via glycolitic Embden–Meyerhof pathway. (iii) NADPH-dependent enzymes known as NADPH methemoglobin reductase and NADPH-flavin reductase reduce metHb. The oxidized $NADP^+$ is reduced to NADPH via glycolitic pentose phosphate pathway. In addition,

RBCs contain superoxide dismutase (SOD) converting $O_2^{-}\cdot$ to H_2O_2 and catalase (CAT) decomposing H_2O_2.

We have been conducting extensive investigations of the development of artificial oxygen carrier using Hb,[10,11] and have been energetically investigating methods to suppress metHb formation without the use of unstable enzymatic systems described above.[12–15] Because ROS reacts with various biological substances resulting in tissue damage,[16–19] we examined reagents including amino acids, polymers, and constituents within RBCs for potential inhibitory effects on metHb formation. Thereby, we discovered an extremely strong inhibitory effect of NADH at much higher concentration than normal level in RBCs. In this study, we strove to optimize the inhibitory effect of NADH on metHb formation and to clarify the mechanisms by measuring the pseudo-enzymatic activities in the absence of actual enzymes. We also prepared artificial RBCs (Hb-vesicles, Hb-V) encapsulating only NADH and HbO_2 but no enzymes, and administered them to rats for *in vivo* assessment for a new role of NADH.

2. RESULTS

Characteristics of Purified HbO_2 that Contains no Enzymes—HbO_2 solution was prepared via a purification process comprising heat treatment of carbonyl hemoglobin (HbCO) and nanofiltration, followed by decarboxylation reaction. In the purified HbO_2 solution, no other protein was detected either by HPLC, SDS-PAGE, or isoelectric focusing. Furthermore, there were no activities of SOD, CAT, NADH-metHb reductase and NADPH-diaphorase, indicating complete elimination of enzymes during purification. This purified HbO_2 solution was used for all the experiments described below.

Screening of Compounds for Potential Inhibitory Effect on HbO_2 Autoxidation—In the HbO_2 solution without enzyme, the metHb level increased to 53% because of autoxidation after incubation at 37°C for 24 hr (**Table 1**). Various compounds including amino acids, polymers and antioxidants that were regarded as having low toxicity were added excessively (1 g/dL) to this test system. However, they had only modest inhibitory effects on metHb formation (32 – 49%). In contrast, NADH alone demonstrated much stronger inhibitory effects on metHb formation (16.4±0.4%) than the effects of other compounds.

Table 1. Comparison of metHb formation of HbO_2 in the presence of potential antioxidants. Purified HbO_2 solution (10 g/dL, 1.6 mM) with an excess amount of a potential antioxidant (1 g/dL) was incubated at 37°C for 24 hr. Mean±S.D. (n = 3)

Potential antioxidant	Concentration (mM) at 1 g/dL	metHb (%)
none added HbO_2	—	53.0 ± 0.4
L-tyrosine	55.2	38.2 ± 0.4
L-arginine	57.4	35.7 ± 2.6
L-glutamine	68.4	42.7 ± 0.4
L-tryptophan	49.0	35.9 ± 1.8
L-lysine	68.4	34.0 ± 1.1
L-histidine	64.4	32.3 ± 0.9
L-asparagine	75.1	38.2 ± 1.4
L-cysteine	82.5	33.2 ± 0.6
L-methionine	67.0	43.2 ± 3.3
glutathione	32.5	39.6 ± 1.5
human serum albumin (Mw 66000)	0.2	30.2 ± 1.4
PEG2000 (Mw 1900 - 2200)	5.0	41.8 ± 0.4
PEG400 (Mw 360 - 440)	25.0	42.5 ± 0.7
PEG200 (Mw 180-220)	50.0	39.0 ± 0.2
hydroxyethyl starch (Mw 70000)	0.1	37.1 ± 0.9
hyaluronic acid sodium salt*	5.0*	35.7 ± 1.9
quercetine	29.6	41.6 ± 0.1
astaxanthin	16.8	38.2 ± 1.0
sodium glucuronate	46.3	50.5 ± 1.0
sodium L-ascorbate	50.5	37.1 ± 0.3
D-glucose	55.5	35.1 ± 0.7
glycerol	108.6	40.2 ± 3.7
nicotinamide	81.9	32.8 ± 0.8
ATP	19.7	44.7 ± 0.5
NADPH	12.0	37.9 ± 1.0
NADH	14.1	16.4 ± 0.4

* The concentration was 0.2 g/dL because of the limited solubility. Molar concentration of the monomeric repeat unit is shown because of the uncertainly of molecular weight of this biomacromolecule.

MetHb Formation in HbO_2 Solution by Oxidants and Inhibitory Effects of NADH—The effects of the addition of oxidants including H_2O_2, $NaNO_2$ and NO to HbO_2 solution were evaluated. For the experiment of NO, 1-hydroxy-2-oxo-3- (*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7) was used. It decomposed in aqueous solution to generate NO. The addition of oxidants increased absorbance at 630 nm that corresponded to metHb. The HbO_2 was oxidized almost completely in 40 min (**Fig. 1**). In contrast, the coexistence of equivalent molar NADH to HbO_2 caused marked decreases in metHb formation. The levels of metHb 40 min after adding H_2O_2, $NaNO_2$, and NOC7 were, respectively, 52%, 47%, and 56% of the case without NADH.

