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Comparative Biochemistry and Physiology, Part A 161 (2012) 265–270

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Oxygenation properties and oxidation rates of mouse hemoglobins that differ in reactive cysteine content

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ARTICLE INFO

Article history:
Received 13 September 2011
Received in revised form 4 November 2011
Accepted 7 November 2011
Available online 16 November 2011

Keywords:
Globins
Hemoglobin
met-hemoglobin
Mus
Oxidative stress

ABSTRACT

House mice (genus *Mus*) harbor extensive allelic variation at two tandemly duplicated genes that encode the β-chain subunits of adult hemoglobin (Hb). Alternative haplotypes differ in the level of sequence divergence between the two β-globin gene duplicates: the Hbb^d and Hbb^p haplotypes harbor two structurally distinct β-globin genes, whereas the Hbb^s haplotype harbors two β-globin duplicates that are identical in sequence. One especially salient difference between the *s*-type Hbs relative to the *d*- and *p*-type Hbs relates to the number of reactive β-chain cysteine residues. In addition to the highly conserved cysteine residue at β93, the *d*- and *p*-type Hbs contain an additional reactive cysteine residue at β13. To assess the functional consequences of allelic variation in β-globin cysteine content, we measured O_2-binding properties and H_2O_2-induced oxidation rates of mono- and dicysteinyl β-Hbs from 4 different inbred strains of mice: C57BL/6J, BALB/cJ, MSM/Ms, and CAROLI/EiJ. The experiments revealed that purified Hbs from the various mouse strains did not exhibit substantial variation in O_2-binding properties, but *s*-type Hb (which contains a single reactive β-chain cysteine residue) was far more readily oxidized to Fe^{3+} metHb by H_2O_2 than other mouse Hbs that contain two reactive β-chain cysteine residues. These results suggest that the possession of an additional reactive cysteine residue may protect against metHb formation under oxidizing conditions. The allelic differences in β-globin cysteine content could affect aspects of redox signaling and oxidative/nitrosative stress responses that are mediated by Hb-S-nitrosylation and Hb-S-glutathionylation pathways.

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1. Introduction

Electrophoretic surveys of wild house mice (genus *Mus*) have revealed striking patterns of allelic polymorphism at two tandemly duplicated genes that encode the β-chain subunits of adult hemoglobin (Hb) (Selander and Yang, 1969; Selander et al., 1969a, b; Miyashita et al., 1985; Kawashima et al., 1995). In natural populations of Eurasian house mice, three main classes of β-globin haplotype have been characterized: Hbb^d, Hbb^p, and Hbb^s (Erhart et al., 1985; Storz et al., 2007; Runck et al., 2009, 2010). These alternative haplotypes differ in the level of sequence divergence between the two β-globin gene duplicates, *HBB-T1* and *HBB-T2*. The Hbb^d and Hbb^p haplotypes harbor two structurally distinct β-globin genes, and in each case, the more highly expressed *HBB-T1* gene encodes the β-chain subunits of the major Hb isoform (isoHb) whereas *HBB-T2* encodes the β-chains of the minor isoHb (Whitney, 1977; Leder et al., 1980). In contrast to the two distinct β-globin duplicates on the Hbb^d and Hbb^p haplotypes, the Hbb^s haplotype harbors two β-globin duplicates that are identical in sequence (Erhart et al., 1985; Storz et al., 2007; Hoffmann et al., 2008). Thus, mice that are homozygous for the Hbb^s haplotype synthesize a single β-chain isoHb during postnatal life.

One especially noteworthy difference between the *s*-type Hbs relative to the *d*- and *p*-type Hbs relates to the number of reactive β-chain cysteine residues. All of the mouse Hbs contain the highly conserved cysteine at β93(F9), which is present in the β-globins of all mammals and birds examined to date (Riggs, 1960; Reischl et al., 2007; Jensen, 2009), but the *d*- and *p*-type Hbs contain an additional reactive cysteine residue at β13(A10). The sulfhydryl –SH group of β93Cys plays an important role in the formation of mixed disulfides with low molecular mass thiols like glutathione (Murakami and Mawatari, 2003; Thomas et al., 2003; Giustarini et al., 2006; Hempe et al., 2007; Colombo et al., 2010) and the role of β93 S-nitrosylation in transducing hypoxic nitric oxide (NO) vasoactivity is a matter of ongoing debate (reviewed by Gladwin and Kim-Shapiro, 2008; Jensen, 2009; Reeder, 2010). Studies of mouse Hbs by Hempe et al. (2007) demonstrated that both β13Cys and β93Cys form mixed disulfides with glutathione under oxidizing conditions and that β13Cys is especially reactive. Thus, mice that express the *d*- and *p*-type Hbs have red cells with an elevated concentration of reactive sulfydryl groups relative to mice that only express *s*-type Hbs, and this results in pronounced differences in the blood-mediated

