Oxygen Association-Dissociation and Stability Analysis on Mouse Hemoglobins With Mutant α- and β-Globins

Stephen J. D'Surney and Raymond A. Popp

Biology Division, Oak Ridge National Laboratory, and the University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, Tennessee 37831

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ABSTRACT

Oxygen association-dissociation and hemoglobin stability analysis were performed on mouse hemoglobins with amino acid substitutions in an α-globin (α89, His to Leu) and a β-globin (β59, Lys to Ile). The variant α-globin, designated chain 5^m in the Hba^{g2} haplotype, had a high oxygen affinity and was stable. The variant β-globin, (β^{s2}) of the Hbb^{s2} haplotype, also had an elevated oxygen affinity and in addition was moderately unstable in 19% isopropanol. Hemoglobins from the expected nine ($Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s} \times Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$) F_2 genotypes can be grouped into five classes of P_{50} values characterized by strict additivity and dependency on mutant globin gene dosage; physiologically, both globin variants gave indistinguishable effects on oxygen affinity. The hemoglobin of normal mice ($Hba^{a}/Hba^{a};Hbb^{s}/Hbb^{s}$) had a P_{50} = 40 mm Hg and the hemoglobin of $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ F_2 mice had a P_{50} = 25 mm Hg (human P_{50} = 26 mm Hg). Peripheral blood from $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$, $Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$ and $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice exhibited normal hematological values except for a slightly higher hematocrit for $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$ and $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice, slightly elevated red cell counts for mice of the three mutant genotypes, and significantly lower values for the mean corpuscular volume and mean corpuscular hemoglobin for $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice.

A number of high oxygen affinity and unstable variants of human hemoglobin have been identified. Currently, at least 19 high oxygen affinity α-chain variants and 50 high oxygen affinity β-chain variants have been characterized in humans (BUNN and FORGET 1986). Hemoglobin Chesapeake, an α-globin variant (α92, Arg to Leu) has a P_{50} of 19 mm Hg (normal for humans is 26 mm Hg), and individuals carrying this variant have greatly elevated red blood cell counts (CLEGG, NAUGHTON and WEATHERALL 1966). Hemoglobin Brisbane, a β-globin variant (β68, Leu to His) also has an elevated oxygen affinity and is mildly unstable (BRENNAN *et al.* 1981). At the molecular level, most high oxygen affinity variants of human hemoglobin can be attributed to amino acid changes in three regions: (1) the $\alpha_1\beta_1$ interface, (2) the C-terminal end of the β-chain and (3) the 2,3-diphosphoglycerate and Bohr effect sites.

There are also a large number of unstable human hemoglobins. Nineteen unstable α-globin variants and 74 unstable β-globin variants have been characterized (BUNN and FORGET 1986). Hemoglobin Bristol (β67, Val to Asp) is a highly unstable hemoglobin variant that comprises 36% of the total hemoglobin in hemoglobin Bristol patients. Severe hemolytic anemia is observed in these patients and large Heinz body inclusions are found in the red blood cells. Hemoglobin Bristol is unstable because the amino acid substitution weakens the heme-to-globin linkage (STEADMAN, YATES and HUEHNS 1970). Other unstable human hemoglobin variants are caused by mutations that interfere with secondary (hemoglobin Ann Arbor: α80, Leu to Arg), tertiary (hemoglobin Baylor: β81, Leu to Arg) and quaternary (hemoglobin Khartoum: β124, Pro to Arg) structures.

In mice, only one high oxygen affinity variant (Hbb^{d4}/Hbb^{d4}) and one unstable β-globin variant (Hbb^{s2}/Hbb^{s2}) have been reported. Both variant globins are products of mutant genes that were induced by treatment of parental mice with *N*-ethyl-*N*-nitrosourea (ENU). The mutant hemoglobin (β145, Tyr to Cys) in Hbb^{d4}/Hbb^{d4} mice has characteristics similar to hemoglobin Rainier in humans (PETERS *et al.* 1985); it has an increased oxygen affinity, reduced Bohr effect, and decreased cooperativity. Severe polycythemia occurs in animals homozygous for the Hbb^{d4}/Hbb^{d4} mutation.

The mutant hemoglobin (β59, Lys to Ile) in mice of the Hbb^{s2} haplotype (LEWIS *et al.* 1985) is moderately unstable (WAWRZYNIAK and POPP 1986), but its oxygen binding properties have not been determined. An ENU-induced mutation at the mouse α-globin locus in the Hba^{g2} haplotype has also been described (POPP *et al.* 1983). In mice of the mutant Hba^{g2} haplotype, the effects of the variant globin (α89, His to Leu) on the stability and oxygen binding characteris-

tics of hemoglobin have not been determined. This report describes the oxygen association-dissociation and stability properties of hemoglobins from parental mice of the Hba^{g2} and Hbb^{s2} haplotypes and their ($Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s} \times Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$) F_2 offspring.

