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Molecular Stability and Function of Mouse Hemoglobins

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ABSTRACT—The hemoglobin types of mouse strains can be distinguished according to patterns observed on cellulose acetate electrophoresis. The two common mouse hemoglobin patterns are single and diffuse. The differences in the patterns result from differences in the β-globin chains of the hemoglobin molecules. Mice with the single hemoglobin pattern have one β-globin type identified as β-single (*Hbb*^{s}), whereas mice with the diffuse hemoglobin pattern have two different β-globin types identified as β-major (*Hbb*^{maj}) and β-minor (*Hbb*^{min}). We examined the functional and stability properties in these mouse hemoglobins, and the oxygen binding properties of red blood cells obtained from mice with four different hemoglobin phenotypes: *Hbb*^{s}/*Hbb*^{s}, *Hbb*^{s}/*Hbb*^{min}, *Hbb*^{min}/*Hbb*^{min} and *Hbb*^{maj}/*Hbb*^{min}. The P_{50}, the partial pressure of oxygen at which hemoglobin is half-saturated, of purified forms of *Hbb*^{s}, *Hbb*^{min} and *Hbb*^{maj} was 14.8 ± 0.4 mm Hg, 13.3 ± 0.6 mm Hg and 13.6 ± 0.5 mm Hg, respectively. The *n* value, determined from the slope of the Hill plot was 2.45 to 2.59 for the mouse hemoglobins. The alkaline Bohr effects of purified *Hbb*^{S}, *Hbb*^{min} and *Hbb*^{maj} were 0.69, 0.61 and 0.60, respectively. The mechanical stability of *Hbb*^{s}, *Hbb*^{min} and *Hbb*^{maj}, expressed by the first order kinetic constant, were 0.098 ± 0.01/min, 0.027 ± 0.013/min and 0.27/min, respectively. The P_{50} of red blood cell suspensions from lines of mice expressing *Hbb*^{s}/*Hbb*^{s}, *Hbb*^{min}/*Hbb*^{min}, *Hbb*^{s}/*Hbb*^{min} and *Hbb*^{maj}/*Hbb*^{min} were 40.2 ± 1.8 mm Hg, 40.4 ± 1.5 mm Hg, 38.9 ± 1.4 mm Hg, and 38.7 ± 0.9 mm Hg, respectively.

INTRODUCTION

Mice are characterized by polymorphisms of the hemoglobin β-chain (*Hbb*) (Whitney, 1978). Three β-chain haplotypes, single (*Hbb*^{s}), diffuse and peculiar, have been described, both in wild and inbred strains (Russell and McFarland, 1974). In mice with a diffuse hemoglobin pattern, 80% of the adult β-globin is β-major (*Hbb*^{maj}), while the remaining 20% is β-minor (*Hbb*^{min}) (Russell and McFarland, 1974).

Mice are widely used in transgenic experiments and as animal models of various human diseases. Studies that involve expression of human hemoglobins in transgenic mice (Behringer *et al*., 1989; Ryan *et al*., 1990) require information on the structural and functional differences between human and mouse hemoglobins. Such studies on the structure-function relationship of mouse hemoglobins may provide important information regarding the regulatory mechanisms of hemoglobin oxygen affinity. In this paper, we measured the functional and stability properties of three different types of mouse hemoglobins, and the oxygen binding properties of red blood cells obtained from mice with four different hemoglobin haplotypes.

