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Genetic differences in hemoglobin function between highland and lowland deer mice

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SUMMARY

In high-altitude vertebrates, adaptive changes in blood–O_2 affinity may be mediated by modifications of hemoglobin (Hb) structure that affect intrinsic O_2 affinity and/or responsiveness to allosteric effectors that modulate Hb–O_2 affinity. This mode of genotypic specialization is considered typical of mammalian species that are high-altitude natives. Here we investigated genetically based differences in Hb–O_2 affinity between highland and lowland populations of the deer mouse (*Peromyscus maniculatus*), a generalist species that has the broadest altitudinal distribution of any North American mammal. The results of a combined genetic and proteomic analysis revealed that deer mice harbor a high level of Hb isoform diversity that is attributable to allelic polymorphism at two tandemly duplicated α-globin genes and two tandemly duplicated β-globin genes. This high level of isoHb diversity translates into a correspondingly high level of interindividual variation in Hb functional properties. O_2 equilibrium experiments revealed that the Hbs of highland mice exhibit slightly higher intrinsic O_2 affinities and significantly lower Cl^- sensitivities relative to the Hbs of lowland mice. The experiments also revealed distinct biochemical properties of deer mouse Hb related to the anion-dependent allosteric regulation of O_2 affinity. In conjunction with previous findings, our results demonstrate that modifications of Hb structure that alter allosteric anion sensitivity play an important role in the adaptive fine-tuning of blood–O_2 affinity.

Key words: high-altitude adaptation, hypoxia, oxygen transport, *Peromyscus*, physiological genetics.

INTRODUCTION

In air-breathing vertebrates, changes in blood–O_2 affinity provide an important line of physiological defense against low O_2 availability (environmental hypoxia) (Eaton et al., 1974; Penny and Thomas, 1975; Monge and León-Velarde, 1991). Fine-tuned adjustments in blood–O_2 affinity may be mediated by changes in the intrinsic O_2 binding affinity of hemoglobin (Hb), changes in the sensitivity of Hb to allosteric effectors that modulate Hb–O_2 affinity, and/or changes in the concentration of allosteric effectors within the erythrocyte (Nikinmaa, 2001; Weber and Fago, 2004; Weber, 2007; Storz and Moriyama, 2008). This last mechanism involves regulatory adjustments of the chemical milieu within the erythrocyte and does not require structural modification of Hb itself. In the mature erythrocytes of most mammalian species, the most potent allosteric effector molecule is 2,3-diphosphoglycerate (DPG), a metabolite of glycolysis, which reduces Hb–O_2 affinity by preferentially binding and stabilizing the deoxygenated, 'tense-state' conformation of the Hb tetramer. Other allosteric effectors that reduce O_2 affinity include Cl^- ions, as well as H^+ and CO_2 which facilitate O_2 unloading in the tissue capillaries *via* the Bohr effect (lowered Hb–O_2 affinity at low pH) (Weber and Fago, 2004).

Identifying the specific mechanisms responsible for changes in blood–O_2 affinity should provide insights into the relative roles of genotypic specialization *vs* phenotypic plasticity in adaptation to environments that differ in O_2 availability. An increase in blood–O_2 affinity that is mediated by structural changes in the Hb protein (a strategy of genotypic specialization) may be characteristic of species that are native to high-altitude environments (Bunn, 1980; Monge and León-Velarde, 1991; Storz, 2007; Weber, 2007). By contrast, in species that inhabit a broad range of different elevational zones, or that must cope with changing O_2 availability on a daily or seasonal basis, it may be more advantageous to retain some capacity for the continuous adjustment of blood–O_2 affinity in response to changes in O_2 availability or changes in metabolic demands (e.g. aerobic thermogenesis during periods of cold stress). One such mechanism of regulatory control involves adjusting erythrocytic concentrations of organic phosphates like DPG. Modulating blood–O_2 affinity in this manner could be considered a mechanism of phenotypic plasticity because the same genotype (manifest as Hb structure) can express a range of different biochemical phenotypes (blood–O_2 affinities) depending on the prevailing environmental circumstances. For example, a reduced erythrocytic concentration of DPG would produce an increased blood–O_2 affinity that helps safeguard arterial O_2 saturation under hypoxia, and this could be reversed upon a return to normoxic conditions.

Under conditions of severe hypoxia, theoretical and experimental results indicate that it is generally advantageous to have an elevated blood–O_2 affinity because of the increased premium on pulmonary O_2 loading (Turek et al., 1973; Eaton et al., 1974; Turek et al., 1978a; Turek et al., 1978b; Bouverot, 1985; Samaja et al., 1986; Samaja et al., 2003; Scott and Milsom, 2006). Consistent with this expectation, elevated blood–O_2 affinities are commonly recorded in terrestrial vertebrates that are native to high altitude environments, and in subterranean mammals that cope with the hypoxic and hypercapnic conditions of closed burrow systems (Jelkmann et al., 1981; Perutz, 1983; Monge and León-Velarde, 1991; Storz, 2007; Weber, 2007). It is unclear whether elevated blood–O_2 affinities are also characteristic of species that inhabit a broad range of elevational zones.