MATERIALS AND METHODS

Mice: Mice homozygous for two mutant hemoglobin haplotypes Hbb^{s2} ($Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$) β59, Lys to Ile, and Hba^{g2} ($Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$) α89, His to Leu, were initially selected for oxygen association-dissociation analysis because their hemoglobins contain amino acid substitutions in the vicinity of the heme-binding sites. The proposed β60 Val to Glu substitution for Hbb^{s2} (LEWIS *et al.* 1985) was incorrect, the correct substitution is β59 Lys to Ile (J. JONES, personal communication). Control mouse strains were C57BL/6 ($Hba^{a}/Hba^{a};Hbb^{s}/Hbb^{s}$) and DBA/2J ($Hba^{g}/Hba^{g};Hbb^{d}/Hbb^{d}$).

Homozygous Hba^{g2} and Hbb^{s2} mice were mated to produce doubly heterozygous ($Hba^{a}/Hba^{g2};Hbb^{s}/Hbb^{s2}$) F_1 mice that were mated to produce F_2 progeny for oxygen association-dissociation and stability analyses. $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$, $Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$, and $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ F_2 mice were selected for peripheral blood hematology analysis using an Ortho ELT-15 Automatic Hematology Analyser (Ortho Diagnostic Systems Inc.) that has been modified specifically for the analysis of mouse blood (POPP *et al.* 1986). The hemoglobin genotypes of all mice were determined by isoelectric focusing of hemolysates in a pH range of 7 to 9 (WHITNEY *et al.* 1979).

Oxygen association-dissociation analysis: Oxygen association-dissociation analysis on mouse hemoglobin was performed on a TCS Hemox Analyzer (FESTA and AKASURA 1979). A Clark oxygen electrode monitors the oxygen concentration in the sample during oxygenation with air and deoxygenation with nitrogen. Analyses were made on 20–70-μl samples of freshly drawn blood suspended in a solution of 4 ml of Hemox buffer at pH 7.4 plus 20 μl of serum albumin solution (additive A) and 10 μl of antifoaming agent (additive B) purchased from TCS Medical Products, Southampton, Pennsylvania. After equilibration at 37° the hemoglobin was fully oxygenated by bubbling air through the blood sample and then completely deoxygenated by bubbling with purified nitrogen. The hemoglobin was slowly oxygenated again by bubbling with air over a 20-min period while recording the oxygen association curve, which was used to read the P_{50} values. Seven or more blood samples were analyzed from mice of each hemoglobin genotype. The oxygen association curves were similar to the oxygen dissociation curves, but the latter were not routinely measured.

Hemoglobin stability in isopropanol: Isopropanol solutions of 0, 15, 16, 17, 18, 19, 20, 22 and 25% (v/v) were made in 0.1 M Tris-HCl buffer and the pH was adjusted to 7.4 with HCl. All isopropanol solutions were at room temperature prior to mixing with the hemolysates. Mice were bled from the supraorbital sinus and the blood (0.5 ml) was suspended in 30 ml of saline citrate solution (0.67% NaCl and 1.0% sodium citrate). The pelleted red cells (centrifuged at 600 × *g* for 10 min) were washed twice in 0.85 % NaCl and lysed in 5 volumes of 0.1 M Tris-HCl, pH 7.4. The lysates were centrifuged at 10,000 × *g* for 30 min to remove cell membranes and the concentration of the clear hemolysate was adjusted to $A_{575\ nm}$ of 1.5 (WAWRZYNIAK and POPP 1986). In each reaction tube, 111 μl of hemolysate was mixed with 1.0 ml of buffered isopropanol. The hemolysate-isopropanol mixture was incubated at 37° in a water bath for 30 min prior to reading the absorbancy of the mixture at 604 nm in a Beckman ACTA III spectrophotometer. Hemolysates from each mouse were run alongside hemolysates of C57BL/6 controls. The formation of a suspension of precipitated protein in the diluted hemolysate-isopropanol mixture interfered with light transmittance at 604 nm and was recorded as $A_{604\ nm}$ values.

TABLE 1

Segregation and assortment of Hba^{g2} and Hbb^{s2} genes among F_2 progeny of $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s} \times Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$ mice

Hemoglobin genotype[a]	No. of mice	
	Observed	Expected
a/a;s/s	7	8.75
a/g2;s/s	20	17.50
g2/g2;s/s	9	8.75
a/a;s/s2	18	17.50
a/g2;s/s2	39	35.00
g2/g2;s/s2	15	17.50
a/a;s2/s2	8	8.75
a/g2;s2/s2	12	17.50
g2/g2;s2/s2	12	8.75

Total = 140 $\chi^2 = 4.55$, $P < 0.75$.