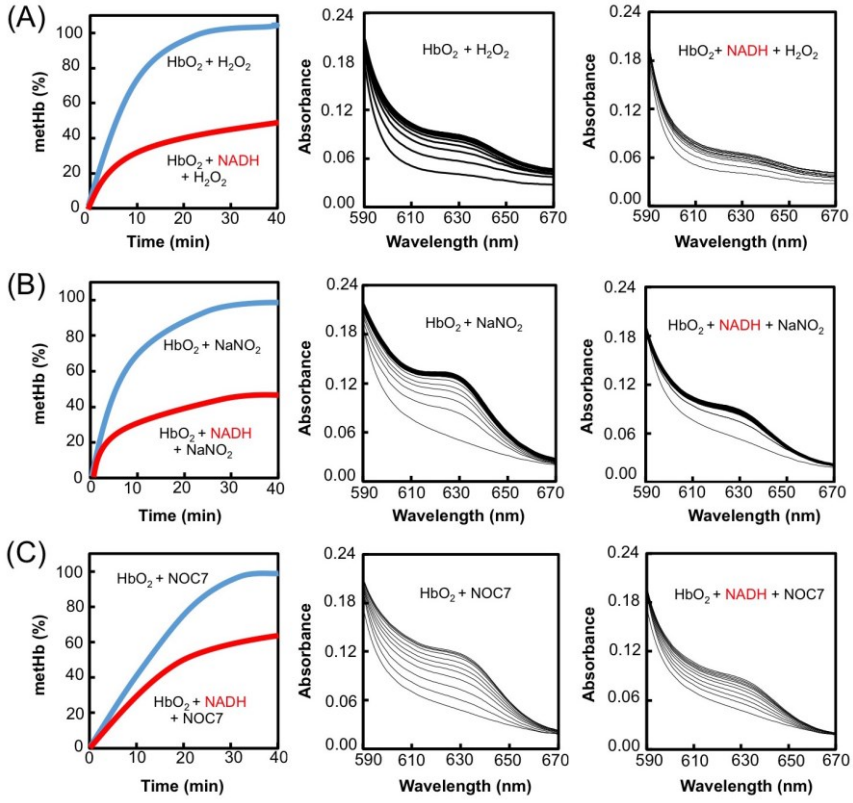

FIGURE 1. Time course of metHb level (%) as well as absorbance spectra of HbO_2 solution with or without NADH after the addition of oxidants: (A) H_2O_2, (B) $NaNO_2$, and (C) NOC7. The initial concentrations of HbO_2, NADH, $NaNO_2$, H_2O_2, and NOC7 were all 15.6 μM in the cuvettes. The level of metHb (%) was calculated based on the increase of absorbance at 630 nm. The absorbance change of Hb without NADH after incubation at 25 °C for 40 min was defined as 100%.

Reactivity between NADH and Oxidants, and Reactivity between NADH and MetHb—Change in the absorbance (λ_{max} = 340 nm) of a NADH solution (0.15 mM) was monitored after incubation with equivalent molar quantity of H_2O_2, NOC7-generating NO, or $NaNO_2$ at 25°C for 10 min. H_2O_2 and NOC7 respectively decreased the absorbance to 85% and 70% from the baseline (**Fig. 2**). Anaerobic conditions prevent NOC7 reaction with NADH. Rate constant *k* for the reaction between H_2O_2 and NADH was determined from the initial slope. It was 1118 $M^{-1}s^{-1}$. No reaction was confirmed in the case of NADH mixied with $NaNO_2$ (data not shown).

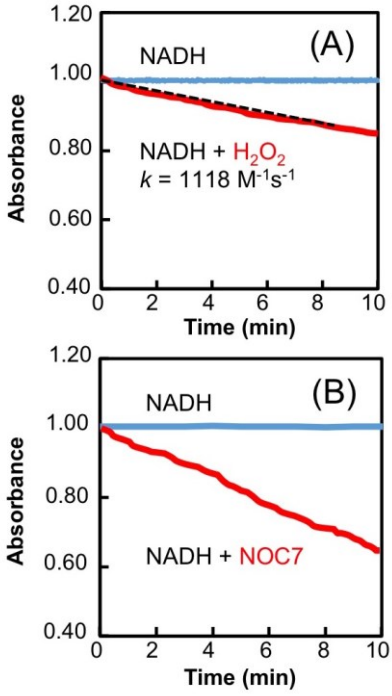

FIGURE 2. Time course of NADH oxidation in the presence of (A) H_2O_2 and (B) NOC7. Change in the absorbance at 340 nm corresponds to NADH was monitored in 0.15 mM NADH solution in the presence of H_2O_2, (0.15 mM) or NOC7 (0.15 mM) at 25 °C for 10 min.

We also tried to detect N_2O using a GC-MS in the reaction system of NOC7 and NADH under the same aerobic condition. However, N_2O was not detected (below the lower limit of quantitation with GC-MS) (data not shown).

NADH and metHb with no enzymes were mixed to ascertain whether a high concentration of NADH can reduce metHb, or not. No reduction of metHb was detected (data not shown).

NADH with Hb Demonstrates Pseudo Enzymatic Activities—Stroma-free hemolysate (SFHL) containing enzymes was prepared as a positive control presenting high CAT and SOD activities (Entry No. 19 in **Table 2**). By contrast, HbO_2, metHb and HbCO prepared from a highly purified Hb solution had no enzymatic activity (Entry Nos. 16–18). Addition of NADH (0.5 - 1.5 mM) to 1.5 mM HbO_2 increased pseudo-CAT and SOD activities (Entry Nos. 1–3). When NADH concentration exceeded equimolar ratio to HbO_2 (Entry Nos. 4 and 5) the activities seemed to reach a plateau. For the condition of Entry No. 3, Michaelis constant K_m and k_{cat} for the pserudo-CAT activity were 1.07±0.28 mM and 18.8±1.7 g Hb/s, respectively. Coexistence of 15 mM tyrosine with HbO_2 and metHb showed slight pseudo-enzymatic activities (Entry Nos. 10–11), but the activities were much weaker than that of NADH with HbO_2 or metHb. Based on the measurement principles, 1.5 mM NADH alone also showed pseudo-enzymatic activities (Entry No. 12), but they were much lower (approximately 10%) than those of the mixture of NADH and HbO_2. Because NADH is known to react with H_2O_2, increasing NADH concentration showed higher pseudo-CAT activity (Entry Nos.12-15). However, pseudo-SOD activity was essentailly low and there seemed no NADH concentration dependence. Although metHb and HbCO with coexistence of NADH demonstrated pseudo-enzymatic activities (Entry Nos. 6 and 7), the activities were minimal, indicating that a ferrous state of Hb and oxygen bound complex are necessary for high pseudo-enzymatic activities.

Table 2. Pseudo-catalase (CAT) and pseudo-superoxide dismutase (SOD) activities in Hb solutions with or without coexistence of NADH or tyrosine (Tyr). Mean ± S.D. (n = 3). The solutions were diluted 1000 times for pseudo-enzymatic activities.