MATERIALS AND METHODS

Materials

C57BL/6 and BALB/C mice used in this study were purchased from Jackson Labs (Bar Harbor, ME, USA) and Charles River (Wilmington, MA, USA), respectively. The hemoglobin phenotypes of C57BL/6 and BALB/C mice are *Hbb*^{s}/*Hbb*^{s} and *Hbb*^{maj}/*Hbb*^{min}, respectively. Mice heterozygous for a deletional form of β-thalassemia on a C57BL/6 background, also obtained from Jackson Labs, express both *Hbb*^{s} and *Hbb*^{min}. The heterozygous mice were bred to homozygosity to obtain mice that produced only *Hbb*^{min}. The typing of mouse hemoglobin phenotypes was done by the method of Whitney (1978), using cellulose acetate electrophoresis with cystamine. On the basis of hemoglobin electrophoretic patterns, mouse RBC were characterized in four different hemoglobin phenotypes; homozygous *Hbb*^{s}/*Hbb*^{s} containing only *Hbb*^{s} hemoglobin; heterozygous *Hbb*^{s}/*Hbb*^{min} containing both *Hbb*^{s} and *Hbb*^{min} hemoglobins; homozygous *Hbb*^{min}/*Hbb*^{min} containing only *Hbb*^{min} hemoglobin and *Hbb*^{maj}/*Hbb*^{min} containing both *Hbb*^{maj} and *Hbb*^{min}. Blood was drawn from the retro-orbital sinus into a heparinized microhematocrit capillary tube (Fisher Scientific, Pittsburgh, PA, USA). Normal human adult hemoglobin (HbA) and human sickle hemoglobin (HbS) were used as references for the mechanical and heat stability tests.

Purification of hemoglobin

Three different mouse hemoglobins, *Hbb*^{s}, *Hbb*^{maj} and *Hbb*^{min} were examined in this study. Mouse globins were separated by reverse phase high pressure liquid chromatography (RP-HPLC), using a Dionex Series 4500i HPLC system (Sunnyvale, CA, USA) (Reilly *et al*., 1994). Approximately 25 μg hemoglobin was injected onto a Vydac

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C4 reversed-phase column (4.6 × 250 mm) (Hibernia, CA, USA) and eluted with a linear gradient of acetonitrile and 0.3% trifluroacetic acid as described by Shelton *et al.* (1984). RP-HPLC showed that hemolysates from homozygous Hbb^s/Hbb^s and homozygous Hbb^{min}/Hbb^{min} mice had single β-globin peaks corresponding to β-single and β-minor, respectively. Therefore, hemolysates of the Hbb^s/Hbb^s and Hbb^{min}/Hbb^{min} RBC were used for experiments without further purification (Fig. 1). To remove 2,3-DPG, the hemolysates were passed through a Sephadex-G-25 (Sigma Chemical Company, St. Louis, MO, USA) column. The Hbb^{maj} was purified from the blood of BALB/C mice (Fig. 1). After lysing the washed cells with a 20 mM Tris-5 mM EDTA solution (pH 7.2), the hemoglobin was dialyzed against a 10 mM phosphate buffer, pH 7.0, for 4 hr. The lysate was applied to a CM-Sephadex column (Sigma), and the hemoglobin was eluted from the column using a gradient of 10 mM phosphate buffer, pH 7.0, to 20 mM phosphate buffer, pH 8.0, as previously described (Adachi *et al.*, 1980). Each chromatographic component was pooled and concentrated using a microconcentrator (Centricon-30, Amicon, Inc., Beverly, MA, USA).

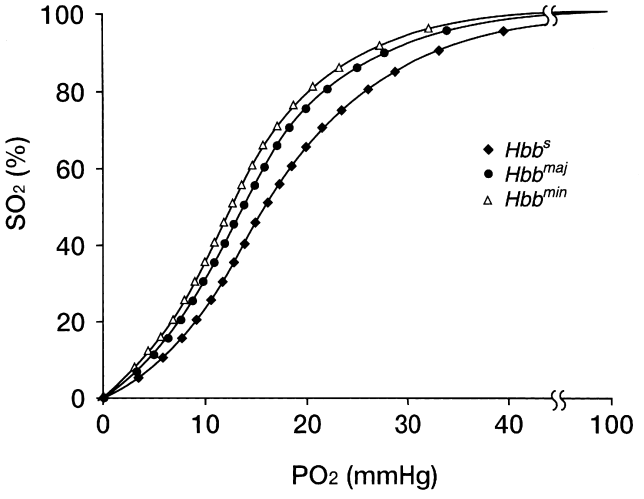

Fig. 1. Oxygen dissociation curves of mouse hemoglobins. OEC for Hbb^S shows a right side shift compared to those of Hbb^{min} and Hbb^{maj}.