[a] The *Hba* alleles are given first and the *Hbb* alleles are given last. *a* = Hba^{a} (homozygous in Hbb^{s2} stock; *g*^{2} = Hba^{g2} (homozygous in Hba^{g2} stock); *s* = Hbb^{s} (homozygous in Hba^{g2} stock); *s2* = Hbb^{s2} (homozygous in Hbb^{s2} stock).

RESULTS

Genetic study: $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$ and $Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$ mice were mated to produce the F_1 and F_2 progeny shown in Table 1. Mice of the nine expected genotypes were observed among the 140 F_2 progeny in ratios expected for a dihybrid cross ($\chi^2 = 4.55$, $P < 0.75$). The largest departure from the predicted number of offspring occurred when 12 doubly homozygous $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice were found among the F_2 progeny where nine were expected.

Oxygen association-dissociation analysis: The average values for the P_{50} measurements on hemoglobins from control and mutant mice are shown in Table 2. Control C57BL/6 and DBA/2J mice had P_{50} values of about 40 mm Hg. Hemoglobins from the nine ($Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s} \times Hbb^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$) F_2 genotypes exhibited a progressive decrease in P_{50} values (increase in oxygen affinity) with increasing gene dosage of mutant α-globin (Hba^{g2}) and β-globin (Hbb^{s2}) alleles; *e.g.*, hemoglobins from mice of the $Hba^{a}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$, P_{50} = 28 mm Hg; $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s2}$, P_{50} = 28 mm Hg; and doubly homozygous mutant ($Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$), P_{50} = 25 mm Hg. Hemoglobins from mice of these genotypes

TABLE 2

Whole blood P_{50} values for mice of various hemoglobin genotypes

Hemoglobin genotype[a]	Mouse stocks and strains	P_{50} value[b] ± SEM
a/a;s/s	C57BL/6	41.6 ± 0.2
g/g;d/d	DBA/2J	38.4 ± 0.3
a/a;s/s	$(Hba^{g2} \times Hbb^{s2})F_2$	38.9 ± 0.6
$a/g^2;s/s$	$(Hba^{g2} \times Hbb^{s2})F_2$	34.7 ± 0.4
$g^2/g^2;s/s$	$(Hba^{g2} \times Hbb^{s2})F_2$	31.5 ± 0.2
$a/a;s/s^2$	$(Hba^{g2} \times Hbb^{s2})F_2$	35.1 ± 0.2
$a/g^2;s/s^2$	$(Hba^{g2} \times Hbb^{s2})F_2$	32.3 ± 0.3
$g^2/g^2;s/s^2$	$(Hba^{g2} \times Hbb^{s2})F_2$	27.8 ± 0.4
$a/a;s^2/s^2$	$(Hba^{g2} \times Hbb^{s2})F_2$	31.2 ± 0.2
$a/g^2;s^2/s^2$	$(Hba^{g2} \times Hbb^{s2})F_2$	28.2 ± 0.4
$g^2/g^2;s^2/s^2$	$(Hba^{g2} \times Hbb^{s2})F_2$	24.5 ± 0.6

[a] The *Hba* alleles are given first and the *Hbb* alleles are given last. a = Hba^a (homozygous in C57BL/6 and Hbb^{s2} stocks); g^2 = Hba^{g2} (homozygous in Hba^{g2} stock); s = Hbb^s (homozygous in C57BL/6 mice and Hba^{g2} stock); s^2 = Hbb^{s2} (homozygous in Hbb^{s2} stock); g = Hba^g (homozygous in DBA/2J mice); d = Hbb^d (homozygous in DBA/2J mice).

[b] Seven to ten determinations for each hemoglobin genotype.

