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Characterization of hydrogen peroxide removal reaction by hemoglobin in the presence of reduced pyridine nucleotides

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Abstract

Hydrogen peroxide removal rates by hemoglobin were enhanced in the presence of reduced pyridine nucleotides. The species which had the activity to oxidize pyridine nucleotides was purified from human blood and identified as hemoglobin A. Hydrogen peroxide removal rates by hemoglobin A without reduced pyridine nucleotides at 0.2 mM hydrogen peroxide were 0.87 ± 0.11 μmol/s/g hemoglobin, and the removal rates using 0.2 mM NADH and NADPH were 2.02 ± 0.20 and 1.96 ± 0.31 μmol/s/g hemoglobin, respectively. We deduced that the removal reaction by hemoglobin included formations of methemoglobin and the ferryl radical and reduction of the latter with pyridine nucleotides. The hydrogen peroxide removal ability by hemoglobin was less than that by catalase but was larger than that by glutathione peroxidase-glutathione reductase system at 0.2 mM hydrogen peroxide. Under acatalasemic conditions, it was suggested that NAD(P)H were important factors to prevent the oxidative degradation of hemoglobin.

Keywords: Takahara disease; Acatalasemic erythrocyte; Hydrogen peroxide removal; Hemoglobin; Reduced pyridine nucleotide; Glutathione peroxidase

1. Introduction

In 1952, Takahara reported that Japanese acatalasemia patients suffered from progressive oral gangrene (Takahara's disease) by infection of hydrogen peroxide-generating bacteria [1]. Takahara's disease formerly occurred in about half of Japanese acatalasemia patients. It is now rare though the reasons are not known at present [2]. As Ogata et al. [3] suggested that the severity of catalase (EC 1.11.1.6) deficiency in blood was an important factor to cause the disease, we were interested in investigating hydrogen peroxide removal ability in acatalasemic erythrocytes. Hydrogen peroxide removal rates in normal and acatalasemic mouse and human erythrocytes were examined [4,5]. We found a hydrogen peroxide removal activity by hemoglobin in hemolysates [5] and deduced that the removal rates by hemoglobin were substantial in acatalasemic human erythrocytes by comparison with the rates by residual catalase in acatalasemic mouse erythrocytes and glutathione peroxidase (EC 1.11.1.9) in human erythrocytes [5,6]. To clarify the property of the hydrogen peroxide removal by hemoglobin, we examined the reaction in the presence of reduced pyridine nucleotides (NAD(P)H), which are present at considerably high concentration in erythrocytes [7].

2. Materials and methods

2.1. Materials

Human blood samples were obtained from healthy adult male volunteers (29–51 years old). Heparin was used as an anticoagulant. Male mice (8 weeks old) of C3H/AnLCs^{a}Cs^{a} (normal) and C3H/AnLCs^{b}Cs^{b} (acatalasemia) strains established by Feinstein et al. [8] were maintained on a laboratory diet (MF diet, Oriental Yeast, Tokyo, Japan) and water ad libitum till experiments. Mouse blood samples were collected.

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DEAE-Sephadex A-50 was purchased from Pharmacia Biotech (Uppsala, Sweden), and CM-cellulose (CM 52) was purchased from Whatman Ltd. (Maidstone, UK). Chemicals of analytical grade were purchased from Sigma Co. Ltd. (St. Louis, MO, USA) or Wako Pure Chemical Ind. (Osaka, Japan).

2.2. General procedures

Protein concentration was determined by Biuret method, using bovine serum albumin as a standard. Hemoglobin contents were determined by the method of Drabkin and Austin [9]. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [10]. Isoelectric focusing was carried out according to the method of Ishino and Ubuka [11]: the slab polyacrylamide gel containing 2% carrier ampholite (Pharmalyte pH 3–10) was prepared in the presence of 10 mM potassium cyanide to analyze ferrous hemoglobin [12]. Authentic hemoglobin A was prepared from human blood using a DEAE-Sephadex column according to the Williams and Tsay method [13]. Methemoglobin was prepared by the oxidation of hemoglobin with potassium ferricyanide [14]. Pyridine nucleotide contents in erythrocytes were measured by the method of Wagner and Scott [7]. Concentration of hydrogen peroxide in stock solution (100 mM) was determined according to the modified method [15]. Ten milliliters of hydrogen peroxide solution was added to 25 ml of 5% sulfuric acid containing 0.25 g of potassium iodide. After 15 min, 1 drop of 3% ammonium molybdate solution was added to the mixture, and then liberated iodine in the mixture was titrated with 0.1N sodium thiosulfate solution using starch as an indicator.

2.3. Preparation of human and mouse hemoglobin solution

Hemoglobin was purified according to the method of Lynch et al. [14]. Erythrocytes were separated from blood at $850 \times g$ for 5 min and washed three times with 10 ml of phosphate-buffered saline (PBS, 140 mM sodium chloride solution and 10 mM potassium phosphate buffer at pH 7.4) [4]. The hemolysate was prepared by addition of 9 volumes of water to packed erythrocytes (0.4 ml). The hemolysate was centrifuged at $10{,}000 \times g$ for 30 min. The supernatant (2 ml) was applied to a CM-cellulose column (1.5×12 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.8). The column was washed with 68 ml of 5 mM potassium phosphate buffer (pH 6.8) and then target species was eluted with 55 ml of 1 M potassium phosphate buffer (pH 6.8). The fractions containing hemoglobin were combined. The concentration of hemoglobin was determined, and the prepared solution was immediately used for experiments. We used the molecular ratio of hydrogen peroxide to hemoglobin in range of 10–1100 for the following experiments.