Entry No.	State of Hb	Hb (mM)	Potential Antioxidant	Potential Antioxidant concentration (mM)	Pseudo-CAT ($\times 10^4$ IU/g Hb)	Pseudo-SOD ($\times 10^2$ U/g Hb)
1	HbO_2	1.5	NADH	0.5	6.2±1.7	2.1±1.7
2	HbO_2	1.5	NADH	1.0	19.2±1.4	7.6±1.5
3	HbO_2	1.5	NADH	1.5	34.3±1.2	15.6±0.9
4	HbO_2	1.5	NADH	3.0	35.2±1.0	16.0±1.2
5	HbO_2	1.5	NADH	15.0	46.8±1.2	20.4±1.4
6	metHb	1.5	NADH	1.5	4.5±1.4	1.2±0.6
7	HbCO	1.5	NADH	1.5	0.5±0.4	1.0±0.5
8	HbO_2	1.5	Tyr	1.5	N.D.	N.D.
9	metHb	1.5	Tyr	1.5	N.D.	N.D.
10	HbO_2	1.5	Tyr	15.0	0.3±0.2	0.2±0.1
11	metHb	1.5	Tyr	15.0	0.2±0.2	0.1±0.1
12	-	-	NADH	1.5	3.6±0.4 *)	1.1±0.7 *)
13	-	-	NADH	3.0	4.1±0.8 *)	1.3±0.6 *)
14	-	-	NADH	30.0	19.2±0.6 *)	0.6±0.6 *)
15	-	-	NADH	45.0	19.9±0.8 *)	1.6±0.5 *)
16	HbO_2	1.5	-	-	N.D.	N.D.
17	metHb	1.5	-	-	N.D.	N.D.
18	HbCO	1.5	-	-	N.D.	N.D.
19	SFHL (HbO_2)**)	1.5	-	-	20.1±1.4	21.6±1.7

*) Assuming Hb concentration of 1.5 mM
**) SFHL, stroma free hemolysate
Tyr, tyrosine; N.D., not detected

NADH Prevents $NaNO_2$-induced NO Emission—By the addition of equivalent molar of $NaNO_2$ to a 1.6 mM HbO_2 solution, NO emission was detected. The concentration reached a plateau in approximately 10 s, followed by a gradual decrease (**Fig. 3**). The theoretical NO concentration was 1600 nM in the mixture of HbO_2 and $NaNO_2$. The addition of equivalent molar of NADH to the solution of $NaNO_2$ and HbO_2 decreased the NO concentration to approximately 60% compared to the case without NADH. NO was not detected when NADH was mixed with $NaNO_2$ or HbO_2.

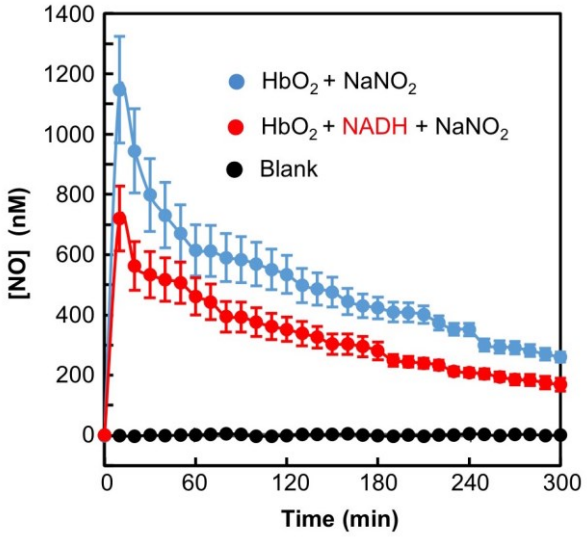

FIGURE 3. Time course of NO concentration in the mixture of HbO_2 and $NaNO_2$ with or without the addition of NADH. Blank: a mixture of HbO_2 and NADH, and a mixture of $NaNO_2$ and NADH. Concentrations of HbO_2, NADH, and $NaNO_2$ were fixed at 1.6 mM.

Artificial RBC Models (Hb-Vs) Encapsulating NADH and HbO_2—We prepared artificial RBC models (Hb-Vs) encapsulating the purified and concentrated HbO_2 solution (40 g/dL, 6.2 mM) and NADH (0, 3.1, 6.2, 12.5, and 25 mM, corresponding to a molar ratio of NADH to Hb of 0, 0.5, 1.0, 2.0, and 4.0, respectively). The levels of metHb were monitored during incubation at 37 °C for 25 h. The metHb formation was suppressed with increasing NADH concentration (**Fig. 4A**). The plots of the metHb level at 24 h versus NADH concentration (**Fig. 4B**) demonstrate that NADH concentrations at an equivalent or higher molar

ratio to Hb have a sufficient inhibitory effect on metHb formation. Pseudo-CAT and pseudo-SOD activities in Hb-Vs co-encapsulating NADH were determined at each NADH/Hb molar ratio as follows: NADH/Hb = 1 (CAT: 17.1±2.0 $\times 10^4$ IU/g Hb, SOD: 12.4±1.5$\times 10^2$ U/g Hb); NADH/Hb = 2 (CAT: 17.8±1.3$\times 10^4$ IU/g Hb, SOD: 12.8±2.2$\times 10^2$ U/g Hb), and NADH/Hb = 10 (CAT: 21.5±1.1$\times 10^4$ IU/g Hb, SOD: 16.8±1.6 $\times$ 10^2 U/g Hb). These pseudo-enzymatic activities in Hb-Vs were lower than those observed in Hb solutions (**Table 2**).

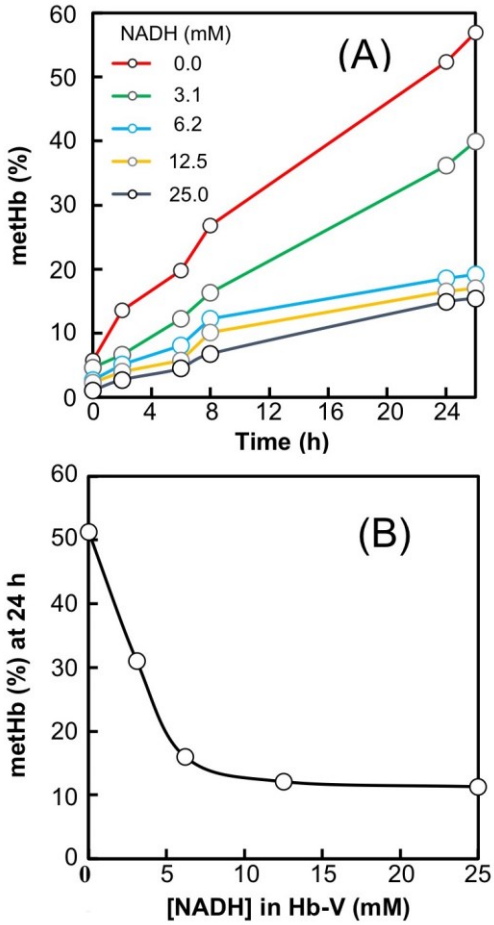

FIGURE 4. Artificial RBC models (Hb-Vs) encapsulating HbO_2 and NADH show suppressed metHb formation depending on the amount of encapsulated NADH during incubation at 37 °C. (A) Time course of metHb formation of Hb-Vs with different NADH co-encapsulation. (B) Relation between level of metHb (%) at 24 h and NADH concentration in Hb-Vs.