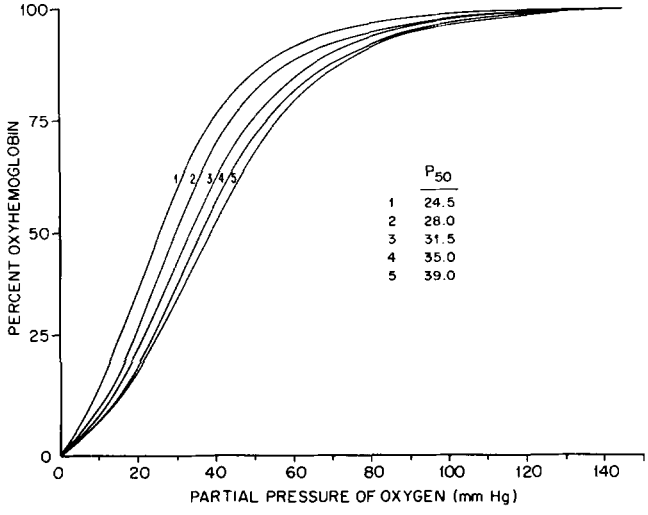

FIGURE 1.—Oxygen association curves and P_{50} values for hemoglobins from F_2 mice of nine genotypes. Curve 1, $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$; 2, $Hba^a/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ and $Hba^{g2}/Hba^{g2};Hbb^s/Hbb^{s2}$; 3, $Hba^a/Hba^{g2};Hbb^s/Hbb^{s2}$, $Hba^{g2}/Hba^{g2};Hbb^s/Hbb^s$ and $Hba^a/Hba^a;Hbb^{s2}/Hbb^{s2}$; 4, $Hba^a/Hba^{g2};Hbb^s/Hbb^s$ and $Hba^a/Hba^a;Hbb^s/Hbb^{s2}$; and 5, control–$Hba^a/Hba^a;Hbb^s/Hbb^s$.

have P_{50} values similar to that of normal adult human hemoglobin (P_{50} = 26 mm Hg).

The oxygen association-dissociation curves of hemoglobins from representative F_2 offspring are shown in Figure 1. The nine hemoglobin genotypes exhibited five oxygen association-dissociation curves; the quantities of the mutant forms of the α- and β-globins are correlated with the P_{50} values for each of these hemoglobins. Cooperativity appears to be undisturbed for the mutant hemoglobins as sigmoidicity is main-

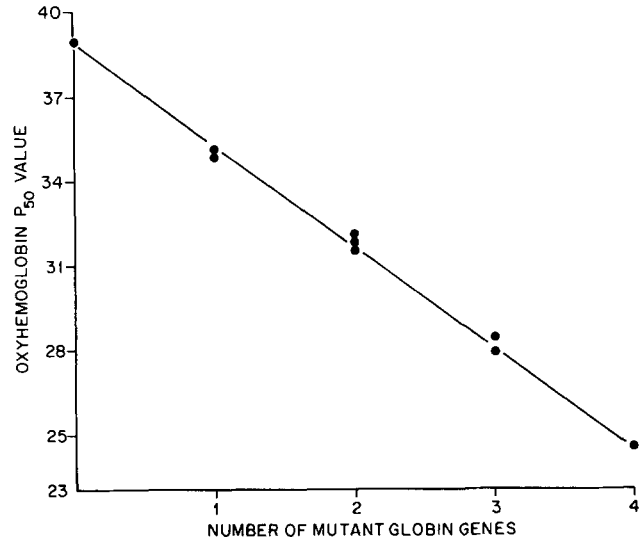

FIGURE 2.—Relationship of the oxygen P_{50} values and the number of mutant globin genes. The P_{50} values were determined from the oxygen association curves shown in Figure 1 and plotted against the number of mutant globin genes in mice of the nine hemoglobin genotypes: 0, $Hba^a/Hba^a;Hbb^s/Hbb^s$ (control); 1, $Hba^a/Hba^{g2};Hbb^s/Hbb^s$ and $Hba^a/Hba^a;Hbb^s/Hbb^{s2}$; 2, $Hba^{g2}/Hba^{g2};Hbb^s/Hbb^s$, $Hba^a/Hba^a;Hbb^{s2}/Hbb^{s2}$ and $Hba^a/Hba^{g2};Hbb^s/Hbb^{s2}$; 3, $Hba^a/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ and $Hba^{g2}/Hba^{g2};Hbb^s/Hbb^{s2}$; and 4, $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ (double mutant).

tained for each of the oxygen association-dissociation curves. The additive effects of both the α-globin (Hba^{g2}) and the β-globin (Hbb^{s2}) mutations are depicted in Figure 2. The left shift in hemoglobin P_{50} values among mice of the nine F_2 genotypes (Table 2) is linearly related to the number of mutant globin genes. The mutant α-globin chain (5^m) in Hba^{g2} mice and the mutant β-globin chain (β^{s2}) in Hbb^{s2} mice appear to be physiologically equivalent in terms of their effect on oxygen binding. The hematological values determined from peripheral blood of normal $Hba^a/Hba^a;Hbb^s/Hbb^s$ and mutant $Hba^{g2}/Hba^{g2};Hbb^s/Hbb^s$, $Hba^a/Hba^a;Hbb^{s2}$s/Hbb^{s2}, and $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice are shown in Table 3. Mild erythrocytosis (elevated red cell counts, RBC) was observed in blood from mice of the three mutant genotypes in comparison to that of controls. Hematocrit (HCT) values were slightly elevated in $Hba^{g2}/Hba^{g2};Hbb^s/Hbb^s$ and doubly homozygous mutant $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice, but were normal in $Hba^a/Hba^a;Hbb^{s2}/Hbb^{s2}$ mice. In addition, the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values were significantly lower in $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice.