The deer mouse, *Peromyscus maniculatus* Wagner 1845, has the broadest altitudinal distribution of any North American mammal (Hock, 1964). This species is found below sea level in Death Valley,

CA, USA (–60 m), and also on the summits of the highest peaks in the Sierra Nevada and Rocky Mountains (4350 m) where the partial pressure of O_2 (P_{O_2}) is less than 60% of the sea level value. The deer mouse therefore represents a good model species for investigating mechanisms of physiological adaptation to different elevational zones. Previous physiological studies have demonstrated a strong, positive correlation between blood–O_2 affinity and the native altitude of different deer mouse populations across western North America (Snyder, 1981; Snyder et al., 1982; Snyder, 1985; Snyder et al., 1988). Moreover, breeding experiments have demonstrated that this variation in blood–O_2 affinity has a strong heritable component and does not simply reflect a reversible acclimatization effect (Snyder, 1982; Snyder et al., 1982). This genetically based variation in blood–O_2 affinity appears to be attributable to structural variation in Hb (Snyder, 1981; Snyder et al., 1982; Chappell and Snyder, 1984; Chappell et al., 1988) as well as regulatory variation in erythrocytic DPG concentration (Snyder, 1982). However, in natural populations of deer mice, the frequency of different Hb structural variants and the intraerythrocytic DPG/Hb ratio both covary with altitude (Snyder, 1982), which makes it difficult to disentangle the relative contributions of structural and regulatory factors to adaptive altitudinal variation in blood–O_2 affinity. There would seem to be ample scope for genetically based variation in Hb–O_2 affinity as electrophoretic surveys of deer mouse populations across western North America have revealed extremely high levels of allelic polymorphism at multiple α- and β-globin genes (Rasmussen et al., 1968; Snyder, 1978; Snyder et al., 1988). More recently, genomic studies have characterized the complete globin gene repertoire of deer mice (Hoffmann et al., 2008b; Storz et al., 2008), and surveys of polymorphism at the nucleotide level have revealed the specific amino acid changes that distinguish the previously characterized α- and β-globin electromorphs (Storz et al., 2007; Storz and Kelly, 2008; Storz et al., 2009). These amino acid polymorphisms exhibit pronounced allele frequency differences between high- and low-altitude populations, and the β-globin variants that predominate at high altitude are associated with suppressed sensitivity to DPG (Storz et al., 2009). However, it is not known how the myriad combinations of α- and β-chain subunit isoforms might contribute to altitudinal differences in Hb–O_2 affinity.

The goals of the present study were (i) to characterize differences in Hb isoform (isoHb) composition between highland and lowland deer mice, and (ii) to test whether these highland and lowland mice exhibit genetically based differences in Hb–O_2 affinity and anion sensitivity. The results of our survey revealed that deer mice harbor a high level of isoHb diversity that is attributable to allelic polymorphism at two tandemly duplicated α-globin genes (HBA-T1 and HBA-T2) and two tandemly duplicated β-globin genes (HBB-T1 and HBB-T2). O_2 binding experiments revealed that the Hbs of highland mice exhibit lower Cl^- sensitivities relative to the Hbs of lowland mice but similarly low sensitivities to DPG. Our experiments also revealed distinct biochemical properties of deer mouse Hb related to the allosteric regulation of O_2 affinity. In deer mouse Hb, Cl^- ions exerted a more pronounced allosteric effect than DPG, and in lowland mice, Cl^- ions exerted a more pronounced allosteric effect in isolation than when present in combination with DPG, suggesting a negative functional interaction.

MATERIALS AND METHODS

Sampling

We sampled a total of 110 deer mice from a pair of high- and low-altitude localities in eastern Colorado, USA, for our comparative study of isoHb composition and Hb function. In August 2004, we collected a total of 75 specimens that were used for an isoelectric focusing (IEF) analysis of Hb polymorphism in conjunction with a survey of DNA sequence polymorphism at the α- and β-like globin genes. In August 2007, we collected an additional 35 specimens for more detailed examination of isoHb composition and experimental studies of Hb function. High-altitude mice were trapped in alpine fell fields and talus fields on the summit of Mt Evans (Clear Creek County, Colorado, 39°15′24″N, 106°10′54″W; 4347 m above sea level) and low-altitude mice were trapped in prairie grassland habitat (Bonny Reservoir, Yuma County, Colorado, 39°37′30″N, 102°10′27″W; 1158 m above sea level). On the summit of Mt Evans, the standard barometric pressure is 68% that of the low-altitude site (456 *vs* 667 Torr).

Mice were live-trapped and handled in accordance with guidelines approved by the University of Nebraska Institutional Animal Care and Use Committee (IACUC no. 07-07-030D) and the National Institutes of Health (NIH publication no. 78-23). After each mouse had been killed, we collected ~0.5–1.0 ml of whole blood by means of cardiac puncture with a 22 gauge hypodermic needle. Blood was collected in cryotubes, snap-frozen in liquid nitrogen, and stored at –80°C prior to use as a source of Hb for functional studies. We collected bone marrow from the femurs of each mouse as a source of globin mRNA. Bone marrow was preserved in RNAlater (Qiagen, Valencia, CA, USA) and stored at –80°C prior to RNA extraction. Finally, we dissected out the heart, liver and kidney and all organs were snap-frozen in liquid nitrogen as a source of genomic DNA. All specimens from the 2007 collection were deposited in the vertebrate collection at the Denver Museum of Nature and Science (Denver, CO, USA; catalog nos ZM11755–ZM11791).