NADH in Hb-Vs Suppresses Oxidant-enhanced MetHb Formation—The level of metHb formation was measured in Hb-Vs after the addition of oxidants, H_2O_2, $NaNO_2$, and NOC7 (**Fig. 5**). In the absence of NADH co-encapsulation, all three oxidants enhanced metHb formation. In contrast, co-encapsulation of NADH showed a clear inhibitory effect on metHb formation. The level of metHb was decreased to approximately one-third that of the case without NADH. This inhibitory effect observed in Hb-Vs was consistent with results obtained for the Hb solution (**Fig. 1**).

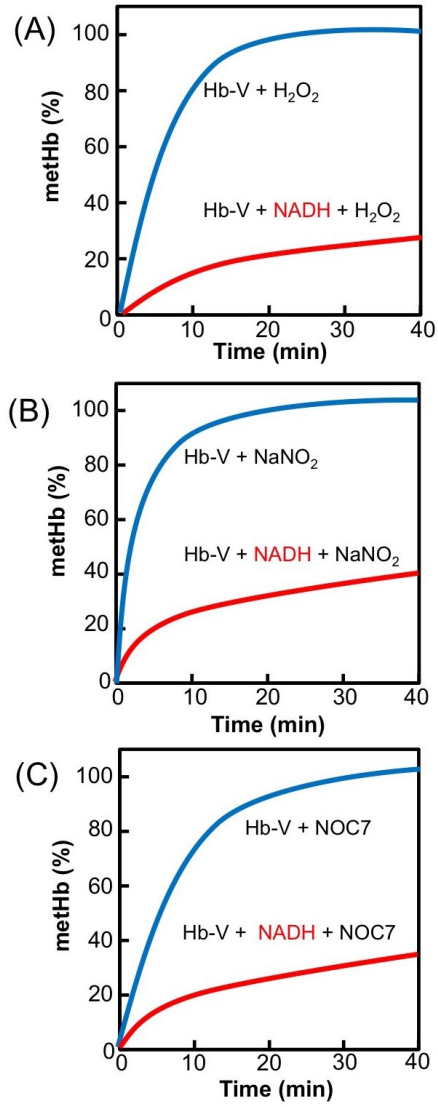

FIGURE 5. Time course of metHb formation in Hb-V with or without co-encapsulation of NADH following the addition of oxidants, (A) H_2O_2, (B) $NaNO_2$, and (c) NOC7, in a cuvette at 25 °C. NADH and Hb concentration in the Hb-V was 6.2 mM. In this experiment, the level of metHb (%) was calculated from the absorbance at 630 nm, assuming that the level at 40 min after the addition of oxidants to the Hb-V without NDAH was 100%.

In Vivo Inhibitory Effects of NADH on MetHb Formation—Hb-V dispersion ([Hb] = 10 g/dL) containing different concentrations of NADH (NADH/Hb molar ratio = 0, 1, or 2) was injected intravenously to rats (10 mL/kg body weight). The level of metHb in Hb-V without NADH increased to about 40% at 7 h after injection. By contrast, in the Hb-V containing NADH, metHb formation was suppressed significantly ($p < 0.001$) to approximately half, thereby prolonging the functional half-life of Hb-V (**Fig. 6**). No significant difference was found in inhibitory effects on metHb formation between the two concentrations of NADH (NADH/Hb molar ratio = 1 and 2). No apparent toxic sign was observed in any rat receiving injections of Hb-V, irrespective of the presence or absence of NADH.

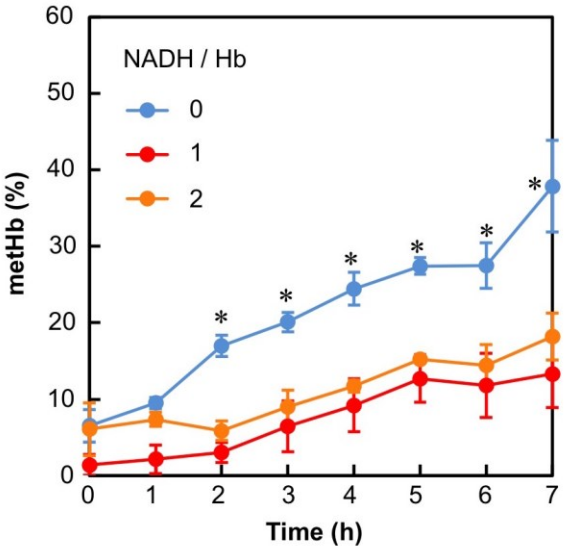

FIGURE 6. Time course of metHb formation in the healthy rats following intravenous injection of the Hb-V containing NADH. Hb-V containing NADH (NADH/Hb molar ratio = 0, 1, and 2) was injected intravenously to Wistar rats ($n = 3$), and blood samples were collected hourly for metHb concentration up to 7 h after injection. Each plot is shown as mean ± S.D. * $p < 0.01$ versus other Hb-Vs containing NADH.

3. DISCUSSION

NADH serves various key roles in basal metabolism, respiration, glycolysis, lipolysis, synthesis of ATP, and other enzymatic reactions. Moreover, NADH is known as a coenzyme for NADH-cytochrome b_5 reductase in RBCs, which is involved in reduction of ferric metHb to ferrous Hb (**Fig. 7A**). Our study demonstrated that NADH alone without enzymes inhibited metHb formation. Moreover, our study elucidated its inhibitory mechanism. We found that NADH alone or coexisting with Hb had a function to eliminate oxidants including H_2O_2, $NaNO_2$, and NO, that are generated in the body and which enhance metHb formation, resulting in metHb formation inhibition **(Fig. 7B)**. NADH with coexistence of Hb shows strong pseudo-CAT and pseudo-SOD activities. NADH reacts with NO in aerobic conditions. They are evidence of a newly identified function of NADH. The function is effective for inhibiting metHb formation independently from known enzymatic pathways. Additionally, we demonstrated the possibility that the new NADH function is applicable for an artificial oxygen carrier (Hb-V).