Stability analysis: Clear hemolysates of blood from $Hba^{g2}/Hba^{g2};Hbb^s/Hbb^s$, $Hba^a/Hba^a;Hbb^{s2}/Hbb^{s2}$, and doubly homozygous mutant $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ F_2 and C57BL/6 ($Hba^a/Hba^a;Hbb^s/Hbb^s$) mice were mixed with increasing concentrations of buffered isopropanol ranging from 0 to 25%. When murine oxyhemoglobin was analyzed on a Beckman ACTA III scanning spectrophotometer, two absorbance peaks

TABLE 3

Hematologic data on mouse blood of C57BL/6 ($Hba^{a/a};Hbb^{s/s}$), $Hba^{g2/g2};Hbb^{s/s}$, $Hba^{a/a};Hbb^{s2/s2}$, and $Hba^{g2/g2};Hbb^{s2/s2}$ genotypes

Hemoglobin genotype	No. of samples	WBC (× 10^6/ml)	RBC (× 10^9/ml)	HGB (g/dl)	HCT (%)	MCV (pg/RBC)	MCH (pg/RBC)	MCHC (g/dl)
$Hba^{a/a}$	7	8.6[a]	10.2	16.3	48.2	47.0	15.9	33.8
$Hbb^{s/s}$		0.5	0.1	0.3	0.6	0.2	0.2	0.4
$Hba^{g2/g2}$	8	3.1	11.2	17.9	51.9	46.3	16.0	34.5
$Hbb^{s/s}$		0.2	0.1	0.3	0.6	0.3	0.3	0.8
$Hba^{a/a}$	7	4.6	10.6	16.1	48.3	45.6	15.2	33.5
$Hbb^{s2/s2}$		0.6	0.2	0.3	1.1	0.7	0.3	0.9
$Hba^{g2/g2}$	7	6.2	11.9	16.8	51.1	43.0	13.8	32.2
$Hbb^{s2/s2}$		0.9	0.4	0.5	1.5	0.8	0.5	1.0

[a] The average for each value is listed first and the ± SEM is listed below each average value.

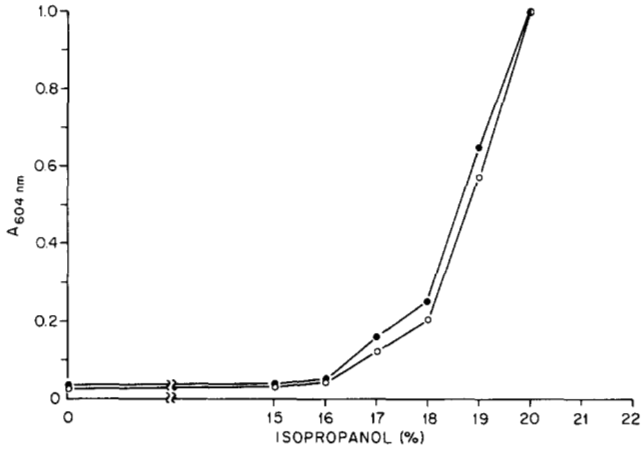

FIGURE 3.—Denaturation profile of hemoglobins from Hba^{g2} and C57BL/6 mice in increasing concentrations of isopropanol. The increase in $A_{604\ nm}$ is an inverse measure of the decrease in transmittance with increasing amounts of precipitate formed during 30-min incubation of hemolysates at 37° in isopropanol. (●) $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$; (○) $Hba^{a}/Hba^{a};Hbb^{s}/Hbb^{s}$. Hemoglobin from $Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$ mice is equally stable or only slightly less stable than C57BL/6 hemoglobin.

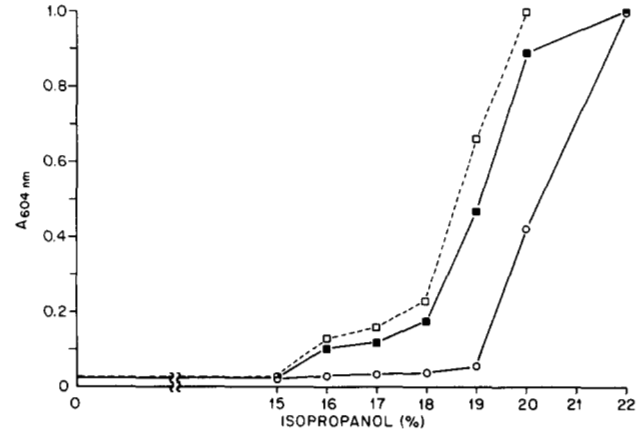

FIGURE 4.—Denaturation profile of hemoglobins from Hbb^{s2} and C57BL/6 mice in increasing concentrations of isopropanol. (○) $Hba^{a}/Hba^{a};Hbb^{s}/Hbb^{s}$ (C57BL/6); (■) $Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$; and (□) $Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$ $A_{604\ nm}$ values extrapolated to represent the expected profile if the Hbb^{s2} mutant β-globin chain comprised 100% (rather than 70%) of the adult hemoglobin.