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doi:10.1016/j.cbpa.2011.11.004

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metabolism of oxidants and thiol reactants such as NO and glutathione (Miranda, 2000; Giustarini et al., 2006; Hempe et al., 2007).

Results of previous studies indicated that the various Hb types of house mice exhibit slight differences in O_2 affinity (Newton and Peters, 1983; Uchida et al., 1998; Runck et al., 2010). However, much more dramatic functional differences between monocysteinyl (*s*-type) β-Hbs and dicysteinyl (*d*- and *p*-type) β-Hbs are manifest under conditions of oxidative stress. Specifically, red cells of mice expressing dicysteinyl β-Hbs are far more susceptible to oxidant-induced hemolysis than are those of mice expressing monocysteinyl β-Hbs (Kruckeberg et al., 1987). Studies by Kruckeberg et al. (1987) demonstrated that susceptibility to red cell hemolysis was positively associated with the rate of membrane lipid oxidation, as measured by the formation of malonyldialdehyde, a fatty acid oxidative breakdown product. Red cells that were susceptible to oxidant-induced hemolysis showed a rapid rate of malonyldialdehyde formation, whereas red cells that were resistant to hemolysis showed a far lower rate of malonyldialdehyde formation. These results suggest that differences in β-globin cysteine content may be responsible for differences in heme oxidation rates, which in turn influences cell survival under oxidative stress. This is because Hbs in the ferrous Hb-Fe^{2+} or ferric Hb-Fe^{3+} (metHb) states react with hydrogen peroxide (H_2O_2) and/or lipid hydroperoxides to form a ferryl Fe^{4+} heme and a protein-centered radical in the reaction with metHb (Alayash et al., 2001). Such products can promote lipid oxidation (Harel and Kanner, 1986; Everse and Hsia, 1997; Umbreit, 2007), a process that may be further enhanced by the release of hemin from metHb subunits (Everse and Hsia, 1997; Reeder, 2010). However, experimental studies of human red cells have revealed a complex association between rates of Hb oxidation and the susceptibility to oxidant-induced hemolysis (Trotta et al., 1981, 1982, 1983). Paradoxically, red cells that accumulated a higher concentration of metHb experienced lower levels of membrane lipid oxidation. Under conditions of oxidative stress, metHb appears to protect against oxidative damage to the red cell membrane by scavenging reactive intermediates that propagate lipid peroxidation chain reactions. The oxidation of β93Cys to cysteic acid has been proposed to play a role in the scavenging of free radicals generated by reactions of ferrous and ferric heme with H_2O_2, thereby protecting other cellular components from oxidative damage (Jia et al., 2007; Reeder, 2010; Widmer et al., 2010).

To assess the functional consequences of allelic variation in β-globin cysteine content, we measured O_2-binding properties and H_2O_2-induced oxidation rates of mono- and dicysteinyl β-Hbs from 4 different inbred strains of mice: C57BL/6J, BALB/cJ, MSM/Ms, and CAROLI/EiJ. The experiments revealed that purified Hbs from the various mouse strains did not exhibit substantial variation in O_2-binding properties, but *s*-type Hb (which contains a single reactive β-chain cysteine residue) was far more readily oxidized to Fe^{3+} by H_2O_2 than other mouse Hbs that contain two reactive β-chain cysteine residues. These results suggest that the possession of an additional reactive cysteine residue may protect against metHb formation under oxidizing conditions. If so, then these allelic differences in Hb oxidation rates may underlie previously documented variation in S-nitrosylation, S-glutathionylation, and the susceptibility to red cell hemolysis under oxidative stress (Kruckeberg et al., 1987; Giustarini et al., 2006; Hempe et al., 2007).