Oxygen equilibrium curves of the hemoglobins

Hemoglobin oxygen equilibrium curves (OEC), at a heme concentration of 100 mM, were determined in potassium phosphate buffer, pH 7.0, at 20°C with a Hemox-Analyzer (TCS, Southampton, PA, USA), an automatic apparatus that consists of a spectrophotometer cuvette fitted with a magnetic stirrer, gas exchange line and oxygen electrode. The gas electrode (oxygen tension; PO_2) and spectrophotometer responses (% oxyhemoglobin; SO_2) are recorded on the X and Y axes, respectively, of an X-Y recorder. The temperature of the cuvette is controlled by an external water bath circulating at a constant temperature (Festa and Asakura, 1979). Ten μl of 1% dimethylpolysiloxane (Union Carbide, NY, USA), an anti-foaming agent, and a stabilizing solution (0.001% hexamethyl-phosphoramide) were added to the solution. The methemoglobin reduction system (Hayashi *et al.*, 1973) was used to prevent the oxidation of the hemoglobin. OEC were plotted according to the Hill equation with log (SO_2/100-SO_2) on the Y axes and log PO_2 on the X axes. The slopes of the lines, Hill's *n* values, were determined in mouse hemoglobins. The alkaline Bohr effect was calculated from the pH dependence of the log P_{50} between pH 6.5 and 7.5. 2,3-DPG and inositol hexaphosphate (IHP) were products of Sigma.

Oxygen equilibrium curves of suspension of red cells

The OEC of red cells suspensions were determined by suspending the RBC in an isotonic TES (*N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer, pH 7.4 at 37°C. Ten μl of 1% dimethylpolysiloxane was added as an anti-foaming agent, and bovine serum albumin was added to yield a final concentration of 0.1% to prevent red cell hemolysis. The concentration of 2,3-DPG in the whole blood samples was measured using the method of Rose and Leibowitz (Rose and Liebowitz, 1970). Hematocrit (Ht) was measured following the centrifugation of the blood samples for 5 min in a Hawksley micro-hematocrit centrifuge.

Mechanical stability of hemoglobins

Mechanical shaking experiments were done as described previously (Asakura *et al.*, 1974). Hemoglobin solutions from Hbb^s/Hbb^s and Hbb^{min}/Hbb^{min} mice and purified Hbb^{maj} were diluted in 0.1 M potassium phosphate buffer (pH 7.0) to a final heme concentration of 100 mM. Two mls of each solution were placed in a 10 × 50 glass vial and shaken with a Model 250 shaker (TCS, Southampton, PA, USA) at 60 Hz for various time intervals at room temperature. Following shaking, the vials were centrifuged at 15,000 × g, and the absorption spectrum of the hemoglobin remaining in the supernatant was recorded from 500 to 700 nm. The mechanical stability of the hemoglobins was expressed by the first order kinetic constant, K.

Heat denaturation experiments

Heat denaturation experiments were done using the oxy-forms of Hbb^S, Hbb^{min}, Hbb^{maj}, human HbA and human HbS. Each hemoglobin was diluted to yield a final heme concentration of 40 mM in 0.1 M phosphate buffer, pH 7.4, containing 5 mM EDTA. A continuous spectrum for each hemoglobin solution was recorded between 500 and 700 nm using a Hitachi U-3410 spectrophotometer (Hitachi, Tokyo, Japan). Hemoglobin solutions were heated to 60°C for 30 min, followed by centrifugation at 15,000 × g for 10 min. The hemoglobin concentration remaining in the supernatant was measured spectrophotometrically. Data are reported as mean ± standard deviation. Turkey-Kramer test was used for the statistical evaluation of the data.