were found at 542 and 572 nm and minimal absorbance (maximal light transmittance) occurred above 600 nm (data not given), so the $A_{604\ nm}$ of the hemolysate-isopropanol reaction mixtures was measured after a 30-min incubation at 37° to compare the relative amounts of insoluble protein formed.

A comparison of the $A_{604\ nm}$ values for Hba^{g2} ($Hba^{g2}/Hba^{g2};Hbb^{s}/Hbb^{s}$) and C57BL/6 ($Hba^{a}/Hba^{a};Hbb^{s}/Hbb^{s}$) hemolysates is shown in Figure 3. For both hemolysates the denaturation profiles with increasing concentrations of isopropanol are nearly identical and both hemoglobins become completely denatured in 20% isopropanol.

In Figure 4 the denaturation profiles of hemoglobin from Hbb^{s2} ($Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$) and C57BL/6 mice are compared. A marked difference in the amount of precipitate between the two hemoglobin samples occurs from 16 to 20% isopropanol. Hemoglobin from Hbb^{s2} mice forms 15-fold more precipitate in 19% isopropanol than C57BL/6 hemoglobin. Since 70% of the adult hemoglobin tetramers in Hbb^{s2} mice contain the variant β^{s2}-globin chain (WAWRZYNIAK and POPP 1985), a hypothetical extrapolation is shown in Figure 4 for hemoglobin containing the β^{s2}-chain in Hbb^{s2} mice.

The denaturation profile of hemoglobin from doubly homozygous ($Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$) F_2 mice is compared to C57BL/6 hemoglobin in Figure 5. The denaturation profile of the doubly mutant hemoglobin is nearly identical to that of $Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$ hemoglobin shown in Figure 4. Since the hemoglobin of Hba^{g2} mice is relatively stable, the instability of hemoglobin of $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice is primarily due to the presence of the β^{s2} variant globin chain.

DISCUSSION

A number of methods exist for determining the oxygen association-dissociation characteristics of he-

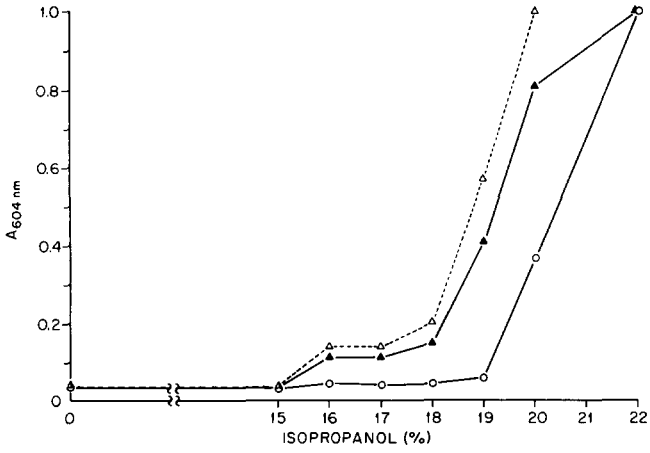

FIGURE 5.—Denaturation profile of hemoglobins from *Hba*^{g2}/*Hba*^{g2};*Hbb*^{s2}/*Hbb*^{s2} and C57BL/6 mice in increasing concentrations of isopropanol. (○) *Hba*^{a}/*Hba*^{a};*Hbb*^{s}/*Hbb*^{s} (C57BL/6); (▲) *Hba*^{g2}/*Hba*^{g2};*Hbb*^{s2}/*Hbb*^{s2}; and (△) *Hba*^{g2}/*Hba*^{g2};*Hbb*^{s2}/*Hbb*^{s2} $A_{604\ nm}$ values extrapolated to represent the expected profile if the *Hbb*^{s2} and the *Hba*^{g2} (assuming that the *Hba*^{g2} mutant α-globin chain is stable) mutant globin chains comprised 100% (rather than 70 and 55%, respectively) of the total adult hemoglobin.