2. Materials and methods

2.1. Samples

We measured O_2-binding properties and oxidation rates of purified hemolysates from 4 different inbred strains of mice: C57BL/6J, BALB/cJ, MSM/Ms, and CAROLI/EiJ. The C57BL/6J and BALB/cJ strains are referable to *Mus musculus domesticus*, the MSM/Ms strain is referable to *M. musculus molossinus*, and the CAROLI/EiJ strain is referable to *M. caroli*. Blood samples from each of the 4 strains were obtained from the Jackson Lab (Bar Harbor, ME, USA). Blood samples from the MSM/Ms strain were procured under a material transfer agreement with the National Institute of Genetics (Mishima, Japan).

2.2. Measurement of O_2-equilibrium curves

Hemolysates were prepared according to standard methods and were stripped of organic phosphates and other ionic cofactors by passing the samples through a mixed bed resin column (MB-1 AG501-X8; BioRad, Hercules, CA, USA). The Hb samples were concentrated by ultrafiltration (cutoff 10,000), dialyzed in CO-equilibrated 10 mM HEPES buffer, pH 7.6, and stored at −80 °C as CO-derivatives. The isoHb composition of hemolysates from each mouse strain was confirmed by using thin-layer isoelectric focusing (PhastSystem, GE Healthcare Biosciences, Piscataway, NJ, USA). Using a modified diffusion chamber, O_2-equilibria of Hb solutions were measured in 10 mM HEPES buffer, pH 7.4, at constant temperature, 37 °C. The met-Hb enzymatic reducing system of Hayashi et al. (1973) was used to maintain Hb in the ferrous state. Changes in the absorbance of Hb solutions were recorded in conjunction with stepwise changes in the partial pressure of O_2 [PO_2] of gas mixtures (prepared using cascaded Wösthoff gas-mixing pumps that perfuse the chamber; Weber, 1981, 1992; Weber et al., 2004).

Values of P_{50} and n_{50} (PO_2 and Hill's cooperativity coefficient, respectively, at 50% oxygenation of the heme groups) were interpolated from linear Hill plots (log ([OxyHb]/[Hb]) vs. log PO_2) based on three to five equilibration steps between 20 and 80% oxygen saturation values.

The P_{50} values for the stripped (i.e. cofactor-free) hemolysates provide an inverse measure of the intrinsic O_2-binding affinities of the major and minor isoHbs occurring in their natural relative concentrations. To assess variation among the different mouse Hbs in the sensitivity to allosteric cofactors, we measured O_2-equilibrium curves for each sample in the absence of added cofactors (stripped hemolysates), in the presence of 2,3-diphosphoglycerate (DPG; 2,3-bisphosphoglycerate), in the presence of Cl^- ions (added as KCl), and in the presence of both cofactors ([Cl^-], 0.10 M; [NaHEPES], 0.1 M; DPG/Hb tetramer ratio, 2.0; [Heme], 0.16 mM).

2.3. Measurement of oxidation rates in the presence of H_2O_2

On the day of the experiment, carboxyHb samples were converted to the oxy form in ~1 h by photodissociation on ice under air or pure O_2. Full conversion to the oxy derivative was checked by UV–visible absorbance spectroscopy. Heme oxidation kinetics of oxyHb were measured at 37 °C by adding H_2O_2 (20 μM final concentration) to a solution of Hb (10 μM heme) in 0.1 M Hepes, pH 7.4. Absorbance was measured every 10 s in the range 350–500 nm using a HP 8453 diode array spectrophotometer. Kinetic traces at 430 nm were used to calculate initial rates (s^{-1}). Statistical differences between rates (values expressed as mean ± s.e.m., with *n* as the number of replicates) were assessed by one-way ANOVA, using a significance threshold of $\alpha = 0.05$.

2.4. Analysis of reaction products by SDS-PAGE

To assess whether the reaction between oxyHb and H_2O_2 promoted disfulfide polymerization, we incubated Hb (200 μM heme) in 0.1 M Hepes buffer pH 7.4, 0.1 M KCl, 100 μM DPG (DPG:Hb_4 2:1) with H_2O_2 (400 μM) at 37 °C for 1 h and overnight. Aliquots were then mixed with SDS sample buffer containing 5 mM NEM to block free thiols, in the presence and absence of 1 mM DTT, and incubated at 100 °C for 5 min before loading on a precast PhastSystem (GE Healthcare) 10–15% SDS-PAGE for analysis of intermolecular disulfide bonds induced by reaction with H_2O_2.