2.4. Measurement of hydrogen peroxide removal by hemoglobin without NAD(P)H

Hydrogen peroxide removal rates by hemoglobin were measured according to the previous method [5]. PBS as a medium was replaced with 0.1 M potassium phosphate buffer to increase the buffering power of the reaction mixture. The mixture of 9.40 ml of 0.1 M potassium phosphate buffer (pH 7.1) and 0.4 ml of hemoglobin solution was incubated at 37 °C for 5 min. Then, 0.2 ml of 3.5 mM hydrogen peroxide was added to the mixture. After 0, 30 and 60 s, 2.0 ml of the reaction mixture (containing less than 140 nmol of hydrogen peroxide) was taken out and put into the test tubes containing 2.0 ml of the reagent solution [consisted of 10 volumes of 0.2 mM *meso*-tetrakis(4-methylpyridyl)-porphinatoiron(III) pentachloride solution, 10 volumes of 41.2 mM *N*,*N*-dimethylaniline in 0.2 M hydrochloric acid, 10 volumes of 8.56 mM 3-methyl-2-benzothiazolinone hydrazone solution in 0.2 M hydrochloric acid and 1 volume of 20 mM EDTA solution]. The mixture was incubated at 25 °C for 1 h, and then the absorbance at 590 nm was measured. Hydrogen peroxide removal rates by hemoglobin were calculated from the decrease of hydrogen peroxide.

2.5. Characteristics of hydrogen peroxide removal by hemoglobin without NAD(P)H

Hydrogen peroxide removal reaction was carried out at 0.0175–0.14 mM hydrogen peroxide at 37 °C. The kinetic parameters were obtained from Lineweaver–Burk plot analysis of the removal rates. Hydrogen peroxide removal reaction at 0.07 mM hydrogen peroxide was also carried out at various pHs. The rates at pH 4.0–5.5 were measured in 0.1 M potassium acetate buffers, and the rates at pH 5.5–7.5 were measured in 0.1 M potassium phosphate buffers. Hydrogen peroxide removal rates in the presence of 0.5 and 1.0 mM sodium azide were examined at 0.0175–0.14 mM hydrogen peroxide. Hydrogen peroxide removal rates under carbon monoxide gas were examined at 0.07 mM hydrogen peroxide. Carbon monoxide gas was gently passed through the hemoglobin solution in 0.1 M potassium phosphate buffer (pH 7.1) for 30 s, and then hydrogen peroxide was added to start the reaction.

2.6. Hydrogen peroxide removal by hemoglobin in the presence of NAD(P)H

2.6.1. Measurement of hydrogen peroxide removal rates

After the reaction by hemoglobin in the presence of NAD(P)H, the reaction mixture was diluted with water to less than 0.02 mM NAD(P)H. Then hydrogen peroxide content in the mixture was determined since high concentration of NAD(P)H (reducing agents) interfered with the color development of hydrogen peroxide [4]. The mixture of 2.76 ml of 0.1 M potassium phosphate buffer (pH 7.1) and 0.06 ml of 10 mM NAD(P)H solution and 0.12 ml of

hemoglobin solution was incubated at 37 °C for 5 min. Then, 0.06 ml of 10 mM hydrogen peroxide was added to the mixture. After 0, 30 and 60 s, 0.2 ml of the reaction mixture was taken out and was immediately put into a test tube containing 1.8 ml of water for dilution of the reaction mixture and 2.0 ml of the reagent solution for determination of hydrogen peroxide. As the control experiments, hemoglobin solution was replaced with equal volumes of denatured hemoglobin solution. The solution was prepared by heating hemoglobin solution at 80 °C for 10 min.

2.6.2. Measurement of pyridine nucleotide oxidation rates depending on hydrogen peroxide

The reaction mixture was prepared and maintained at 37°C as above. Then, 0.06 ml of 10 mM hydrogen peroxide was added to the mixture, and decrease of the absorbance at 340 nm was recorded for 1 min. Pyridine nucleotide oxidation rates (A) were calculated. Pyridine nucleotide oxidation rates (B) were obtained by replacing hemoglobin solutions with equal volumes of denatured hemoglobin solution. Pyridine nucleotide oxidation rates (C) were obtained by replacing 10 mM hydrogen peroxide solution with equal volumes of water. Hydrogen peroxide dependent pyridine nucleotide oxidation rates by hemoglobin solution were calculated as (A) − (B) − (C).