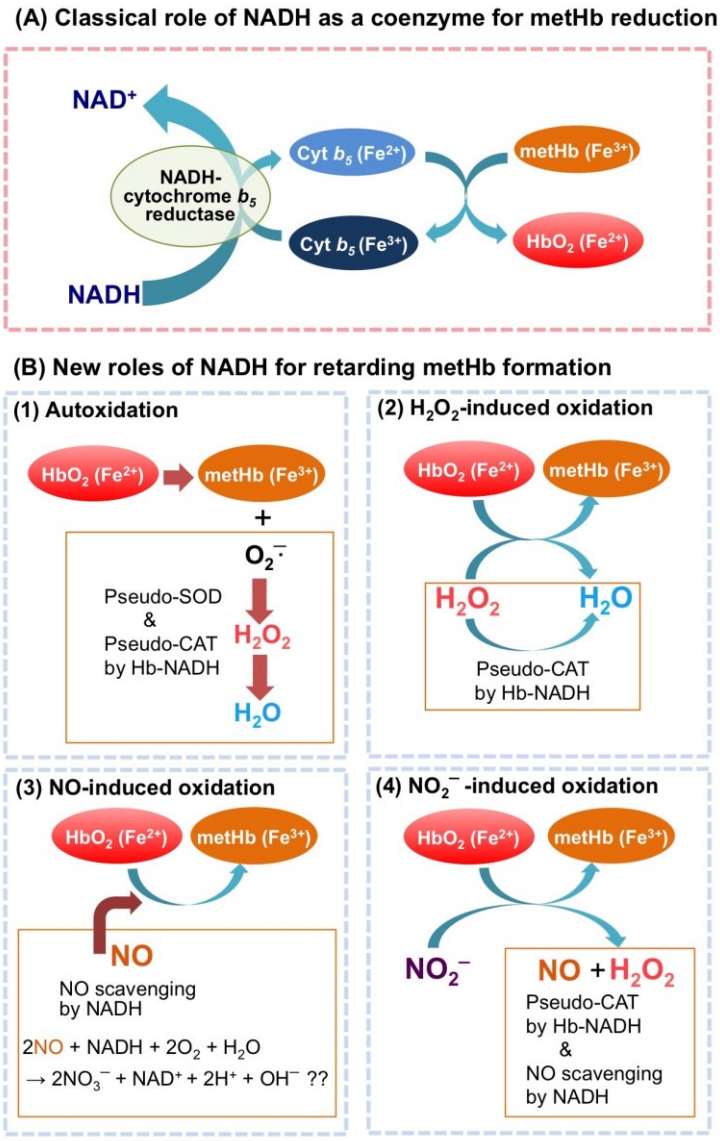

FIGURE 7. (**A**) NADH, known as a coenzyme for NADH-cytochrome b_5 reductase, is involved in reduction of ferric metHb to ferrous Hb via cytochrome b_5. (Cyt b_5) (**B**) Mechanistic hypotheses of inhibitory effects of NADH on metHb formation induced by autoxidation and addition of oxidants. (1) HbO_2 autoxidizes to form metHb and $O_2^{-}\cdot$. NADH with coexistence of HbO_2 demonstrates pseudo-SOD and -CAT activities that eliminate $O_2^{-}\cdot$ and H_2O_2, resulting in inhibition of metHb formation. (2) HbO_2 is oxidized by H_2O_2. NADH with coexistence of HbO_2 has pseudo-CAT activity to eliminate H_2O_2 resulting in inhibition of metHb formation. (3) HbO_2 is oxidized by NO to metHb. NADH reacts directly with NO in aerobic condition, resulting in inhibition of metHb formation in part. (4) NO_2^{-} does not react directly to NADH. However, H_2O_2 and NO produced during reaction between HbO_2 and NO_2^{-} can be eliminated by NADH in the same way as reactions in (2) and (3), resulting in inhibition of metHb formation.

The mechanism of autoxidation of HbO_2 is well known:[20] HbO_2 gradually autoxidizes to form metHb releasing $O_2^{-}\cdot$.

$$Hb(Fe^{2+})O_2 \rightarrow metHb(Fe^{3+}) + O_2^{-}\cdot \quad \textbf{(Eq. 1)}$$

$O_2^{-}\cdot$ is known to further react to HbO_2.

$$Hb(Fe^{2+}) + O_2^{-}\cdot + 2H^{+} \rightarrow metHb(Fe^{3+}) + H_2O_2 \quad \textbf{(Eq. 2)}$$

$O_2^{-}\cdot$ is converted to H_2O_2 via disproportionation reaction, enhancing oxidation of HbO_2.

$$2O_2^{-}\cdot + 2H^{+} \rightarrow H_2O_2 + O_2 \quad \textbf{(Eq. 3)}$$

$$2Hb(Fe^{2+})O_2 + H_2O_2 + 2H^{+} \rightarrow 2metHb(Fe^{3+}) + 2H_2O + O_2 \quad \textbf{(Eq. 4)}$$

All potential antioxidants examined in our screening demonstrated some inhibitory effects on autoxidation of Hb (**Table 1**), probably because ROS are expected to react with the compounds to some degree. Among them, NADH demonstrated the strongest inhibitory effect. It is consistent with our results that H_2O_2 is eliminated via direct interaction with NADH as follows[21] shown below.

$$2H_2O_2 + 2NADH \rightarrow 2NAD^{+} + 3H_2O + 1/2O_2 \quad \textbf{(Eq. 5)}$$

$$(2H_2O_2 + 4NADH \rightarrow 4NAD^{+} + 4H_2O) \quad \textbf{(Eq. 6)}$$

With a structure resembling NADH, NADPH can show the same reactions as Eq. 5 and 6. The effect of NADPH, however, was not as strong as NADH, which might be attributed to its degradation in an early phase of 25 h incubation at 37°C, presumably because NADPH lacks thermal stability.[22]

NADH with coexistence with HbO_2 demonstrated a stronger pseudo-CAT activity than NADH did alone, indicating that the coexistence with HbO_2 can enhance the H_2O_2 elimination effect.[23] The following mechanism hypothesis can explain how NADH demonstrates a pseudo-

CAT activity. MetHb reacts to H_2O_2 at first, generating ferryl Hb radical (Fe^{4+}=O) (Eq. 7), which then interacts with NADH, providing it with two electrons to eliminate H_2O_2 and to convert ferrylHb to metHb (Eq. 8).

$$\text{H-metHb}(Fe^{3+}) + H_2O_2 \rightarrow \cdot\text{ferrylHb}(Fe^{4+}{=}O) + H_2O + H^+ \quad \textbf{(Eq. 7)}$$

$$\cdot\text{ferrylHb}(Fe^{4+}{=}O) + \text{NADH} + 2H^+ \rightarrow \text{H-metHb}(Fe^{3+}) + NAD^+ + H_2O \quad \textbf{(Eq. 8)}$$

A similar mechanism has also been reported previously for pseudo-CAT activity of tyrosine with Hb.[14] However, NADH demonstrated much greater activity than tyrosine. Because combination of NADH with HbO_2 showed higher activities than with metHb, partially oxidized metHb of four subunits might be involved in the high pseudo-enzymatic activities. Although the mechanism of pseudo-SOD activity of NADH has not yet been elucidated, it can reduce the amount of $O_2^{-}\cdot$, inhibiting the reaction of $Hb(Fe^{2+}) + O_2^{-}\cdot$ (Eq. 2), and enhancing the reaction of Eq. 3, a disproportionation reaction to H_2O_2. Furthermore, the pseudo-CAT activity contributes to elimination of H_2O_2, leading to overall retardation of metHb formation.