Molecular genetic analysis of isoHb diversity

Having previously characterized the genomic structure of the α- and β-globin gene clusters of *P. maniculatus* (Hoffmann et al., 2008b; Storz et al., 2008), we designed locus-specific primer sets that enabled us to conduct population-level surveys of nucleotide polymorphism at two paralogs that encode the α-chain subunits of adult Hb (HBA-T1 and HBA-T2) and two paralogs that encode the corresponding β-chain subunits (HBB-T1 and HBB-T2). The HBA-T1 and HBA-T2 genes are separated by 5.0 kb on chromosome 8 (Storz et al., 2008), and the HBB-T1 and HBB-T2 genes are separated by 16.2 kb on chromosome 1 (Hoffmann et al., 2008b) (Fig. 1). We cloned and sequenced both alleles of the HBA-T1 and HBA-T2 genes in 17 highland mice and 17 lowland mice (68 sequences per gene, 136 sequences total). Likewise, we cloned and sequenced both alleles of the HBB-T1 and HBB-T2 genes in a subset of 13 highland mice and 13 lowland mice from the same panel (52 sequences per gene, 104 sequences total). We therefore obtained complete diploid genotypes for both HBA paralogs and both HBB paralogs from the same individual mice, and we were able to resolve the ‘haplotype phase’ of all heterozygous sites. This means that for each single-locus diploid genotype, we were able to identify which mutations occurred together on the same allele (in *cis*), and which occurred on different alleles of the same gene (in *trans*). Summaries of the DNA polymorphism data were reported previously (Storz et al., 2007; Storz and Kelly, 2008; Storz et al., 2009). Here we used the same DNA polymorphism data to inform our assessment of isoHb diversity at the protein level. Specifically, conceptual translations of coding sequences from all α- and β-like globin genes – including the complete set of HBA and HBB sequences from the population survey – were used to construct a reference database for

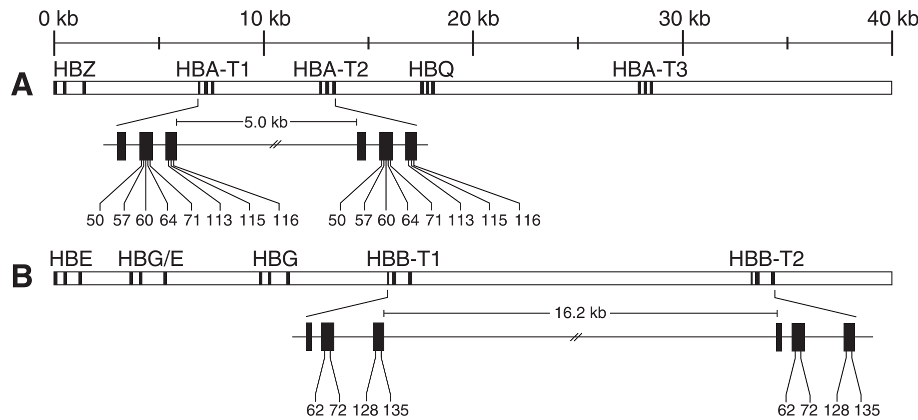

Fig. 1. Genomic structure of the α- and β-globin gene clusters (A and B, respectively) of *Peromyscus maniculatus*. (A) The tandemly duplicated adult α-globin genes (HBA-T1 and HBA-T2) segregate 8 amino acid polymorphisms that exhibit significant allele frequency differences between highland and lowland populations. Lines denote nucleotide positions of the 8 replacement changes in HBA exons 2 and 3, and numbers refer to the corresponding amino acid positions in the encoded α-chain polypeptide. As described in the text, the two HBA genes segregate the same 8 amino acid polymorphisms because of a history of interparalog gene conversion. (B) The tandemly duplicated adult β-globin genes (HBB-T1 and HBB-T2) segregate 4 amino acid polymorphisms that exhibit significant allele frequency differences between highland and lowland populations. Lines denote nucleotide positions of the 4 replacement changes in HBB exons 2 and 3, and numbers refer to the corresponding amino acid positions in the encoded β-chain polypeptide. Similar to the case with the HBA genes, the two HBB genes segregate the same 4 amino acid polymorphisms because of a history of interparalog gene conversion.

proteomic analysis of isoHb composition (see below). By integrating genetic and proteomic data, we were able to determine whether diploid genotypes at the adult HBA and HBB genes could account for the subunit isoHb composition of each specimen, or whether functional Hb tetramers incorporated the products of additional members of the α- or β-globin gene families.