RESULTS AND DISCUSSION

Oxygen equilibrium curves of hemoglobins

The oxygen binding properties of the three different mouse hemoglobins, Hbb^S, Hbb^{min} and Hbb^{maj} are shown in Table 1 and Fig. 2. The mean P_{50}, the partial oxygen pressure at which hemoglobin is 50%-saturated, for Hbb^S was significantly higher than that of Hbb^{min} and Hbb^{maj} ($p < 0.001$). The addition of 2 mM 2,3-DPG or 1 mM IHP shifted the OEC toward the right (Table 1). The Hill's *n* value was 2.45 to 2.59 for the mouse hemoglobins. The Hill's *n* value, which is an indication of hemoglobin subunit cooperativity, for mouse hemoglobins is slightly lower than the average value of 2.8-3.0 for normal human hemoglobin (Bunn and Forget, 1986). Riggs (1960) and Riggs and Herner (1962) reported that the alkaline Bohr effect was 0.9 in mice, which is much larger than that of human and larger mammals' hemoglobin. However, we determined the alkaline Bohr effect of purified Hbb^S, Hbb^{min} and Hbb^{maj} to be 0.69, 0.61 and 0.60, respectively (Table 1 and Fig. 3). Our results are consistent with those reported by Smith *et al.* (1966), who found a Bohr effect of 0.6. The Bohr factor of human hemoglobin is 0.6 (Bunn and Forget, 1986), therefore there is little deference in the Bohr effect between mouse and human hemoglobins.

Table 1. Oxygen affinity and Bohr factor of mouse hemoglobins

	Hbb^S	Hbb^{min}	Hbb^{maj}
P_{50} (mm Hg)			
No additives	14.8 ± 0.4* (7)	13.3 ± 0.64 (11)	13.6 ± 0.53(8)
+2 mM 2,3-DPG	20.5 (2)	17.3 (2)	18.5 (2)
+1 mM IHP	58.3 (2)	46.4 (2)	52.4 (2)
Hill's *n* value	2.59 ± 0.18 (4)	2.45 ± 0.03 (3)	2.57 (2)
Bohr factor	0.69 (2)	0.61 (2)	0.61 (2)

The number in parentheses is number of measurements.
2,3-DPG: 2,3-diphosphoglycerate. IHP: inositol hexaphosphate.
Hill's *n* value: the slope of Hill plot.
* $p < 0.001$ vs. Hbb^{min} or Hbb^{maj}

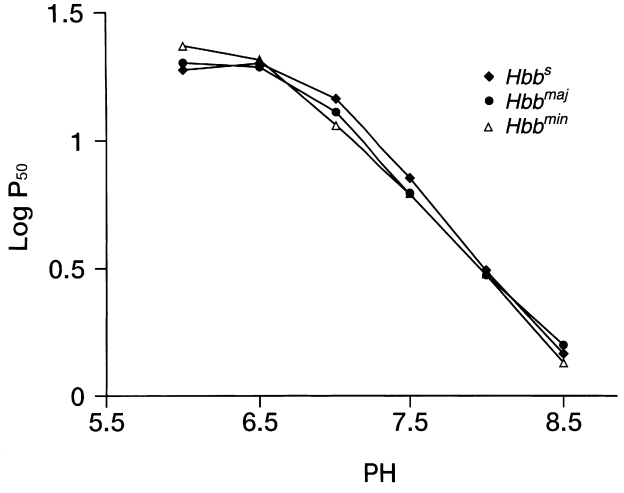

Fig. 2. The effect of pH on oxygen affinity of mouse hemoglobins. The slope of the curve between pH 6.5 and 7.5 is a measure of the alkaline Bohr effect. There were no significant differences between the slopes of the three mouse hemoglobins.

Oxygen equilibrium curves of red cell suspensions

The mean 2,3-DPG and P_{50} values in mouse RBC with Hbb^S/Hbb^S, Hbb^S/Hbb^{min}, Hbb^{min}/Hbb^{min} and Hbb^{maj}/Hbb^{min} mice are shown in Table 2. The mean 2,3-DPG concentration in Hbb^S/Hbb^S mice was significantly higher than that of mice with other hemoglobins. Although the differences in P_{50} values were not statistically significant, RBC from Hbb^{maj}/Hbb^{min} and Hbb^{min}/Hbb^{min} mice tended to have lower mean P_{50} values compared to Hbb^S/Hbb^S and Hbb^S/Hbb^{min} mice.

Newton and Peters (1983) reported that RBC from mice with Hbb^{maj}/Hbb^{min} tend to have a lower P_{50} than RBC from mice with Hbb^S/Hbb^S. This is consistent with our results and is reasonable, because both the P_{50} for hemoglobin solution from Hbb^{maj}/Hbb^{min} RBC and the 2,3-DPG concentration in Hbb^{maj}/Hbb^{min} RBC were lower than those of Hbb^S/Hbb^S RBC in this study. In mice with Hbb^S/Hbb^{min}, the percentage of Hbb^S was significantly greater than Hbb^{min} and the P_{50} for the hemoglobin solution of Hbb^S was higher than that of Hbb^{min}. This could explain why RBCs from mice with Hbb^S/Hbb^{min} tended to have a higher P_{50} value than did mice with Hbb^{min}/Hbb^{min}.