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3. Results

3.1. Patterns of β-globin sequence variation

In contrast to the C57BL/6J strain, which carries a pair of tandemly duplicated β-globin genes that are identical in sequence, the BALB/cJ, MSM/Ms, and CAROLI/EiJ strains each possess structurally distinct *HBB-T1* and *HBB-T2* genes (Fig. 1). On the Hbb^d haplotype of BALB/cJ, the *HBB-T1* and *HBB-T2* genes (which encode the β-chain subunits of the major and minor isoHbs [d_{major} and d_{minor}], respectively) are distinguished from one another by nine amino acid substitutions. Similar to the case with the Hbb^d haplotype, the *HBB-T1* and *HBB-T2* genes on the Hbb^p haplotype of MSM/Ms encode the β-chain subunits of the major and minor isoHbs (p_{major} and p_{minor}), respectively, and are distinguished from one another by ten amino acid substitutions. The Hbb^d and Hbb^p haplotypes share identical *HBB-T1* sequences ($d_{major} = p_{major}$), but they are distinguished by two amino acid substitutions at *HBB-T2*: β22(B4)Glu→Ala and β23(B5)Val→Ile ($d_{minor} \rightarrow p_{minor}$, in both cases). Thus, any functional difference between the β-chain isoHbs produced by the Hbb^d and Hbb^p haplotypes would have to be attributable to the two amino acid differences that distinguish the minor isoHbs, d_{minor} and p_{minor}. The *HBB-T1* and *HBB-T2* genes of CAROLI/EiJ are distinguished from one another by eight amino acid substitutions. Whereas the single Hb of C57BL/6J contains a single reactive cysteine at β93, the major and minor β-chain isoHbs expressed by the other three strains contain an additional fast-reacting cysteine residue at β13. The *s*-type β-globin sequence of C57BL/6J is distinguished from the *HBB-T1* and *HBB-T2* sequences of the other three strains by eight to eleven amino acid differences, including the presence of Gly instead of Cys at β13 (Fig. 1, Table 1). Aside from the β13Gly/Cys change, the only other consistent difference between the *s*-type Hb of C57BL/6J and the major Hbs of the other three strains involves the replacement of Ala for Thr at β139(H17) (Fig. 1).

3.2. Hb-O_2 affinity

O_2 equilibrium curves revealed that the purified hemolysates of the various mouse strains have fairly similar O_2-binding properties. Relative to the Hbs of BALB/cJ, MSM/Ms, and CAROLI/EiJ, the *s*-type Hb of C57BL/6J was characterized by a slightly higher O_2-affinity (lower P_{50}) in the presence and in the absence of DPG and DPG + Cl^- ions (Fig. 2, Table 2). In each strain, O_2 equilibrium measurements revealed that Cl^- ions exert a more potent allosteric effect than DPG. Hb-O_2 affinity was always reduced to a greater extent in the presence of 0.1 mol l^{-1} Cl^- ions than in the presence of DPG at twofold molar excess over tetrameric Hb. This is indicated by the fact that $\Delta\log P_{50\ (KCl\text{-stripped})}$ values were roughly two-fold higher than the corresponding $\Delta\log P_{50\ (DPG\text{-stripped})}$ values (Table 2). In each strain, Hb-O_2 affinity was reduced to a similar extent by KCl alone and by KCl in combination with DPG ($\Delta\log P_{50\ [KCl\text{-stripped}]}$ values were highly similar to $\Delta\log P_{50\ ([DPG+KCl]\text{-stripped})}$ values; Table 2). Thus, there does not appear to be any significant competition for binding sites between the monovalent and polyvalent anions.

Table 1
Amino acid differences between orthologs of *HBB-T1* and *HBB-T2* in pairwise comparisons between different stains of house mice. See Fig. 1 for information about the nature of the substitutions.