For 10 of the specimens that were used in the experimental analysis of Hb function (N=5 highland mice and N=5 lowland mice), we also used reverse-transcriptase PCR (RT-PCR) to amplify HBA and HBB cDNAs which were then cloned and sequenced. For each mouse, we sequenced 12 clones containing products of HBA-specific RT-PCR and 12 clones containing products of HBB-specific RT-PCR. Thus, full-length inserts representing cDNAs of all expressed HBA and HBB genes were sequenced at 12× coverage and the haplotype phase of all variable sites was determined experimentally. Total RNA was extracted from hematopoietic bone marrow cells using the RNeasy Plus Mini Kit (Qiagen), and cDNA was generated from 1 μg RNA by reverse transcription. For RT-PCR, we used the SuperScript III Platinum One-Step RT-PCR system with Platinum *Taq* DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions were as follows: 1 cycle at 50°C for 10 min, followed by 40 cycles at 94°C for 15 s, 55°C for 30 s and 68°C for 2 min, and a final extension cycle at 68°C for 7 min. We then cloned gel-purified RT-PCR products into pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). In the case of the β-globin genes, we used the same RT-PCR primer pair for HBB-T1 and HBB-T2 (forward: GACTTGCAACCTCAGAAACAGAC, reverse: GSCCAAAGG-CCTTCATCATTT). In the case of the α-globin genes, we used the same primer pair for HBA-T1 and HBA-T2 (forward: CTGAT-TCTCACAGACTCAGGAAG, reverse: CCAAGAGGTACAGG-TGCGAG), and we designed a distinct primer pair for a third α-like globin gene, HBA-T3 (forward: CACTTCTGATTCTCACAGA-CTCAG, reverse: CCAAGAGGTACAGGTGCGAG). The HBA-T3 gene is highly divergent relative to the HBA-T1 and HBA-T2 paralogs and it is characterized by amino acid substitutions at a number of highly conserved sites (Storz et al., 2008). We therefore suspected that HBA-T3 might be transcriptionally inactive (at least in erythroid cell lineages), despite the presence of an intact reading frame and conserved splice junctions.

The triplicated HBA genes have experienced a history of non-reciprocal recombinational exchange (Hoffmann et al., 2008a; Storz et al., 2008), a process known as interparalog gene conversion. As a result of this ‘copy-and-paste’ process of sequence exchange, a small fraction of alleles segregating at the HBA-T1 and HBA-T2 genes have been partially ‘converted’ by the downstream HBA-T3 gene. Consequently, HBA-T1 and HBA-T2 segregate a number of low-frequency converted alleles (containing several hundred bp of T3-derived sequence) that harbor unusual combinations of amino acid mutations. If these alleles are expressed, and if their products are incorporated into functional Hb tetramers, then the resultant amino acid polymorphism could make a significant contribution to standing genetic variation in Hb function. The objectives of the RT-PCR experiments were (i) to determine whether the HBA-T3 gene is expressed; (ii) to determine whether converted HBA-T1 and HBA-T2 alleles are expressed; and (iii) to determine whether there are any expressed globin genes that were not accounted for in the original surveys of polymorphism in the adult HBA and HBB genes. All cDNA sequences were deposited in GenBank under the accession numbers GU972787–GU972811.

Proteomic analysis of isoHb diversity

For all 35 specimens that were used in the functional experiments, the Hb isoform composition of each hemolysate was characterized by means of IEF (PhastSystem, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Hemolysates from a subset of 18 specimens (including the 10 specimens that were used in the RT-PCR experiments) were also subjected to 2D gel electrophoresis and mass spectrometry analyses to identify the constituent isoHb components. The 2D gel analysis involved separation of tetrameric isoHbs by means of native gel electrophoresis, followed by the separation of dissociated α- and β-globin monomers by means of acetic acid–urea–Triton X-100 (AUT) gel electrophoresis. Globin chains excised from each AUT gel were digested with trypsin, and the resultant peptides were identified by means of tandem mass spectrometry (MS/MS). The peak lists of the MS/MS data were

generated by Distiller (Matrix Science, London, UK) using the charge state recognition and de-isotoping with default parameters for quadrupole time-of-flight data. Database searches of the resultant MS/MS spectra were performed using Mascot v1.9.0 (Matrix Science). Specifically, peptide mass fingerprints derived from the MS/MS analysis were used to query the reference database of α- and β-globin sequences derived from the DNA-based survey. The following search parameters were used: no restriction on protein molecular weight or isoelectric point, and methionine oxidation allowed as a variable peptide modification. Mass accuracy settings were 0.15 Da for peptide mass and 0.12 Da for fragment ion masses. We identified all significant protein hits that matched more than one peptide with $P<0.05$.

Experimental analysis of Hb–O_2 binding equilibria

We measured O_2 binding properties of stripped hemolysates from a total of 35 wild-caught mice ($N=20$ high altitude mice and $N=15$ low altitude mice). We measured O_2 equilibria for 9 individual specimens, and in the remaining cases we measured O_2 equilibria for pooled samples of 2–8 specimens that had identical IEF band profiles. O_2 binding data for 4 of the individually assayed specimens were reported in a previous study (Storz et al., 2009).