moglobin and are based on the spectral differences between 400 and 800 nm of both oxyhemoglobin and deoxyhemoglobin. The oldest method made use of a tonometer to deoxygenate and oxygenate hemoglobin in a rotating V-shaped flask at 37° (BENESCH, MACDUFF and BENESCH 1965). The procedure required large amounts of blood sample and oxygenation and deoxygenation were performed manually. The development of automated oxygen association-dissociation analyzers permitted microliter samples of blood to be used. The Aminco Hem-O-Scan Analyser (NEWTON and PETERS 1983) uses as little as 2 μl of blood placed between a glass slide and a gas permeable membrane to allow oxygenation and deoxygenation of a thin film of blood where the pH and temperature are maintained in a heated, humidified chamber flushed with a mixture of O_2, N_2 and CO_2. The more advanced model TCS Hemox ODC Analyzer, which uses a Clark oxygen electrode, measures oxygen association-dissociation properties of hemoglobin in microliter samples of blood suspended in a pH 7.4 buffered physiological solution maintained at 37° in a cuvette through which gases are bubbled to oxygenate and deoxygenate the hemoglobin. The Hemox Analyzer is easy to operate and produces highly accurate oxygen association-dissociation curves with as little as 2 μl of blood (FESTA and AKASURA 1979).

In adult *Hbb*^{s2} (*Hbb*^{a}/*Hbb*^{a};*Hbb*^{s2}/*Hbb*^{s2}) mice, 70% of the adult hemoglobin contains the variant β^{s2} β-globin polypeptide (WAWRZYNIAK and POPP 1985). In adult *Hba*^{g2} (*Hba*^{g2}/*Hba*^{g2};*Hbb*^{s}/*Hbb*^{s}) mice, 55% of the adult hemoglobin contains the variant α-globin chain 5^{m} (POPP *et al.* 1983). Both of these α- and β-globin chain variants are present at these same levels in doubly homozygous *Hba*^{g2}/*Hba*^{g2};*Hbb*^{s2}/*Hbb*^{s2} mice (our unpublished results). The variant chains are not present in 100% of the adult hemoglobin because mice express two adult α-globin and two adult β-globin genes (LYON, BARKER and POPP 1988).

In *Hba*^{g2}, *Hbb*^{s2}, and (*Hba*^{g2}/*Hba*^{g2};*Hbb*^{s}/*Hbb*^{s} × *Hba*^{a}/*Hba*^{a};*Hbb*^{s2}/*Hbb*^{s2}) F_2 mice, both mutant α- and β-globin chains are physiologically equivalent; that is, the oxygen affinity of hemoglobin with both globin variants is increased in proportion to the mutant gene dosage regardless of genotypic combination (Table 2, Figures 1 and 2). When both mutant globins are present in the same animal, they exert their effect on oxygen affinity in an additive manner. Although both mutant globins are physiologically similar, the presence of 55% α-chain 5^{m} *vs.* 70% β^{s2} in hemoglobins *Hba*^{g2} and *Hbb*^{s2} mice, respectively, implies that hemoglobin with α-chain 5^{m} at the molecular level has a 30% greater oxygen affinity than hemoglobin with the β^{s2}-globin. Minor differences in oxygen association-dissociation properties among normal C57BL/6 and DBA/2J mice (Table 2) may reflect a composite of globin chain variant oxygen affinities in addition to minor physiological differences such as intracellular levels of 2,3-diphosphoglycerate and pH.

Hemoglobin stability can be measured by a number of methods such as isopropanol precipitation (CARRELL and KAY 1972; WAWRZYNIAK and POPP 1986), heat denaturation (DACIE *et al.* 1964), zinc denaturation (DACIE *et al.* 1967), and *p*-chloromercurobenzoate denaturation (HUEHNS 1970). These methods will identify moderately to highly unstable hemoglobin variants, but quantitative results are subject to variability in reaction rate and measurement of the quantity of precipitate formed. For each of these procedures, unstable hemoglobins denature and precipitate under conditions that do not cause denaturation and precipitation of normal hemoglobin. The quantity of precipitate formed is proportional to the degree of instability, but methods to quantitate the amount of precipitate formed have not been standardized. In this report, we have measured interference of the transmittance of light at a wavelength at which native hemoglobin has minimal absorbance to determine the relative amounts of precipitated protein and plot the "denaturation profile" of mouse hemoglobin in increasing concentrations of buffered isopropanol.

The spectrophotometric method to detect the formation of flocculated hemoglobin is fast and simple to perform, and consistent results are obtained provided that incubation time and temperature are highly controlled. The test is highly sensitive and quantitative, and even mildly unstable hemoglobins can be detected when they constitute a sizable fraction of the total hemoglobin. Maximum absorbance (precipitation) of mouse hemoglobin usually occurs at 22% isopropanol; decreased absorbance is consistently ob-

served at 25% isopropanol due to aggregation and settling of the flocculated precipitate which allows for increased light transmittance in the supernatant at 604 nm.