Hbb^{maj}/Hbb^{min} mice have 80% Hbb^{maj} and 20% Hbb^{min}. The P_{50} for the Hbb^{maj} and Hbb^{min} were lower than that for Hbb^S, which explains why the P_{50} of RBC from Hbb^{maj}/Hbb^{min} mice was lower than that from Hbb^S/Hbb^S mice.

Newton and Peters (1983) also reported an increased hematocrit in Hbb^{maj}/Hbb^{min} mice. They postulated that this increase was the result of a lower RBC P_{50} in Hbb^{maj}/Hbb^{min} mice than in Hbb^S/Hbb^S mice, which caused a decrease in oxygen delivery to the tissues. In our study, we did not find a significant difference in the hematocrit values between Hbb^{maj}/Hbb^{min} and Hbb^S/Hbb^S mice, although the P_{50} of the Hbb^{maj}/Hbb^{min} mice was lower than that of the Hbb^S/Hbb^S mice. In Hbb^{min}/Hbb^{min} mice, both the P_{50} and hematocrit were decreased, suggesting a decrease in oxygen transport.

Table 2. Red blood cell P_{50} values, 2,3-DPG and hematocrit in mice with four different mouse hemoglobin phenotypes

Hemoglobin phenotype	P_{50} (mm Hg)	Hematocrit (%)	2,3-DPG (nmole/ml RBC)
Hbb^S/Hbb^S	40.2 ± 1.8 (6)	48.6 ± 3.4 (9)	9382.3 ± 519.0* (9)
Hbb^S/Hbb^{min}	40.4 ± 1.5 (7)	46.3 ± 3.1 (8)	8557.5 ± 515.3 (8)
Hbb^{min}/Hbb^{min}	38.9 ± 1.4 (8)	31.6 ± 2.7** (5)	8546.8 ± 826.1 (5)
Hbb^{maj}/Hbb^{min}	38.7 ± 0.9 (5)	48.6 ± 3.2 (5)	8036.6 ± 445.8 (5)

The number in parentheses is number of measurements.
* $p < 0.01$ vs. Hbb^{maj}/Hbb^{min}, and $p < 0.05$ vs. Hbb^{min}/Hbb^{min}
** $p < 0.001$ vs. Hbb^S/Hbb^S, Hbb^S/Hbb^{min} or Hbb^{maj}/Hbb^{min}

Table 3. Mechanical and heat stability of mouse and human hemoglobins

Hemoglobin types	Shake test k (/min)	Heat stability test % remaining Hb
Hbb^{S}	0.098 ± 0.01* (8)	14.8 (2)
Hbb^{min}	0.027 ± 0.013** (5)	18.4 (2)
Hbb^{maj}	0.27 (2)	10.0 (2)
Human HbA	0.021 ± 0.006** (5)	71.2 (2)
Human HbS	0.188 ± 0.054 (4)	70.5 (2)

The number in parentheses is number of measurements.
* $p < 0.001$ vs. *Hbb*^{min}, HbA, HbS
** $p < 0.001$ vs. *Hbb*^{S}, or HbS

Mechanical and heat stability of hemoglobins

Since this is the first report to examine the stability of various mouse hemoglobins by mechanical shake and heat denaturation methods, we used human HbA and HbS for comparison. As shown in Table 3, the k-values (Ohnishi *et al.*, 1974), which represent the first order denaturation constants, indicated that mouse *Hbb*^{maj} was the least stable among all hemoglobins tested and similar to that of human HbS. On the other hand, mouse *Hbb*^{min} was most stable. The mechanical stability of *Hbb*^{S} was intermediate between human HbA and HbS, while that of *Hbb*^{min} was similar to HbA. In the heat stability tests, mouse *Hbb*^{maj} was the least stable among the three mouse hemoglobins tested. All mouse hemoglobins were less stable to heat denaturation than human HbA and HbS.

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