Hemolysates were prepared according to standard methods and were stripped of organic phosphates and other ionic cofactors by means of a mixed bed resin MB-1 AG501-X8 (BioRad, Hercules, CA, USA), concentrated by ultrafiltration (molecular weight cutoff 10,000), dialyzed in CO-equilibrated 10 mmol l^{-1} Hepes buffer, pH 7.6, and stored at –80°C as a CO derivative. O_2 equilibria of Hb solutions were measured in the presence of 0.1 mol l^{-1} Hepes buffer, pH 7.40, at 37°C. The met-Hb enzymatic reducing system described by Hayashi et al. (Hayashi et al., 1973) was used to prevent oxidation of ferrous heme to its non-reactive ferric form. O_2 equilibrium curves were measured using a modified diffusion chamber where changes in the absorbance of thin-layer Hb solutions (4 μl) were recorded in conjunction with stepwise changes in the O_2 tension of gas mixtures inside the chamber [prepared using cascaded Wösthoff gas-mixing pumps (Weber, 1981; Weber, 1992; Weber et al., 2004)]. Values of P_{50} (the P_{O2} at 50% saturation of the heme groups) and n_{50} (Hill's cooperativity coefficient at 50% saturation) were interpolated from the linear portion of Hill plots [log([OxyHb]/[Hb]) *vs* logP_{O2}] based on at least 4 equilibration steps between 30% and 70% oxygenation. The P_{50} values provide an inverse measure of the O_2 affinities of the hemolysates with the constituent isoHbs occurring in their natural relative concentrations. To assess genetic variation in the allosteric regulation of Hb–O_2 affinity, we measured O_2 equilibrium curves for each sample in the absence of allosteric cofactors (stripped hemolysates), in the presence of DPG alone, in the presence of Cl^- alone (added as KCl), and in the presence of both cofactors ([Cl^-], 0.10 mol l^{-1}; [NaHepes], 0.1 mol l^{-1}; DPG/Hb tetramer ratio, 2.0; [Heme], 0.10–0.16 mmol l^{-1}). The apparent binding constant and the number of binding sites of allosterically bound Cl^- ions were determined in hemolysates from samples B and I (the pooled samples representing the most common IEF band profiles in the high- and low-altitude populations, respectively) by measuring O_2 equilibria at different KCl concentrations in the range 0.01–0.44 mol l^{-1}. As tested with replicate applications of the same Hb sample (Weber, 1992), this method yields highly reproducible P_{50} values (mean ± s.e.m. 4.73±0.04, $N=6$). Equilibrium Cl^- binding constants were estimated from the free Cl^- concentration at half-saturation when fitting logP_{50} as a function of Cl^- concentration according to a hyperbolic function. The maximal linear slope of the logP_{50} *vs* log[Cl^-] plot was used to calculate the number of allosterically bound Cl^- ions per heme. Free Cl^- concentration was measured using a model 926S Mark II chloride analyzer (Sherwood Scientific Ltd, Cambridge, UK).

Table 1. O_2 affinity (P_{50}, Torr) and cooperativity coefficients (n_{50}) of deer mouse hemolysates, measured in 0.1 mol l^{-1} Hepes buffer at pH 7.40 (±0.02), 37°C, obtained by linear regression of Hill plots ($R>0.999$)

	Stripped		DPG		KCl		DPG+KCl	
Sample	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}
High altitude								
A (N=3)	7.17	2.13	9.17	2.25	13.69	2.20	15.76	2.24
B (N=8)	8.17	2.51	9.00	2.32	12.51	2.25	13.17	2.66
C (N=5)	7.92	2.00	9.66	2.11	13.68	2.17	14.68	2.40
D (N=1)	7.53	2.20	10.88	1.94	13.73	2.47	14.90	2.32
E (N=1)	6.70	1.77	7.47	2.01	11.09	2.10	11.71	2.20
F (N=1)	7.01	1.93	7.90	2.36	11.31	2.15	12.74	2.15
G (N=1)	7.18	2.32	8.94	2.45	12.45	2.38	12.57	2.54
Mean	7.38	2.12	9.00	2.21	12.64	2.25	13.65	2.36
s.e.m.	0.20	0.09	0.42	0.07	0.42	0.05	0.56	0.07
Low altitude								
H (N=2)	8.27	2.19	8.45	2.32	14.27	2.49	13.41	2.46
I (N=8)	7.90	2.33	8.96	2.29	14.70	2.40	13.38	2.42
J (N=1)	8.47	2.16	9.62	2.47	14.66	2.53	13.82	2.40
K (N=1)	8.56	2.30	11.57	2.58	16.55	2.58	13.74	2.61
L (N=1)	7.59	2.32	9.39	2.38	13.61	2.34	14.47	2.23
M (N=1)	7.42	2.34	9.32	2.47	13.35	2.52	13.72	2.50
N (N=1)	7.15	2.28	9.88	2.58	14.14	2.61	14.76	2.63
Mean	7.91	2.27	9.60	2.44	14.47	2.50	13.83	2.46
s.e.m.	0.21	0.03	0.37	0.04	0.40	0.04	0.18	0.05

The sample sizes in parentheses refer to the number of individual hemolysates contained in each pooled sample. O_2 equilibria were measured in the absence of allosteric cofactors (stripped hemolysates), in the presence of DPG alone, in the presence of Cl^- alone (added as KCl), and in the presence of both cofactors ([Cl^-], 0.10 mol l^{-1}; [NaHepes], 0.1 mol l^{-1}; DPG/Hb tetramer ratio, 2.0; [Heme], 0.10–0.16 mmol l^{-1}).