2.6.3. Characteristics of pyridine nucleotide oxidation depending on hydrogen peroxide

The reaction was examined at 0.07–2.0 mM hydrogen peroxide in the presence of 0.2 mM NAD(P)H and at 2 mM hydrogen peroxide in the presence of 0.025–0.2 mM NAD(P)H to obtain the kinetic parameters. The reactions at pH 4.0–7.5 were examined at 0.07 mM hydrogen peroxide in the presence of 0.2 mM NAD(P)H. The reactions in the presence of 0.5 and 1.0 mM sodium azide were examined at 0.2–2 mM hydrogen peroxide in the presence of 0.2 mM NAD(P)H. The reactions in the presence of 0.025–0.1 mM potassium cyanide were examined at 0.2–2 mM hydrogen peroxide in the presence of 0.2 mM NAD(P)H. The reactions under nitrogen (0% of oxygen), air (21%) and oxygen gas (100%) were examined as follows. The gas was gently passed through 2.82 ml of 0.1 M potassium phosphate buffer (pH 7.1) containing 0.2 mM NAD(P)H in a cuvette with a screw cap at 37 °C for 4 min. Hemoglobin solution (0.12 ml) was added, and the gas was further passed through it for 30 s. Then, 0.06 ml of 10–100 mM hydrogen peroxide solution was added to start the reaction, and the cuvette was immediately sealed with the cap. The reaction under carbon monoxide gas was examined at 2 mM hydrogen peroxide in the presence of 0.2 mM NAD(P)H.

2.7. Hydrogen peroxide removal in acatalasemic mouse hemolysates

As hydrogen peroxide was decomposed by a large amount of catalase in normal hemolysates, hemolysates from acatalasemic mouse were used to examine hydrogen peroxide removal reaction in hemolysates in the presence of NAD(P)H. Pyridine nucleotide oxidation rates depending on hydrogen peroxide were measured by replacing hemoglobin solution with equal volumes of 1% acatalasemic hemolysate.

2.8. Isolation of the active species for pyridine nucleotide oxidation depending on hydrogen peroxide from human hemolysates

Pyridine nucleotide oxidation activity depending on hydrogen peroxide was checked at 2 mM hydrogen peroxide in the presence of 0.2 mM NADH. Human hemolysate was prepared by addition of 6 volumes of water to packed erythrocytes (5 ml). After centrifugation of hemolysate at 10,000 × *g* for 30 min, the supernatant (20 ml) was applied to a CM-cellulose column (2.2 × 45 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.8). The column was washed with 490 ml of 5 mM potassium phosphate buffer (pH 6.8) followed by eluting with 210 ml of 1 M potassium phosphate buffer (pH 6.8). Fifteen-milliliter aliquots of the eluate were collected. The fractions which showed the activity were pooled. The solution was dialyzed with 2 l of 0.05 M Tris buffer (pH 8.0) for 2 h. The dialyzed solution (30 ml) was applied to a DEAE-Sephadex column (2 × 55 cm) equilibrated with 0.05 M Tris buffer (pH 7.6). The column was washed to elute species with activity using 540 ml of 0.05 M Tris buffer (pH 7.6) and then with 210 ml of 0.05 M Tris buffer containing 0.5 M sodium chloride (pH 7.6). Fifteen-milliliter aliquots of the eluate were collected. The fractions which showed the activity were pooled and used for the experiments.

2.9. Hydrogen peroxide removal by the active species in the presence of ascorbic acid or glutathione

Reaction of the purified species in the presence of 0.1 mM ascorbic acid was carried out at 0.2 mM hydrogen peroxide. The reaction mixture was diluted with 9 volumes of water to minimize the effect by ascorbic acid, and concentration of hydrogen peroxide in the mixture was determined. The reaction of the purified species in the presence of 2 mM reduced glutathione was carried out at 0.2 mM hydrogen peroxide. The reaction mixture was put into the test tube containing 0.2 ml of 100 mM *N*-ethylmaleimide to remove glutathione [4], and concentration of hydrogen peroxide in the mixture was determined.

2.10. Glutathione reductase activity and hydrogen peroxide removal rates by glutathione peroxidase in erythrocytes

Glutathione reductase activity (EC 1.6.4.2) in hemolysates was measured at 0.167 mM NADPH in the presence of 5 μM FAD and 2.2 mM oxidized glutathione by the method of Goldberg and Spooner [16]. The reaction was also

Table 1
Kinetic parameters of hydrogen peroxide removal reaction by hemoglobin[a]

	K_m (μM)	V_{max} (μmol/s/g Hb)
Mouse hemoglobin	105 ± 27 (5)	2.79 ± 0.16*
Human hemoglobin	93 ± 15 (4)	1.46 ± 0.12
Human methemoglobin	52 ± 18 (3)*	1.64 ± 0.24

Values were expressed as mean ± S.D. Number in parentheses indicated number of replicates.

[a] Hydrogen peroxide removal rates were measured in the presence of 0.0175–0.14 mM hydrogen peroxide.

* Significantly different from that of human hemoglobin ($P<0.01$).

examined at 0.017–0.167 mM NADPH to obtain the kinetic parameters. Hydrogen peroxide removal rates by glutathione peroxidase under simulated in vivo conditions were measured according to the previous method [6]. Glutathione contents in erythrocytes were measured, and then the removal rates by glutathione peroxidase were measured at the same concentration of glutahione in the presence of 0.14 mM hydrogen peroxide.

2.11. Statistics

Data obtained by the present study were analyzed with Student's *t*-test.

3. Results

3.1. Hydrogen peroxide removal by hemoglobin and methemoglobin without NAD(P)H

The kinetic parameters of the removal reaction catalyzed by mouse hemoglobin were indicated in Table 1. The optimal pH of the reaction was 5.0. Sodium azide acted as a competitive inhibitor of hydrogen peroxide removal reaction though we previously reported that sodium azide did not inhibit the reaction. The reaction at 0.07 mM hydrogen peroxide was inhibited 37.5 ± 5.0% ($n=3$) in the presence of 1 mM sodium azide. The inhibition constant was 0.81 ± 0.06 mM ($n=3$). We deduced that the differences from previous results [5] were mainly due to unexpected decomposition of sodium azide and pH change in the reaction mixture because of the weak buffering power of PBS. Carbon monoxide inhibited 72.1 ± 7.7% ($n=4$) of the original rates.