NO is produced endogenously by NOS. A large quantity of vascular endothelium-derived NO is produced. Under inflammatory conditions, overproduction of NO occurs because of inducible NOS. NO reacts to HbO_2, thereby enhancing metHb production.

$$Hb(Fe^{2+})O_2 + NO \rightarrow \text{metHb}(Fe^{3+}) + NO_3^- \quad \textbf{(Eq. 9)}$$

Our study demonstrated that NO from NOC7 interacts with NADH only in aerobic conditions. The reaction of Eq. 10 producing N_2O was reported for a certain type of hemeprotein from soil bacteria.[24] However, in our experiment, N_2O was not detected with GC-MS analysis, ruling out the possibility of a reaction of Eq. 10. Consequently, it is considered that NO is converted to

NO_3^- via the reaction of Eq. 11.

$$2NO + NADH + H^+ \rightarrow N_2O + NAD^+ + H_2O \quad \textbf{(Eq. 10)}$$

$$2NO + NADH + 2O_2 + H_2O \rightarrow 2NO_3^- + NAD^+ + 2H^+ + OH^- \quad \textbf{(Eq. 11)}$$

Probably, metHb formation was retarded partly by elimination of NO in this reaction. In addition, NO can react with oxygen in the solution, producing nitrite anion.[25]

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad \textbf{(Eq. 12)}$$

Therefore, additions of both NO and $NaNO_2$ enhance metHb formation because of the reaction involving NO_2^- (Eq. 13).

$$4NO_2^- + 4Hb(Fe^{2+})O_2 + 4H^+ \rightarrow 4NO_3^- + 4metHb(Fe^{3+}) + O_2 + 2H_2O + 4H^+ \quad \textbf{(Eq. 13)}$$

Regarding Eq. 13, the following elementary reactions are known to be involved.[26]

$$[Hb(Fe^{2+})O_2]^{2+} + NO_2^- \rightarrow [Hb(Fe^{2+})O_2NO_2]^+ \quad \textbf{(Eq. 14)}$$

$$[Hb(Fe^{2+})O_2NO_2]^+ + NO_2^- \rightarrow [Hb(Fe^{2+})OONO^-]^+ + NO_3^- \quad \textbf{(Eq. 15)}$$

$$[Hb(Fe^{2+})OONO^-]^+ + 2H^+ \rightarrow Hb(Fe^{3+}) + NO + H_2O_2 \quad \textbf{(Eq. 16)}$$

Both H_2O_2 and NO, respectively produced from the reaction of Eq. 16, are expected to promote metHb formation via reactions of Eqs. 4 and 9. NADH reracts with H_2O_2 and NO (Eqs. 5, 6, and 11) and Hb-NADH shows pseudo enzymatic activities, demonstrating inhibitory effects on NO_2^--induced metHb formation. **Figure 7B** summarizes the mechanistic hypotheses of inhibitory effects of NADH on metHb formation.

We have newly identified NADH's own distinctive function, *i.e.,* inhibitory effects on metHb formation, which requires no enzymes. NADH alone also showed pseudo-enzymatic activities based on the measurement principle. However, NADH coexisting with HbO_2 showed

much higher pseudo-enzymatic activities. Cashon *et al.*[27] reported that NADH binds to the cleft of β-subunits in deoxyHb where 2,3-diphosphoglyrerate binds, but it does not bind to HbO_2. Because our experiment was performed with HbO_2, we speculate that the binding site of NADH is not the cleft of β-subunits but somewhere else through a weak interaction. Further study is required to clarify the interaction of NADH and HbO_2 by calorimetric analysis and docking simulation.

We encapsulated only NADH and Hb, but no enzymes into highly biocompatible liposome to produce artificial RBCs (Hb-Vs) for the evaluation of inhibitory effects of the NADH-Hb system on metHb formation. *In vitro* experiments using incubation at 37 °C demonstrated NADH concentration-dependent inhibition on the metHb formation rate. For effective inhibition, NADH at molar equivalent to Hb or greater concentration was necessary. In fact, this NADH concentration is approximately 100 times greater than that present in human RBCs (NAD^+, 0.065 mM; NADH, 0.00028 mM; 0.06528 mM total).[28] In the case of metHb formation enhanced by H_2O_2, $NaNO_2$, or NO, co-encapsulation of NADH at a molar equivalent to Hb showed effective suppression of metHb formation. These effects are induced both by the own reactivity of NADH to the oxidants and pseudo-enzymatic activities of NADH-Hb eliminating oxidative substances. Pseudo-CAT and pseudo-SOD activities were lower in the Hb-V dispersion than in the Hb solution, probably because oxidants such as H_2O_2 and pyrogallol, which were externally added to measure enzymatic activities, could not penetrate the lipid membrane of Hb-Vs: encapsulation protected Hb. Also, we performed intravenous injection of NADH-co-encapsulated Hb-V in rats for *in vivo* validation. NADH prevented metHb formation in the Hb-V to a significant degree. These results show a novel effect of NADH: it prevents

metHb formation without corresponding enzymes.