	BALB/cJ	MSM/Ms	CAROLI/EiJ
HBB-T1			
C57BL/6J	3	3	12
BALB/cJ		0	10
MSM/Ms			10
HBB-T2			
C57BL/6J	11	13	14
BALB/cJ		2	7
MSM/Ms			9

3.3. Oxidation rates

Kinetic measurements revealed that the monocysteinyl β-Hb from the strain C57BL/6J is more readily oxidized to ferric heme by H_2O_2 than the dicysteinyl β-Hbs from the strains BALB/cJ, MSM/Ms, and CAROLI/EiJ (Fig. 3A,B). These experiments revealed no significant variation in oxidation rates among the dicysteinyl β-Hbs from BALB/cJ, MSM/Ms, and CAROLI/EiJ.

To assess whether the oxidation of cysteine –SH groups during incubation with H_2O_2 promoted the formation of intermolecular disulphide bonds, we analyzed reaction products by SDS-PAGE. The absence of any high-molecular weight band (~30,000; data not shown), indicated that covalent intermolecular polymerization of Hb polypeptide chains did not occur under the experimental conditions that we used.

4. Discussion

Results of our experiments revealed that Hbs from the various mouse strains are similar with respect to O_2-binding properties, but *s*-type Hb from C57BL/6J (which contains a single reactive β-chain cysteine) was far more readily oxidized to Fe^{3+} by H_2O_2 than the Hbs from BALB/cJ, MSM/Ms, and CAROLI/EiJ (which contain two reactive β-chain cysteines). These results indicate that the possession of

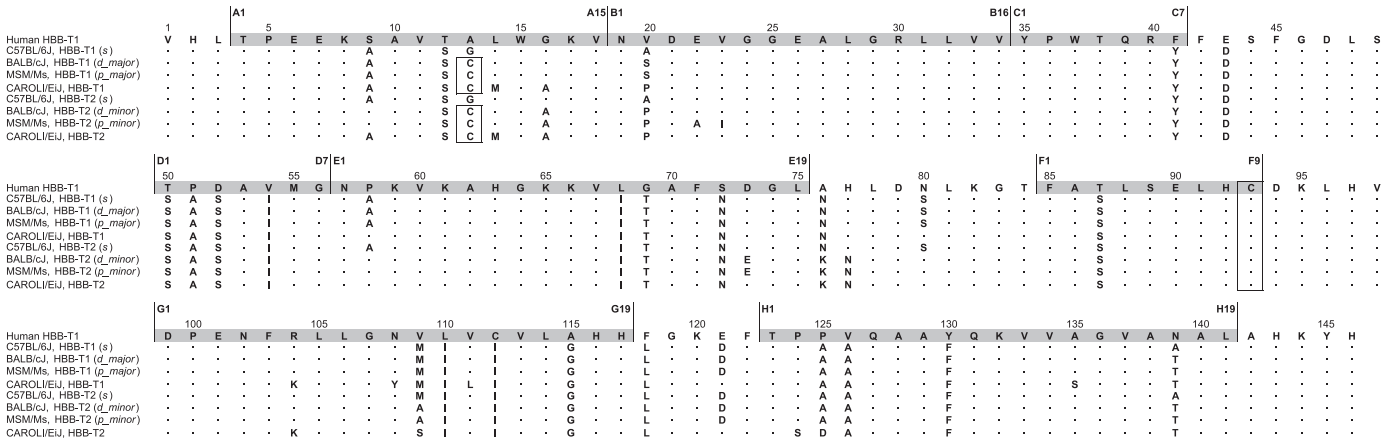

Fig. 1. Structural alignment of house mouse β-globin sequences. Reactive cysteine residues at sites 13 and 93 are shown in boxes.