In the reaction catalyzed by human hemoglobin and methemoglobin, the kinetic parameters were indicated in Table 1. The optimal pH of the removal activity was 4.5.

3.2. Hydrogen peroxide removal by mouse hemoglobin in the presence of NAD(P)H

3.2.1. Hydrogen peroxide removal rates

Hydrogen peroxide removal by denatured mouse hemoglobin was not observed. Hydrogen peroxide removal rates by mouse hemoglobin at 0.2 mM hydrogen peroxide in the absence of NAD(P)H were 1.73 ± 0.32 μmol/s/g hemoglobin ($n=9$), and the rates in the presence of 0.2 mM NADH and NADPH were 3.54 ± 0.12 and 3.56 ± 0.25 μmol/s/g hemoglobin ($n=3$), respectively.

3.2.2. Pyridine nucleotide oxidation rates depending on hydrogen peroxide

Pyridine nucleotide oxidation with hydrogen peroxide in the presence of denatured mouse hemoglobin or with mouse hemoglobin did not occur. Pyridine nucleotide oxidation with hydrogen peroxide was only observed by the addition of mouse hemoglobin. Kinetic parameters of the oxidation reaction by mouse hemoglobin are listed in Table 2. In the reaction at 0.2 mM hydrogen peroxide in the presence of 0.2 mM NADH and NADPH, the oxidation rates by mouse hemoglobin were 3.62 ± 0.30 and 3.85 ± 0.24 μmol/s/g hemoglobin ($n=5$), respectively. Comparison between hydrogen peroxide removal rates and the pyridine nucleotide oxidation rates suggested that one molecule of pyridine nucleotide reacted with one molecule of hydrogen peroxide. The optimal pH of the reaction was 5.0. Inhibitions by saturated carbon monoxide, saturated oxygen and 0.5 mM sodium azide at 2 mM hydrogen peroxide in the presence of 0.2 mM NADH were 82.4 ± 0.3%, 22.6 ± 7.2% and 18.7 ± 4.5% ($n\geq 3$), respectively. The inhibition parameters were summarized in Table 3.

Table 2
Kinetic parameters of pyridine nucleotide oxidation depending on hydrogen peroxide

	NADH[a]		NADPH[a]		H_2O_2[b]	
	K_m (μM)	V_{max} (μmol/s/g Hb)	K_m (μM)	V_{max} (μmol/s/g Hb)	K_m (μM)	V_{max} (μmol/s/g Hb)
Acatalasemic mouse hemolysate	20.6 ± 3.2 (3)*	8.99 ± 0.63*	19.7 ± 3.2 (3)*	9.54 ± 0.40*	259 ± 10 (4)	9.15 ± 0.40*
Mouse hemoglobin	20.9 ± 3.7 (4)*	9.53 ± 0.78*	20.3 ± 2.7 (4)*	9.77 ± 0.42*	250 ± 20 (5)	9.62 ± 0.67*
Human hemoglobin A	12.7 ± 1.0 (3)	6.17 ± 0.47	14.3 ± 2.3 (3)	6.10 ± 0.48	273 ± 31 (3)	6.54 ± 0.54
Human methemoglobin A	11.9 ± 2.0 (3)	6.25 ± 0.24	9.2 ± 1.3 (3)*	6.42 ± 0.26	140 ± 11 (4)*	6.31 ± 0.18

Values were expressed as mean ± S.D. Number in parentheses indicated number of replicates.

[a] The oxidation rates were measured at 2.0 mM hydrogen peroxide in the presence of 0.025–0.2 mM NADH or NADPH.

[b] The oxidation rates were measured at 0.07–2.0 mM hydrogen peroxide in the presence of 0.2 mM NADH.

* Significantly different from that of human hemoglobin A ($P<0.05$). There is no significant difference between acatalasemic mouse hemolysate and mouse hemoglobin.

Table 3
Inhibition of pyridine nucleotide oxidation depending on hydrogen peroxide

	Dioxygen inhibition type inhibition constant (mM)	Azide inhibition type inhibition constant (mM)	Cyanide inhibition type inhibition constant (mM)
Mouse hemoglobin	Competitive (4) 0.26 ± 0.06 (K_i)	Mixed[a] (4) 0.79 ± 0.08 (K_i) 2.83 ± 0.30 (K_i')	Not determined
Human hemoglobin A	Competitive (3) 0.30 ± 0.04 (K_i)	Mixed (3) 0.98 ± 0.05 (K_i) 3.97 ± 0.25 (K_i')	Mixed (3) 0.038 ± 0.004 (K_i) 0.154 ± 0.021 (K_i')
Human methemoglobin A	Not determined	Mixed (3) 0.16 ± 0.04 (K_i)* 1.28 ± 0.61 (K_i')*	Mixed (3) 0.035 ± 0.010 (K_i) 0.196 ± 0.040 (K_i')

Values were expressed as mean ± S.D. Number in parentheses indicated number of replicates.
[a] Mixed meant competitive and noncompetitive inhibition of the reaction. The inhibition constants were indicated as K_i and K_i'.
* Significantly different from that of human hemoglobin ($P < 0.001$).