Our study outcomes are applicable for artificial oxygen carriers (transfusion alternatives) using Hb. Practical application of a long shelf life artificial oxygen carrier without blood type antigen or any pathogen is expected to be beneficial to the blood donation and transfusion systems.[29–31] Hb shows various toxicities once it is released from RBCs into circulating blood. Therefore, the procedures to increase molecular size with intermolecular cross-linking (polymerization) or polymer conjugation have been developed. However, they have not been approved because of remaining adverse events associated with molecular Hb. We have been developing artificial RBCs (Hb-Vs) encapsulating purified and concentrated Hb in liposomes that mimic corpuscular structure of RBCs to shield toxicities of molecular Hbs.[10–11] For clinical application, Hb must be virus-free. The Hb purification process includes heat treatment (60 °C) for virus inactivation and nanofiltration for virus removal. Although these treatments guarantee the utmost safety of Hb-V from infection, unstable enzymes originally present in RBCs are eliminated completely.[32] Consequently, metHb formation after intravenous injection of Hb-Vs has persisted as a challenging task. Our *in vivo* results indicate that the addition of NADH might resolve the issue of metHb formation in the Hb-V to some extent. Under some pathological conditions, biological defense mechanisms produce more ROS and RNS. Co-encapsulation of NADH to the Hb-V would provide a protective function against ROS and RNS. However, autoxidation of HbO_2 in Eq.1 cannot be inhibited. Further research is required to find a method to reduce ferric metHb to ferrous Hb to maintain a lower level of metHb.

4. CONCLUSION

Our study has demonstrated that, with the coexistence of HbO_2, NADH shows a hidden anti-oxidative function at a higher concentration than normal. Moreover, it retards metHb formation. The physiological role of this hidden function of NADH in a normal condition is not known, however, it would be conceivable that such anti-oxidative function of Hb-NADH works in a genetic disease such as deficiency of an enzymatic system. Moreover, this procedure is expected to provide twice the functional longevity of artificial oxygen carriers, Hb-Vs, greatly extending their practical usage.

5. MATERIALS AND METHODS

Purification of Hb from Human RBCs—Experiments using human derived Hb were approved by the ethical committee of Nara Medical University. Hb was purified from RBCs following the previous reports.[32–33] Outdated packed RBCs were provided from the Japanese Red Cross Society. After the supernatant plasma fraction was removed from the centrifuged sample, an approximately equivalent volume of saline was added for redispersion. Then it was again centrifuged, followed by removal of the supernatant. This process was repeated three times to obtain the purified and condensed RBC fraction. Distilled water for injection (Otsuka Pharmaceutical) was added gradually to induce hypotonic hemolysis. Then the Hb solution was permeated across ultrafiltration membrane (cut-off molecular weight, 1000 kDa, Biomax, Millipore Corp.) to remove stroma components. Subsequently, carbon monoxide gas was

ventilated for the conversion from HbO_2 to HbCO; then the solution was heated at 60°C for 12 h. After it was cooled, denatured insoluble protein mass was removed by centrifugation. Ultrafiltration (cut-off molecular weight, 1000 kDa) and nanofiltration was performed (Viresolve; Millipore Corp.). Using an ultrafiltration membrane (cut-off molecular weight, 8 kDa), deionization and concentration were repeated to obtain purified HbCO solution (40 g/dL, metHb < 5%, pH 7.4). Presence or absence of residual enzymatic activities in the Hb solution was ascertained following the procedures described later.

Screening of Compounds for Inhibitory Effect on HbO_2 Autoxidation—The experiment was performed with the following 26 compounds. Nine amino acids (L-tyrosine, L-arginine, L-glutamine, L-tryptophane, L-lysine, L-histidine, L-asparagine, L-cysteine, and L-methionine), two antioxidant agents (quercetin and astaxanthin), and compounds regarded as having low toxicity including sodium L-ascorbate, D-glucose, sodium gluconate, glycerol, nicotinamide, ATP, and NADPH were purchased from Sigma-Aldrich Corp. Human serum albumin (Albumin 25% I.V. 5 g/20 mL-BENESIS®) was purchased from Japan Blood Products Organization. PEG2000 was from Merck. Hydroxyethyl starch was from Otsuka Pharmaceutical Co. Ltd. , PEG400, PEG200, hyaluronic acid sodium salt and glutathione were from Wako Pure Chem. Ind. Ltd. NADH was purchased from Oriental Yeast Co., Ltd. The purity of NADH was 98.7% according to the manufacturer and the impurity consists of NAD^+ and water.

The HbCO solution was diluted to 10 g/dL with saline and poured in an eggplant flask. It was exposed to visible light (halogen lamp: LPL Videeoligtvl-302, LPL Co., Ltd.) under an oxygen stream for conversion of HbCO to HbO_2. To HbO_2 solution, each of 26 compounds

except hyaluronic acid at 1 g/dL was added. Because of low solubility of hyaluronic acid, it was dissolved at 0.2 g/dL. The solution was incubated at 37°C for 24 h. The spectra from 300 nm to 500 nm were measured using a spectrophotometer (V-660; Jasco Corp.) equipped with an integral sphere unit (ISV-722) to minimize the influence of light scattering. The level of metHb was determined based on the ratio of absorbance at 405 nm (λ_{max} of metHb) relative to that at 430 nm (λ_{max} of deoxyHb).

MetHb Formation by Oxidants and Inhibitory Effect of NADH—Three oxidants that promote metHb formation were used: 1) 1-hydroxy-2-oxo-3-(*N*-methyl- 3-aminopropyl)-3-methyl-1-triazene (NOC7; Dojindo Molecular Technologies Inc.), 2) $NaNO_2$ (Wako Pure Chemical Industries Ltd.), and 3) 30 wt% H_2O_2 (Wako Pure Chemical Industries Ltd.). The HbO_2 solution (30 μL, 10.1 g/dL) prepared with the method described above was diluted with 3 mL of PBS (pH 7.4; Gibco Life Technologies Co.) (final Hb concentration, 100.8 mg/dL, 15.6 μM); then it was incubated with each of the oxidants at the molar equivalent to that of HbO_2 at 37°C. The metHb-specific absorbance at 630 nm was measured to evaluate the effect of NADH on metHb formation. In addition, the time course of spectrum from 590 to 670 nm was measured.

Reactivity of NADH with Oxidants and MetHb—NADH was dissolved in PBS (160 μM). The NADH-specific absorbance at 340 nm was monitored with each of the oxidants, NOC7, $NaNO_2$, and H_2O_2 at 25°C for 10 min in aerobic conditions. In addition, the time course of the spectrum from 590 to 670 nm was measured. To confirm the NO reaction in an anaerobic condition, incubation with NOC7 was performed in a Thumberg cuvette ventilated with N_2 gas in advance. For detection of N_2O, a mixed sample of NOC7 and NADH in aerobic conditions in a

sealed glass container was collected with a gastight syringe and was analyzed using GCMS-QP2010 (Shimadzu Corp.) equipped with a column (CP-PoraBOND Q, Varian-Finnigan), performed by Kobelco Research Institute Inc.