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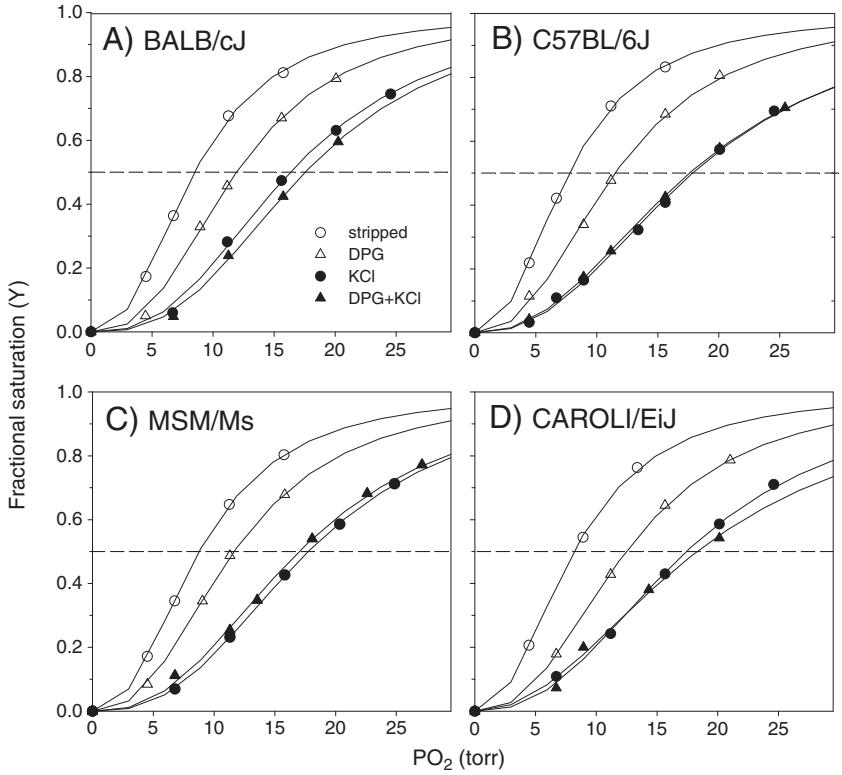

Fig. 2. O_2-equilibrium curves for stripped Hbs from four inbred strains of house mice: (A) BALB/cJ, (B) C57BL/6J, (C) MSM/Ms, and (D) CAROLI/EiJ. O_2-equilibria were measured at 37 °C, in 0.10 M NaHEPES buffer, at pH 7.40, in the presence and absence of allosteric cofactors [(Cl-), 0.1 M; DPG/Hb tetramer ratio, 2.0], at 0.16 mM heme concentration, and using the met-Hb reductase system of Hayashi et al. (1973).

two additional reactive cysteine residues per Hb tetramer may protect against metHb formation under oxidizing conditions.

4.1. Variation in Hb-O_2 affinity

O_2-equilibrium measurements of the purified hemolysates revealed that the *s*-type Hb of C57BL/6J was characterized by a slightly higher O_2-affinity than the *d*- and *p*-type Hbs of BALB/cJ, MSM/Ms, and CAROLI/EiJ (Fig. 2, Table 2). Our results are consistent with previous studies of house mouse Hbs (Uchida et al., 1998), which also showed (under different buffer conditions) a relatively weak effect of DPG on Hb-O_2 affinity. Relative to Hbs of the deer mouse, *Peromyscus maniculatus*, a similarly sized myomorph rodent, the house mouse Hbs were characterized by similar O_2 affinities in the absence of added effectors (stripped) (range of $P_{50\ [37°,\ pH\ 7.4]}$ values = 7.63–8.76 for house mice and 6.70–8.56 for deer mice). However, house mouse Hbs are characterized by substantially lower O_2 affinities in the presence of DPG and Cl^- ions (range of $P_{50\ [37°,\ pH\ 7.4]}$ values = 16.87–18.45 for house mice and 11.71–15.76 for deer mice; Storz et al., 2009, 2010). One unusual property that is shared between the Hbs of deer mice and house mice is that in both cases Cl^- ions exert a stronger allosteric effect than DPG. This is an important finding because DPG is a much more potent allosteric effector than Cl^- ions in the overwhelming majority of mammalian Hbs. The only documented exceptions include Hbs from select lineages of artiodactyls, carnivores, moles, and prosimian primates (Bunn, 1971; Taketa et al., 1971; Bunn et al., 1974; Campbell et al., 2010).

Table 2
O_2 affinity (P_{50}, torr; mean ± s.e.m.) and cooperativity coefficients (n_{50}) for purified hemolysates measured in 0.1 M Hepes buffer at pH 7.40, 37 °C, in the presence of the met-Hb reducing system (Hayashi et al., 1973). Measurements were conducted in the absence of DPG and KCl (stripped), and in the presence of DPG (DPG/Hb tetramer ratio = 2.0) or 0.1 M KCl, or both effectors. [Heme], 0.16 mM. $N = 3$ replicate measurements for C57BL/6J, BALB/cJ, and MSM/Ms.