3.2.3. Hydrogen peroxide removal in acatalasemic mouse hemolysates

Hydrogen peroxide removal rates in the hemolysates at 0.2 mM hydrogen peroxide were 2.01 ± 0.24 μmol/s/g hemoglobin ($n = 5$). Hydrogen peroxide removal rates in the hemolysates at 0.2 mM hydrogen peroxide in the presence of 0.2 mM NADH were 3.82 ± 0.35 μmol/s/g hemoglobin ($n = 3$). Kinetic parameters of pyridine nucleotide oxidation reaction in the hemolysates were indicated in Table 2. These parameters were the same as those of the rates by mouse hemoglobin.

3.2.4. Isolation of the reactive species for the oxidation of pyridine nucleotide depending on hydrogen peroxide from human hemolysates

The active species in human hemolysates was eluted along with hemoglobin from a CM-cellulose column. The eluates between 585 and 630 ml were gathered and then purified by a DEAE-Sephadex column. The active species was eluted along with hemoglobin, and the eluates between 255 and 300 ml were gathered. Summary of the purified activity is shown in Table 4. SDS-polyacrylamide gel electrophoresis and isoelectric focusing at each step were indicated in Fig. 1A and B, respectively. The purified active species consists of two kinds of subunit and the p*I* is 7.1. The visible spectrum of the purified species, λ_{max} (ε_{mM}) 344 (102), 415 (444), 541(53.3), 576 (56.0) nm, is consistent with that of hemoglobin A [17]. The results indicated that the purified active species was hemoglobin A. Hydrogen peroxide removal rates by the purified active species at 0.2 mM hydrogen peroxide in the absence of NAD(P)H were 0.87 ± 0.11 μmol/s/g hemoglobin, and those in the presence of 0.2 mM NADH and NADPH were 2.02 ± 0.20 and 1.96 ± 0.31 μmol/s/g hemoglobin ($n = 4$), respectively. The oxidation rates of NADH and NADPH were 1.95 ± 0.30 and 2.05 ± 0.28 μmol/s/g hemoglobin ($n = 3$), respectively. Hydrogen peroxide removal rates in the presence of 0.1 mM ascorbic acid were 1.24 ± 0.16 μmol/s/g hemoglobin ($n = 3$). Hydrogen peroxide removal rates in the presence of 2 mM reduced glutathione were 0.83 ± 0.16 μmol/s/g hemoglobin.

Table 4
Purification of activity on pyridine nucleotide oxidation depending on hydrogen peroxide from human hemolysates

Step	Volume (ml)	Total activity	Specific activity	Yield (%)	Purification	A_{541}/A_{280}[a]
Hemolysate	15.0	3530	2.56	100	1.00	0.42
CM-Cellulose	45.0	3030	2.81	85.8	1.10	0.45
DEAE-Sephadex	45.0	2230	2.80	63.2	1.09	0.45

[a] Ratio of absorbances at 541 and 280 nm.

The kinetic parameters of the activity in the presence of NAD(P)H were listed in Table 2. The optimal pH was 4.5. Inhibitions of the reaction by saturated carbon monoxide, saturated oxygen, 0.5 mM sodium azide and 0.05 mM potassium cyanide at 2 mM hydrogen peroxide in the presence of 0.2 mM NADH were $83.9 \pm 1.5\%$, $28.8 \pm 3.8\%$, $15.2 \pm 2.9\%$ and $30.7 \pm 2.8\%$ ($n = 3$), respectively. The parameters were summarized in Table 3.

3.2.5. Hydrogen peroxide removal by human methemoglobin A

Hydrogen peroxide removal rates at 0.2 mM hydrogen peroxide in the absence of reduced pyridine nucleotides were 0.95 ± 0.20 μmol/s/g hemoglobin, and the removal rates in the presence of 0.2 mM NADH and NADPH were 1.90 ± 0.26 and 2.00 ± 0.34 μmol/s/g hemoglobin ($n = 3$), respectively. The kinetic parameters of pyridine nucleotide oxidation rates depending on hydrogen peroxide are summarized in Table 2. The pH dependence was indicated that the optimal pH was 4.5. Inhibition of the reaction by saturated carbon monoxide, 0.5 mM sodium azide and 0.05 mM potassium cyanide at 2 mM hydrogen peroxide in the presence of 0.2 mM NADH were $1.5 \pm 2.1\%$, $38.3 \pm 5.7\%$ and $28.5 \pm 3.5\%$ ($n = 3$), respectively. The parameters were summarized in Table 3.