A α-globin alleles

	2	5	9	10	12	13	15	19	21	23	30	34	36	43	44	50	51	52	57	58	60	63	64	71	72	76	78	82	87	106	111	113	115	116	120	140	141	f (high) HBA-T1	f (high) HBA-T2	f (low) HBA-T1	f (low) HBA-T2
Consensus	L	D	N	I	A	A	G	G	G	E	E	C	F	F	P	P	G	S	G	H	A	A	G	S	H	L	A	A	H	L	A	H	A	D	A	Y	R				
Seq 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	0.500	0.500	0.206	0.265
Seq 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	-	-	-	0.029
Seq 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	0.029	-	-	-
Seq 4	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	0.029	-	-
Seq 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	E	.	.	.	-	0.029	-	-
Seq 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	S	.	.	.	.	0.176	0.235	0.118	0.118
Seq 7	.	E	.	V	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.059	-	0.029	-
Seq 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	0.088	-	0.088
Seq 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.059	-	0.176	0.059
Seq 10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	0.088	-	-	0.029
Seq 11	.	E	.	V	.	.	S	.	.	.	.	S	.	.	.	H	.	.	.	.	.	.	D	G	.	.	.	.	.	.	.	L	S	.	.	.	.	-	-	0.029	-
Seq 12	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	0.029	-
Seq 13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	A	.	G	.	D	G	.	.	.	.	.	.	.	L	S	.	.	.	.	0.059	0.088	0.294	0.264
Seq 14	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	A	.	G	.	D	G	.	.	.	.	.	.	.	L	S	.	.	.	.	-	-	-	0.059
Seq 15	.	.	.	.	.	.	S	.	H	.	.	S	.	.	.	.	.	.	T	Q	.	.	D	D	N	.	G	.	.	.	.	Q	-	E	.	.	.	-	-	-	0.029
Seq 16	.	E	.	V	T	V	S	.	R	.	G	E	H	.	L	H	.	.	T	Q	.	T	D	D	N	Q	G	D	.	.	.	L	S	.	.	.	.	0.029	-	-	-
Seq 17	.	E	.	V	T	V	S	.	R	.	G	E	H	.	.	H	.	.	T	Q	.	.	D	D	N	.	.	.	.	Q	R	Q	-	E	.	.	C	-	-	0.029	-
Seq 18	.	E	.	V	T	V	S	.	R	.	G	E	H	L	.	H	.	.	T	Q	.	.	D	D	N	.	.	.	.	Q	R	Q	-	E	.	S	.	-	0.029	-	-
Seq 19	V	E	.	V	T	V	S	D	H	.	G	E	H	.	.	H	.	.	T	Q	.	.	D	D	N	.	G	.	.	Q	S	Q	-	E	.	.	G	-	-	0.029	-
Seq 20	.	.	.	V	T	V	N	.	H	.	G	E	H	.	.	H	S	.	T	Q	S	.	D	D	N	.	G	.	P	.	.	Q	-	E	V	.	.	-	-	0.029	-
Seq 21	.	E	.	V	T	V	S	.	H	.	G	E	H	.	.	H	S	.	T	Q	.	.	D	D	N	.	G	.	.	.	S	Q	-	E	.	.	.	-	-	0.029	-
Seq 22	.	.	.	.	T	V	S	.	.	.	G	E	H	.	.	H	S	.	T	Q	.	.	D	D	N	.	G	.	.	.	S	Q	-	E	.	.	.	-	-	-	0.059

B β-globin alleles

	2	3	8	12	15	48	61	62	65	66	72	74	87	88	102	117	122	128	134	135	142	145	146	f (high) HBB-T1	f (high) HBB-T2	f (low) HBB-T1	f (low) HBB-T2
Consensus	H	L	K	T	W	L	K	G	K	K	G	G	S	L	N	H	F	A	V	A	A	Y	H				
Seq 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.577	0.808	0.192	0.231
Seq 2	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.038	-	-	-
Seq 3	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	0.038	-	-
Seq 4	.	.	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.038	-	-	-
Seq 5	.	.	.	A	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	.	0.038	-	-	-
Seq 6	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	0.038	-
Seq 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	0.038	-	-	-
Seq 8	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	-	0.038	-	-
Seq 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	0.038	-	-	-
Seq 10	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	0.038	-	-	-
Seq 11	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	T	.	.	0.038	-	-	-
Seq 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	0.038	-	-	-
Seq 13	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.115	0.077	-	-
Seq 14	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	-	0.038	-	-
Seq 15	.	.	.	.	.	.	.	A	.	.	S	.	.	.	.	.	.	S	.	S	.	.	.	-	-	0.692	0.692
Seq 16	.	P	.	.	.	.	.	A	.	.	S	.	.	.	.	.	.	S	.	S	.	H	.	-	-	-	0.038
Seq 17	.	.	.	.	.	.	R	A	.	.	S	.	.	.	.	.	.	S	.	S	.	.	.	-	-	0.038	-
Seq 18	.	.	.	.	.	.	.	A	E	.	S	.	.	.	.	.	.	S	.	S	.	.	.	-	-	-	0.038
Seq 19	.	.	.	.	.	.	.	A	.	.	S	.	.	.	.	.	.	S	.	S	.	.	Y	-	-	0.038	-