The denaturation profile of Hba^{g2} hemoglobin (Figure 3) indicates that the mutant α-globin chain 5^{m} is relatively stable in comparison to α-globin chain 1 in C57BL/6 mouse hemoglobin. In contrast, Figure 4 shows that the β^{s2}-globin in Hbb^{s2} mouse hemoglobin is moderately unstable, as reported in an earlier study (WAWRZYNIAK and POPP 1986). Figure 4 also depicts an extrapolation of the denaturation profile of Hbb^{s2} hemoglobin expected if all of the hemoglobin contained the variant β^{s2}-globin chain instead of 70% of the adult hemoglobin. The shift in the denaturation profile of C57BL/6 hemoglobin shown in Figures 3 and 4 most likely resulted from slight variations in incubation temperature and time between completion of incubation and spectrophotometric analysis and demonstrates the need for simultaneous controls for each analysis. Some of this variation can be controlled by placing the reactions on ice immediately after incubation prior to spectrophotometric analysis. Peak precipitation of C57BL/6 hemoglobin occurred in 20 or 22% isopropanol in all experiments. Hbb^{s2} hemoglobin is moderately unstable in contrast to the highly unstable human hemoglobin variants Christchurch and Belfast (BUNN and FORGET 1986). Red cells from individuals with these hemoglobin variants contain Heinz body inclusions and the elevated hemolysis causes hemolytic anemia. In $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice, the denaturation profile is nearly identical to that of $Hba^{a}/Hba^{a};Hbb^{s2}/Hbb^{s2}$ mice (compare Figures 4 and 5). There appears to be no interactive effect between the moderately unstable β^{s2}-globin chain and the relatively stable α-chain 5^{m} in the $\alpha_2{}^{5m}\beta_2{}^{s2}$ hemoglobin tetramers formed in $Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2}$ mice. In these mice, the expected quantity of the $\alpha_2{}^{5m}\beta_2{}^{s2}$ tetramer would be 0.55×0.70, or 38% of the total hemoglobin. The quantity found by isoelectric focusing was 36% (data not presented), which suggests that the tetrameric hemoglobin containing both the mutant α- and β-globins is quite stable under normal physiological conditions.

The α- and β-globin variants do not abolish the cooperativity of oxygen binding but they do alter the kinetics of transition of deoxyhemoglobin to oxyhemoglobin (left shift in P_{50} values) (Figure 1). The tertiary structures of α- and β-globins of human and other mammalian hemoglobins are highly conserved. By homology, the α89, His to Leu substitution in α-chain 5^{m} of Hba^{g2} mice, though near the proximal heme-binding histidine residue, is on the hydrophilic, external surface of the α-globin molecule between two residues involved in the $\alpha_1\beta_2$ contact in the hemoglobin tetramer. This region is important in regulating allosteric cooperativity during the transition of oxyhemoglobin to deoxyhemoglobin via the breaking and forming of salt bridges (ionic interactions). The α89, His to Leu substitution in Hba^{g2} mice may alter such interactions which can result in an increased oxygen binding (BUNN and FORGET 1986; BALDWIN 1980; PERUTZ 1980).

In Hbb^{s2} mice, the site of the β59, Lys to Ile amino acid substitution lies near the hydrophobic heme pocket, but does not involve any residues that associate with the nonpolar porphyrin ring in the heme group. The positively charged lysine residue which projects its side chain toward the similarly charged Fe^{+2}-ligand binding, coordination axis was replaced by a neutrally charged isoleucine moiety. The increased oxygen affinity in Hbb^{s2} hemoglobin may result from reducing the dielectric constant in the vicinity of the iron and distal histidine region, which has a local positive charge (LAUFER, WARREN and MCIVOR 1983). A reduction of the positive charge in this area resulting from the lysine to isoleucine substitution could facilitate oxygen binding to the sixth coordination position of the iron atom and increase the rate of transition of deoxyhemoglobin to oxyhemoglobin via subsequent heme-heme interaction.

The most plausible explanation for the instability of Hbb^{s2} hemoglobin is that the decrease in local positive charge reduces the hydrophobicity of the heme pocket in the vicinity of the distal histidine residue, and isopropanol causes a more rapid denaturation of globin tertiary structure resulting in the formation of methemoglobin, via irreversible hemichrome formation followed by expulsion of the heme and precipitation of globin (BUNN and FORGET 1986). If this explanation is applicable for β^{s2}-globin, then this mouse hemoglobin variant is unique among both human and mouse hemoglobin variants described to date.

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