For confirmation of the absence of a reaction between metHb and NADH, a metHb solution was prepared from the purified HbO_2 based on the method reported by Di Iorio.[34] A 30 μL of the metHb solution (10 g/dL) was added to 3 mL of PBS ([Hb]=101 mg/dL, 15.6 μM), and the equivalent molar of NADH was added. The time course of change in 630 nm absorbance was measured for comparison with the case without NADH.

Pseudo-Enzymatic Activities of NADH-Hb Mixed System—$K_3Fe(CN)_6$ and methylele blue were purchased from Sigma-Aldrich. Pyrogallol was from Wako Pure Chemical Industries Ltd. The following four enzymatic and pseudo-enzymatic activities were measured. 1) The activity of NADH-metHb reductase was measured as activity of NADH-ferricyanide reductase.[35–36] The activity was measured by monitoring the change in 340 nm absorbance reflecting the oxidation rate of NADH as a substrate consumed by the reduction of $K_3Fe(CN)_6$. 2) The activity of NADPH diaphorase corresponding to NADPH metHb reductase and NADPH-flavin reductase in RBCs was measured based on reduction of methylene blue with NADPH as a substrate.[37] The activity was measured by monitoring the change in 340 nm absorbance reflecting the NADPH reduction rate. 3) CAT activity was measured in 1000 times diluted Hb solution based on the change in 230 nm absorbance of H_2O_2.[38] Michaelis constant K_m and k_{cat} of equimolar NADH to Hb were measured using Lineweaver-Burk plot. 4) SOD activity was measured in 1000 times diluted Hb solution using pyrogallol autoxidation method.[39–40] Pyrogallol autoxidizes with

generating superoxide; then the solution becomes yellow with 420 nm absorbance. This reaction is inhibited by the presence of SOD. The SOD activity was measured based on the inhibitory effect. The analytes included purified HbO_2, HbCO, and metHb solutions with or without NADH or L-tyrosine. In addition, stroma-free hemolysates (SFHL) prepared from blood of three individuals were examined for all enzymatic activities.

NO Emissions by Mixing HbO_2 and $NaNO_2$ and Effects of NADH—NO concentration was examined using a polarographic electrode (NO-502; Eiko Kagaku Ltd.). To a 30 mL glass container equipped with an electrode containing 18 mL of PBS was added with an 18 μL of HbO_2 solution (10 g/dL) to 1.6 mM concentration. When NO concentration as a baseline became steady after mixing, $NaNO_2$ in PBS solution was added to the equivalent molar to HbO_2. NO concentration was measured every 10 s for 5 min. Addition of NADH at 1.6 mM was tested in the same manner. As blank controls, the sample containing either NADH alone, HbO_2 alone, $NaNO_2$ alone, a mixture of NADH and HbO_2, or a mixture of NADH and $NaNO_2$ were also examined.

Preparation of Hb-Vs Co-encapsulating NADH and Their Autoxidation Rates—As an allosteric effector, pyridoxal 5'-phosphate (PLP; Sigma-Aldrich Corp.) neutralized with NaOH at the equivalent molar to the Hb, was added to a 10 mL of the purified and concentrated HbCO solution (40 g/dL, 6.2 mM). Subsequently, NADH at 0, 3.1, 6.2, 12.4, or 24.8 mM (corresponding to 0, 0.5, 1.0, 2.0, or 4.0 as molar ratio to Hb) was added. Lipid constituents of Hb-Vs included 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol and 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (purchased from Nippon Fine Chem., Co, Ltd.), and 1,2-

distearoyl-*sn*-glycero-3- phosphatidylethanolamine-*N*-PEG_{5000} (purchased from NOF Corp.). After vesicle sizes were controlled using the method reported previously,[13] unencapsulated Hb in supernatant was removed by ultracentrifugation (50,000 × *g*, for 1 h), and the Hb-V precipitate was redispersed with PBS to 10 g/dL Hb concentration. HbCO was converted to HbO_2 with exposure to the visible light under oxygen stream as described above. The Hb-V solutions with various NADH concentrations were incubated in the thermostat bath at 37°C to measure metHb formation rate for 25 h. In addition, the Hb-Vs were examined for CAT and SOD activities in the manner described above.

Inhibitory Effect of NADH on MetHb Formation in Hb-V by oxidants—Following the experimental procedure of Hb solution described above, Hb-V diluted with PBS ([Hb] = 15.6 μM) was incubated with NOC7, $NaNO_2$, or H_2O_2 at the equivalent molar to Hb at 37°C for the evaluation of the change in metHb-specific 630 nm absorbance.

In Vivo Effect of NADH on MetHb Formation in Hb-V—Animal experiment protocols were approved by the Animal Care and Use Committees of Nara Medical University. Male Wistar rats (9 animals, 290–420 g body weight; Oriental Bioservice Co.) were used. The animals were anesthetized with 1% isoflurane (Pfizer Japan Inc.) inhalation (FiO_2 = 21%; 1.5 L/min flow rate) while maintaining spontaneous respiration. A polyethylene catheter (outer diameter, 0.8 mm, inner diameter, 0.5 mm, SP-31; Natsume Seisakusho Co., Ltd.) filled with 10 IU/mL heparin (Mochida Pharmaceutical Co. Ltd.) in saline was inserted into the left femoral artery and vein.[41] Three Hb-Vs without NADH (control), and with NADH at the molar equivalent to Hb and twice equivalent molar to Hb (*n* = 3) were examined. After intravenous injection of Hb-V

(10 mL/kg, 1 mL/min), blood samples (approximately 100 μL) were collected from the artery every hour into heparin-coated glass capillaries (Hirschmann Laborgeraete GmbH & Co. KG). The capillaries were centrifuged at 12,000 rpm for 1 min (Model 3220; Kubota Corp.). Because of this process, RBCs were precipitated, and a plasma supernatant containing Hb-Vs was obtained. A 20 μL of the plasma supernatant was added to a 3 mL of PBS in a Thumberg cuvette, sealed with the rubber plug, and bubbled with N_2 gas for deoxygenation for 5 min. Consequently, the solution contains two constituents of metHb and deoxyHb. The ultraviolet–visible absorption spectrum was measured for the determination of metHb formation rate based on the previously reported method.[13] Anesthesia was maintained during the experiment. After the final blood sample collection at 7 h, the rats were euthanized by overdose anesthesia and bleeding from the abdominal aorta.

Statistical Analysis—All plots in the graphs of *in vivo* experiemnts are expressed as average ± S.D. (n = 3). Two-way ANOVA multiple analyses were conducted using software (Prism 7 for Mac OS X; GraphPad Software Inc.) for comparisons among the groups. Differences were inferred as significant for $p < 0.01$.

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