Fig. 2. Amino acid polymorphism in the α-globin gene duplicates, HBA-T1 and HBA-T2 (A), and the β-globin gene duplicates, HBB-T1 and HBB-T2 (B), in highland and lowland deer mice. Frequencies (*f*) of the various protein alleles in each gene × population (high-/low-altitude) combination are shown in the rightmost columns. In panel A, the shaded alleles harbor gene conversion tracts derived from a third α-like globin gene, HBA-T3. Products of these converted alleles do not appear to be incorporated into functional Hb tetramers (see text for details).

RESULTS

Molecular genetic analysis

The survey of DNA sequence variation revealed a high level of amino acid polymorphism in the HBA-T1 and HBA-T2 genes (Fig. 2A). As shown in Fig. 2A, many identical protein alleles are shared between the HBA-T1 and HBA-T2 paralogs. This shared polymorphism between the two tandemly duplicated α-globin genes is attributable to a history of interparalog gene conversion (Storz and Kelly, 2008; Storz et al., 2008). At both paralogs, the most common alleles exhibited pronounced frequency differences between the high- and low-altitude population samples (Fig. 2A). The two most common allele classes were distinguished from each other by 8 amino acid changes (Fig. 3A): 50(CD8)Pro/His, 57(E6)Gly/Ala, 60(E9)Ala/Gly, 64(E13)Gly/Asp, 71(E20)Ser/Gly, 113(GH1)His/Leu, 115(GH3)Ala/Ser and 116(GH4)Glu/Asp (the notation in parentheses indicates the sequential number of each residue in α-helices A–H, the interhelical segments, or terminal extensions). At both HBA paralogs, the most common allele in the high-altitude population sample was characterized by the 8-site amino acid combination PGAGSHAE and the most common allele in the low-altitude sample was characterized by the alternative 8-site combination HAGDGLSD (Fig. 3A). A total of eight HBA-T1 and HBA-T2 alleles were recovered that harbored conversion tracts derived from the HBA-T3 paralog (sequences 15–22 in Fig. 2A). These chimeric sequences were highly distinct, differing from the consensus sequence at 12–24 amino acid sites. HBA-T1 and HBA-T2 alleles that harbored conversion tracts derived from HBA-T3 were never recovered in the RT-PCR experiments, nor were the products of these alleles identified in the MS/MS analysis, suggesting that sequences harboring conversion tracts from the HBA-T3 gene do not encode functional α-chain subunits of adult Hb.

The HBB-T1 and HBB-T2 genes also harbored high levels of amino acid polymorphism (Fig. 2B). Similar to the pattern observed at the α-globin genes, the HBB-T1 and HBB-T2 genes segregate the same pair of functionally distinct protein alleles because of a history of interparalog gene conversion (Hoffmann et al., 2008b; Storz et al., 2009). As with the α-globin genes, the most common alleles at the HBB-T1 and HBB-T2 genes exhibited pronounced frequency differences between the high- and low-altitude population samples (Fig. 2B). The two most common allele classes were distinguished from each other by 4 amino acid changes: 62(E6)Gly/Ala, 72(E16)Gly/Ser, 128(H6)Ala/Ser and 135(H13)Ala/Ser. At both HBB paralogs, the most common HBB allele in the high-altitude population sample was characterized by the 4-site amino acid combination GGAA and the most common allele in the low-altitude sample was characterized by the alternative 4-site combination ASSS (Fig. 3B).

Proteomic analysis of isoHb diversity

IEF analysis revealed that deer mice typically express 3–4 distinct isoHb components (Fig. 4), all of which could be accounted for by allelic polymorphism at the α- and β-globin genes. Comparisons between highland and lowland mice revealed that hemolysates of

A

α-chain isoHbs	50(CD8)	57(E6)	60(E9)	64(E13)	71(E20)	113(GH1)	115(GH3)	116(GH4)
α^{I}	Pro	Gly	Ala	Gly	Ser	His	Ala	Glu
α^{II}	His	Gly	Ala	Gly	Ser	His	Ala	Glu
α^{III}	Pro	Gly	Ala	Gly	Ser	His	Ala	Asp
α^{IV}	His	Gly	Ala	Gly	Ser	His	Ala	Asp
α^{V}	Pro	Gly	Ala	Gly	Ser	Leu	Ser	Asp
α^{VI}	His	Gly	Ala	Asp	Gly	Leu	Ser	Asp
α^{VII}	His	Ala	Gly	Asp	Gly	Leu	Ser	Asp

B

β-chain isoHbs	62(E6)	72(E16)	128(H6)	135(H13)
β^{I}	Gly	Gly	Ala	Ala
β^{II}	Ala	Gly	Ala	Ala
β^{III}	Ala	Ser	Ser	Ser