3.2.6. Reduced pyridine nucleotide contents in erythrocytes

Average contents of NADH and NADPH in normal mouse erythrocytes were 31.2 ± 13.1 and 28.7 ± 1.5 μM ($n = 8$), respectively. Average contents of NADH and NADPH in acatalasemic mouse erythrocytes were 33.3

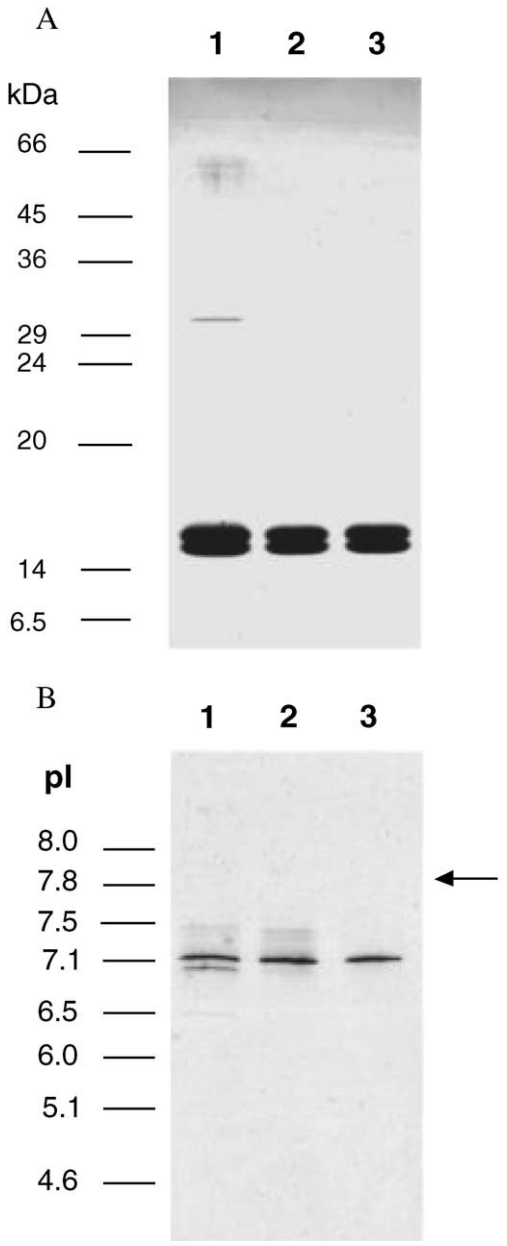

Fig. 1. Pyridine nucleotide oxidation activity depending on hydrogen peroxide purified from human hemolysates. Lane 1, human hemolysates; lane 2, fractions from a CM-cellulose column; lane 3, fractions purified with a DEAE-Sephadex column chromatography. After electrophoresis, gels were stained with Coomassie brilliant blue R-250. (A) SDS-polyacrylamide electrophoresis was carried out by using 15% polyacrylamide gel. The molecular size markers were indicated on left side. (B) Isoelectric focusing was carried out by using 2% carrier ampholite (pH 3–10). The arrow indicated the position of sample application. p*I* markers were indicated on left side.

± 10.1 and 28.0 ± 2.0 μM ($n = 4$), respectively. These values were of the same level in normal ones. Average contents of NADH and NADPH in human erythrocytes were 29.4 ± 8.2 and 29.3 ± 2.5 μM ($n = 4$), respectively. These contents in human erythrocytes were consistent with those reported in Ref. [7].

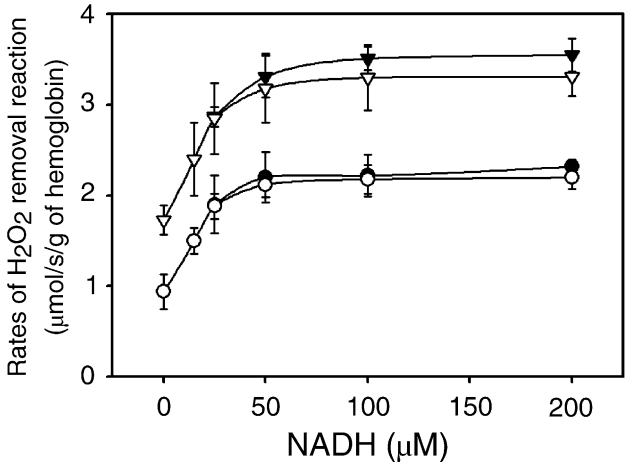

Fig. 2. Effect of pyridine nucleotide on hydrogen peroxide removal rates by hemoglobin. Hydrogen peroxide removal rates and NADH oxidation rates were measured at 0.2 mM hydrogen peroxide in the presence of 0–0.2 mM NADH. (▽) Hydrogen peroxide removal rates by mouse hemoglobin; (▼) pyridine nucleotide oxidation rates by mouse hemoglobin; (○) hydrogen peroxide removal rates by human hemoglobin A; (●) pyridine nucleotide oxidation rates by human hemoglobin A.

3.2.7. Effect of reduced pyridine nucleotide content on hydrogen peroxide removal rates by hemoglobin

Both hydrogen peroxide removal rates and pyridine nucleotide oxidation rates caused by mouse and human hemoglobin were examined at 0.2 mM hydrogen peroxide in the presence of 0–0.2 mM NADH. The results were indicated in Fig. 2. It showed that hydrogen peroxide removal rates by hemoglobin were enhanced in the presence of relatively small amounts of NAD(P)H.

3.2.8. Glutathione reductase activity and hydrogen peroxide removal rates by glutahione peroxidase in erythrocytes

Average glutathione reductase activities in acatalasemic and normal mouse hemolysates were 0.220 ± 0.012 and 0.213 ± 0.031 μmol/s/g hemoglobin ($n = 3$), respectively. The average activity in human hemolysates was 0.132 ± 0.021 μmol/s/g hemoglobin ($n = 4$) and was consistent with that reported in Ref. [16]. Kinetic parameters of glutathione reductase were indicated in Table 5. Hydrogen peroxide removal rates by glutathione peroxidase under simulated in vivo conditions in mouse and human hemolysates were 5.23 ± 0.57 and 0.25 ± 0.11 μmol/s/g hemoglobin, respectively.