	C57BL/6J	BALB/cJ	MSM/Ms	CAROLI/EiJ
P_{50} (torr)				
Stripped	7.63 ± 0.09	8.44 ± 0.09	8.76 ± 0.04	8.10
DPG	10.95 ± 0.42	11.88 ± 0.17	11.64 ± 0.07	12.48
KCl	17.15 ± 0.49	16.27 ± 0.30	17.74 ± 0.06	17.46
DPG + KCl	16.87 ± 0.48	17.53 ± 0.29	17.03 ± 0.27	18.45
Δ *log* P_{50}				
DPG-stripped	0.16	0.15	0.12	0.19
KCl -stripped	0.35	0.29	0.31	0.33
(DPG + KCl) - stripped	0.34	0.31	0.29	0.36
(DPG + KCl) - KCl	−0.01	0.03	−0.02	0.02
(DPG + KCl) - DPG	0.19	0.17	0.17	0.17
n_{50}				
Stripped	2.24 ± 0.03	2.46 ± 0.05	2.40 ± 0.02	2.28
DPG	2.44 ± 0.084	2.65 ± 0.09	2.50 ± 0.04	2.53
KCl	2.36 ± 0.166	2.68 ± 0.12	2.67 ± 0.02	2.57
DPG + KCl	2.45 ± 0.011	2.78 ± 0.13	2.56 ± 0.09	1.91

4.2. Variation in oxidation rates

Results of our experiments revealed far higher rates of H_2O_2-induced heme oxidation in mouse strains with monocysteinyl β-globins (C57BL/6J) than in mouse strains with dicysteinyl β-globins (BALB/cJ, MSM/Ms, and CAROLI/EiJ). This dichotomy in the rate of heme oxidation is mirrored by previously documented variation among mouse strains in red cell survival under oxidative stress: strains like C57BL/6J with monocysteinyl β-globins proved highly resistant to oxidant-induced hemolysis, whereas strains like BALB/cJ with dicysteinyl β-globins proved far more susceptible to hemolysis (Kruckeberg et al., 1987). Our results, combined with the experimental results of Kruckeberg et al. (1987), suggest that the elevated oxidation rate of the monocysteinyl β-chain Hb is associated with increased resistance to hemolysis under conditions of oxidative stress. This is consistent with the hypothesis of Trotta et al. (1981,

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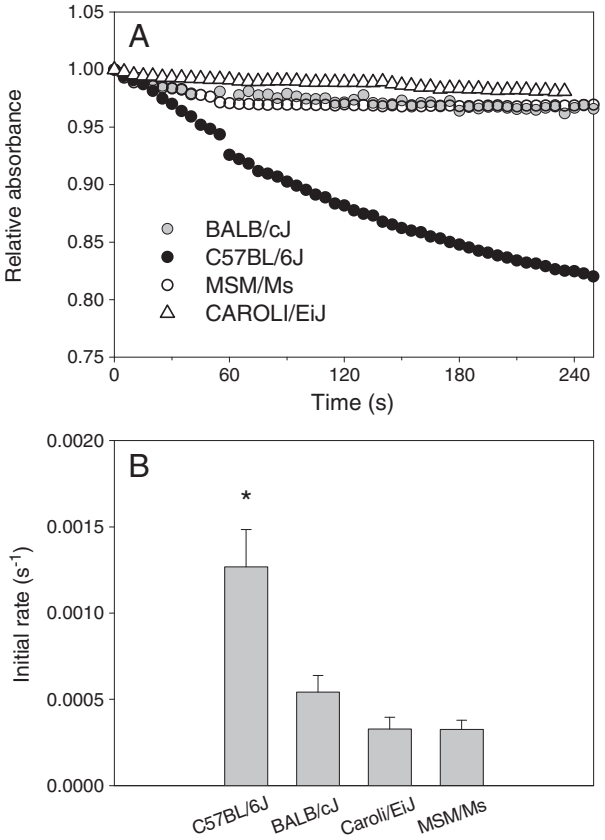

Fig. 3. Oxidation of purified Hbs from mouse strains BALB/cJ, C57BL/6J, MSM/Ms, and CAROLI/EiJ. (A) Representative kinetic traces at 430 nm and (B) derived initial rates of oxidation (s^{-1}) of oxyHb (10 μM heme) to metHb by H_2O_2 at a 1:2 molar ratio of heme:H_2O_2 at 37 °C in 0.1 M Hepes, pH 7.4. Values are expressed as mean ± s.e.m. Statistically significant differences ($P<0.05$) are denoted by an asterisk. Number of replicates (n): BALB/cJ (*5*), C57BL/6J (*4*), MSM/Ms (*4*), and CAROLI/EiJ (*5*).