Table 5
Kinetic parameters of NADPH-gluthathione reductase activity[a]

	K_m for NADPH (μM)	V_{max} (μmol/s/g of Hb)
Mouse erythrocytes	5.11 ± 0.40 (3)	0.251 ± 0.012*
Human erythrocytes	6.10 ± 0.71 (3)	0.152 ± 0.021

Values were expressed as mean ± S.D. Number in parentheses indicated number of replicates.

[a] Gluthione reductase activity was measured at 0.017–0.167 mM in the presence of 5 μM FAD and 2.2 mM oxidized glutahione.

* Significantly different from that of human erythrocytes ($P < 0.001$).

4. Discussion

Our aim is to clarify the characteristic features of hydrogen peroxide removal reaction by hemoglobin, which showed the considerable role in acatalasemic human erythrocytes [5,6], and then compare hydrogen peroxide removal ability by hemoglobin with the abilities by catalase and glutathione peroxidase-glutathione reductase system since there is no report concerning the ability of hemoglobin. We reexamined hydrogen peroxide removal rates by mouse and human hemoglobins. The removal reaction was inhibited by the addition of carbon monoxide and sodium azide. The oxidized hemoglobin, methemoglobin, catalyzed hydrogen peroxide removal reaction, and the property of its reaction were similar to that of hemoglobin. The optimal pH of the reaction by hemoglobin was acidic (pH 4.5–5). Though the optimal pHs of the reactions were acidic for hemoglobin and methemoglobin, cytoplasm in ordinary cells was neutral (pH 7.0–7.3) [18] and the following experiments were done under neutral conditions (pH 7.1). The results confirmed that the reaction by hemoglobin worked under these neutral conditions.

Several researchers reported peroxidase reaction by human hemoglobin, of which optimal pH was acidic [19]. About the mechanism of reaction of metmyoglobin with hydrogen peroxide without reducing agents, King and Winfield [20] suggested that metmyoglobin was oxidized to the ferryl radical ($^{\cdot}$Mb–Fe(IV)=O) and then the radical reverted to modified methemoglobin, which suffered two-electron oxidation. The modified methemoglobin repeated this oxidation–reduction cycle several times. Yusa and Shikama indicated that myoglobin was oxidized by hydrogen peroxide to metmyoglobin and then to the ferryl radical [21–23]. Giulivi and Davies [24] reported on the preparation of the ferryl radical and its high reactivity. Kelman et al. [25] suggested that it was considered a source for cellular and tissue damage.

Our first examination was to estimate the hydrogen peroxide removal reaction by mouse hemoglobin in the presence of NAD(P)H. The removal rates by hemoglobin were enhanced in the presence of NAD(P)H, which were oxidized during the reaction. Kinetic parameters of the oxidation indicated that pyridine nucleotide oxidation depending on hydrogen peroxide was occurring in hemoglobin solution. Hydrogen peroxide removal and pyridine nucleotide oxidation rates at 0.2 mM hydrogen peroxide suggested that one molecule of hydrogen peroxide reacted with one molecule of reduced pyridine nucleotides.

Secondly, we examined hydrogen peroxide removal rates and pyridine nucleotide oxidation rates in acatalasemic mouse hemolysates to make sure that NAD(P)H in erythrocytes were oxidized depending on hydrogen peroxide. The result revealed that hydrogen peroxide removal rates in acatalasemic mouse hemolysates were enhanced in the presence of NAD(P)H and property of hydrogen-peroxide-dependent oxidation in the hemolysates was the same as that in mouse hemoglobin solution.

So, to examine the property of this activity to oxidize NAD(P)H, we specify the activity in human hemolysates by using ion-exchange column chromatography. The activity was assigned to hemoglobin A which catalyzed hydrogen peroxide removal reaction even without NAD(P)H. The removal reaction was enhanced by NAD(P)H and ascorbic acid, but not by reduced glutathione. The reaction by hemoglobin A indicated that one molecule of hydrogen peroxide reacted with one molecule of pyridine nucleotides. The reaction was inhibited by carbon monoxide, dioxygen, sodium azide and potassium cyanide; inhibition by cyanide ion was more effective than that by azide ion ($P<0.001$).

On the other hand, hydrogen peroxide removal reaction by methemoglobin A in the presence of NAD(P)H indicated that the property by methemoglobin A was not so different from that by hemoglobin A. Thus, we deduced that hemoglobin was oxidized to methemoglobin as Eq. (1) and then to the ferryl radical of hemoglobin (Eq. (2)) by hydrogen peroxide as in the reported case of myoglobin [20–23] and then the latter reduced to methemoglobin with NAD(P)H (Eq. (3)). It is reasonable that carbon monoxide and dioxygen stabilize hemoglobin by coordinating to heme (Fe (II)) of hemoglobin to inhibit the first reaction (Eq. (1)) and azide and cyanide ions disturb formation of the ferryl radical by coordinating to hemin (Fe (III)) of methemoglobin to inhibit the second reaction (Eq. (2)). As reactions of (2) and (3) cycle, it is also reasonable that it results in the stoichiometric reactions between hydrogen peroxide and NAD(P)H.

$$2H-Hb-Fe(II)O_2+H_2O_2+2H^+ = 2H-Hb-Fe(III)^+ + 2H_2O + O_2 \quad (1)$$

$$H-Hb-Fe(III)^+ + H_2O_2 = {}^{\cdot}Hb-Fe(IV)\!=\!O + H_2O + H^+ \quad (2)$$

$${}^{\cdot}Hb-Fe(IV)\!=\!O + NAD(P)H + 2H^+ = H-Hb-Fe(III)^+ + NAD(P)^+ + H_2O \quad (3)$$

(H–Hb indicated a subunit of hemoglobin.)

Preliminarily, visible spectroscopic measurements of the methemoglobin systems at 25 °C showed the similar spectra of iron porphyrin during the reaction with and without pyridine nucleotide: λ_{max} 417 (Soret), 545 and 580 nm. As the spectra are corresponded to that of ferryl porphyrin ($^{\cdot}$Hb–Fe(IV)=O and H–Hb–Fe(IV)=O) [24], it suggested formation of the ferryl radical of hemoglobin during the reaction. Further study is currently progressing.

To confirm that hydrogen peroxide removal by hemoglobin was enhanced by NAD(P)H in erythrocytes, we measured reduced pyridine nucleotide contents in erythrocytes and hydrogen peroxide removal rates by hemoglobin at various concentrations of NAD(P)H. As shown in Fig. 2, NAD(P)H in erythrocytes could enhance hydrogen peroxide

removal by hemoglobin. As an average concentration of ascorbic acid in serum was 0.06 ± 0.01 mM [26], ascorbic acid might also enhance the removal rates by hemoglobin.

Comparison of hydrogen peroxide removal reactions with and without NAD(P)H indicated that properties, optimal pHs and inhibitions were similar for both reactions, except that NAD(P)H made the reaction two to four times faster. We deduced that hydrogen peroxide removal reaction without NAD(P)H was formation of methemoglobin (Eq. (1)), followed by the formation of the ferryl radical (Eq. (2)) as in the above equations. Then, the ferryl radical would slowly and spontaneously revert to the modified methemoglobin by self-reduction without reductants, as that of metmyoglobin did [20–24]. As the ferryl radical is very active and reduction of the ferryl radical to methemoglobin essentially requires electrons, some of the compound would oxidize another hemoglobin or other biomolecules when reductants are deficient [20,24,25]. It suggests that sufficient amounts of reductants in erythrocytes are necessary to prevent the oxidation reactions by the ferryl radical.

To compare hydrogen peroxide removal rates by hemoglobin using NAD(P)H with the rates by glutathione peroxidase–glutathione reductase system, we first examined hydrogen peroxide removal rates by glutathione peroxidase–glutathione reductase system in erythrocytes. It is well known that hydrogen peroxide removal by glutathione peroxidase uses reduced glutathione as another substrate. Oxidized glutathione is reduced with NADPH in the presence of glutathione reductase, and the hydrogen peroxide removal by glutathione peroxidase cycles. When NADPH oxidation rates by glutathione reductase were compared to hydrogen peroxide removal rates by glutathione peroxidase, the rates by glutathione reductase in human and mouse hemolysates were 1/2 and 1/20 of hydrogen peroxide removal rates by glutathione peroxidase under simulated in vivo conditions, respectively. It indicates that hydrogen peroxide removal rates by glutathione peroxidase–glutathione reductase system in both erythrocytes are eventually limited by the rates of glutathione reductase though the removal rates by glutathione peroxidase under simulated in vivo conditions in human and mouse erythrocytes are 0.25 ± 0.11 and 5.23 ± 0.57 μmol/s/g hemoglobin, respectively.

Second, we compared NADPH oxidation rates by glutathione reductase with NAD(P)H oxidation rates depending on hydrogen peroxide by hemoglobin. The kinetic parameters indicated that the affinity of glutahione reductase for NADPH in human and mouse erythrocytes was higher than that of hemoglobin, but the (maximum) rates by glutathione reductase in both erythrocytes were about one-tenth of the rates by hemoglobin in the presence of 0.2 mM hydrogen peroxide. It indicates that hydrogen peroxide removal ability by hemoglobin is larger than that by glutathione peroxidase–glutathione reductase system in the presence of considerably high concentration of hydrogen peroxide and that hemoglobin consumes most of NADPH in both erythrocytes under the condition though the Michaelis constants for NADPH are close to NADPH contents in erythrocytes.

Here, we compared hydrogen peroxide removal rates by catalase with the rates by hemoglobin using NAD(P)H. From catalase activity (k/g Hb: 429 ± 67 and 117 ± 10) in human and mouse erythrocytes [5,6], hydrogen peroxide removal rates by catalase in human and mouse erythrocytes at 0.2 mM hydrogen peroxide were about 43 and 7 times as faster as the rates by hemoglobin, respectively. It indicated that the most part of hydrogen peroxide was removed by catalase in normal erythrocytes. However, in acatalasemic mouse erythrocytes, the removal rates by residual catalase activity (k/g Hb: 10.5 ± 1.7) at 0.2 mM hydrogen peroxide are about one half of the rates by hemoglobin. It suggested that some of hemoglobin were oxidized by hydrogen peroxide and removed hydrogen peroxide by using NAD(P)H under the acatalasemic conditions. These findings might be explained that methemoglobin concentration in acatalasemic mouse blood is considerably higher than that in normal mouse blood [27] and excretion of heme degradation products in the urine is five times as much as that in normal mouse urine [28].

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