1982, 1983) that, under conditions of oxidative stress, an increased concentration of metHb may confer a protective effect at the cellular level by inhibiting lipid peroxidation.

4.3. Structural mechanisms of thiol reactivity and heme oxidation

In mouse Hb, the especially fast reactivity of β13Cys relative to β93Cys appears to be attributable to an increased solvent accessibility and an increased electron density around the sulfur atom of the β13Cys thiol group due to the formation of a hydrogen bond with the carbonyl of β10(A7)Ala (Miranda, 2000). The β13Gly → Cys residue change likely confers protection against H_2O_2-induced heme oxidation because H_2O_2 reacts with the thiol instead of the heme. Since Hb is primarily oxidized by H_2O_2 in the deoxy-ferrous state, the initial oxidation of Fe^{2+} to Fe^{3+} should be inversely proportional to O_2 affinity (Alayash et al., 1999). However, our experiments revealed that the Hb type with the highest oxidation rate was also characterized by the highest O_2 affinity (lowest P_{50}) at physiological pH (Table 2), so the observed differences in oxidation rates cannot be explained by differences in Hb-O_2 affinity.

4.4. Possible adaptive significance of the allelic variation in oxidation rates

Electrophoretic surveys of β-globin polymorphism in natural populations of *Mus musculus* and *M. domesticus* have revealed that the Hbb^d and Hbb^s haplotypes are consistently present at intermediate frequencies in population samples from across the species' range, a pattern that is not mirrored by other unlinked autosomal genes (reviewed by Storz et al., 2007). This striking uniformity of allele frequencies has led a number of authors to conclude that the two-locus β-globin polymorphism may be maintained by some form of balancing selection (Berry, 1978). This hypothesis is supported by analyses of nucleotide variation at the *HBB-T1* and *HBB-T2* genes, which have revealed remarkably high levels of nucleotide diversity within species (Storz et al., 2007) and the pervasive sharing of Hbb^d and Hbb^s haplotypes among multiple Eurasian species in the subgenus *Mus* (Runck et al., 2009). However, despite the abundance of indirect evidence for the selective maintenance of the Hbb^d and Hbb^s haplotypes, there is currently no widely accepted mechanistic explanation for the possible existence of fitness variation among mice with different β-globin genotypes. One possibility is that the coding mutations in the *HBB* genes do not directly contribute to fitness variation, but are selectively maintained due to close physical linkage with other (possibly noncoding) sites that represent the true target of balancing selection (Runck et al., 2010). This seems especially plausible since the alternative *s*- and *d*-type alleles at the two tandemly linked *HBB* genes are associated with differences in Hb concentration (Peters et al., 2010). Our results and those of other recent studies (Giustarini et al., 2006; Hempe et al., 2007) suggest that the adaptive significance of the two-locus β-globin polymorphism could relate specifically to allelic differences in oxidation rate (mediated by β-globin cysteine content), and may therefore revolve around a signaling function of the Hb-metHb redox couple. Since the intraerythrocytic concentration of reactive sulfydryl groups influences the availability of reduced glutathione for enzymatic detoxification reactions (Di Simplicio et al., 1998; Murakami and Mawatari, 2003; Giustarini et al., 2006; Dalle-Donne et al., 2007; Hempe et al., 2007; Colombo et al., 2010), allelic variation in β-globin cysteine content may contribute to variation in the cellular response to pathogenic infection and oxidative/nitrosative stress. The differences in met-Hb formation that we documented between strains of mice that express mono- and dicysteinyl β-chain Hbs suggest that the extensive variation in Hb cysteine content among different vertebrate taxa (Reischl et al., 2007) may be associated with equally extensive variation in the redox activity of red blood cells.

Acknowledgments

We thank three anonymous reviewers for helpful comments and suggestions, and we thank Mai-Britt Hemmingsen for valuable assistance in the lab. This work was funded by grants from the National Institutes of Health/National Heart, Lung, and Blood Institute (R01 HL087216 and HL087216-S1) and the National Science Foundation (IOS-0949931).

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