Fig. 3. Amino acid sequences that define the most commonly observed α- and β-chain isoHbs in deer mice from Colorado. (A) Seven allelic α-globin variants defined by different 8-site amino acid combinations. In the sample of highland mice, the most common allele at the HBA-T1 and HBA-T2 genes matched the α^{I} sequence, and in the sample of lowland mice, the most common allele at these same genes matched the α^{VII} sequence. (B) Three allelic β-globin variants defined by different 4-site amino acid combinations. In the sample of highland mice, the most common allele at the HBB-T1 and HBB-T2 genes matched the β^{I} sequence, and in the sample of lowland mice, the most common allele at these same genes matched the β^{III} sequence. Shading denotes the amino acid variants that predominate in the lowland population.

lowland mice generally contained a greater abundance of the major isoHb with the lowest isoelectric point (pI ~7.0), whereas those of the highland mice contained a greater number of minor isoHb components (Fig. 4). The mass spectrometry analysis did not reveal the presence of any subunit isoHbs that were not already characterized at the DNA level. There were no peptide matches corresponding to α-like globin genes other than HBA-T1 and HBA-T2, nor were there any matches to β-like globin genes other than HBB-T1 and HBB-T2.

O_2 binding properties of deer mouse Hbs

O_2 equilibrium curves revealed a high level of interindividual variation in Hb–O_2 affinity measured under varying conditions and a considerable overlap in the range of values for highland and lowland mice (Table 1). Comparisons between samples from the different elevational zones revealed that, on average, Hbs of the highland mice were characterized by slightly higher O_2 affinities in the absence of added effectors (stripped) and in the presence of DPG alone, and by significantly higher affinities in the presence of 0.1 mol l^{-1} KCl alone (Mann–Whitney U=6.00, P=0.017; Fig. 5A). Furthermore, O_2 equilibrium measurements revealed that Hb–O_2 affinity was 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 (Fig. 5B, Fig. 6).

We investigated Cl^- sensitivities of the high- and low-altitude Hb samples by measuring the effect of varying Cl^- concentrations on P_{50} values. The results of this experiment permit an assessment of the apparent equilibrium constant for the reaction of Hb with Cl^- ions and the number of deoxygenation-linked Cl^- binding sites per Hb tetramer. As shown in Fig. 7, Cl^- binds with a lower affinity to the Hbs of highland mice: estimated dissociation constants were 0.127±0.027 mol l^{-1} and 0.104±0.025 mol l^{-1} for the Hbs of highland and lowland mice, respectively. However, slopes of the logP_{50} *vs* log[Cl^-] plots – which reflect the number of deoxygenation-linked ions bound per heme – were similar in the high- and low-altitude hemolysates (0.23 and 0.22, respectively; inset in Fig. 7), indicating the presence of a single deoxygenation-linked Cl^- binding site per tetrameric Hb in both samples.

In four of the Hb samples from lowland mice (samples H, I, J and K; Table 1), Cl^- ions alone exerted a slightly greater allosteric effect than when present in conjunction with DPG (Fig. 6), suggesting an inhibitory effect of the weaker effector (DPG) on the stronger one (Cl^- ions). In highland mice, by contrast, DPG and Cl^- ions had synergistic effects on Hb–O_2 affinity, as the anion-induced increase in P_{50} was greater in the contemporaneous presence of DPG and KCl than in the presence of either anion in isolation (Fig. 5B). In the comparison between highland and lowland mice, the difference in the allosteric effects of Cl^- *vs* DPG+Cl^- was statistically significant ($\Delta\log P_{50([DPG+KCl]–KCl)}$=0.03 *vs* –0.03 in highland and lowland mice, respectively; Mann–Whitney U=5.00, P=0.011).

In summary, the Hbs of highland mice were characterized by a suppressed Cl^- sensitivity relative to lowland mice. These differences

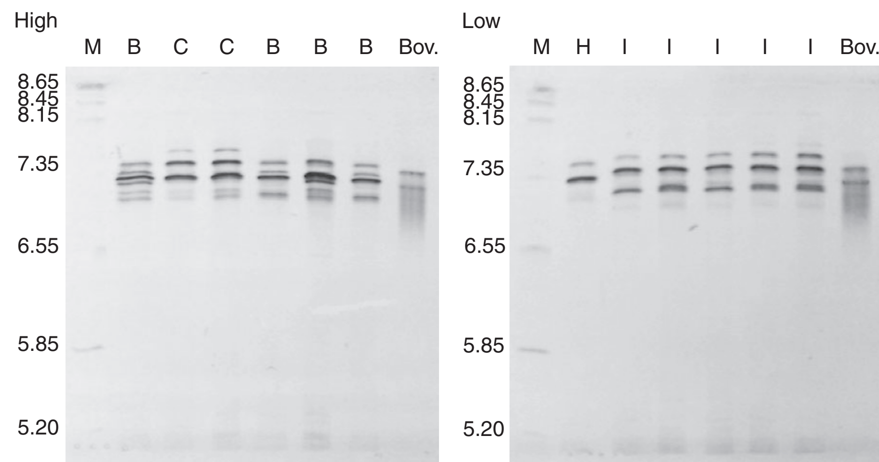

Fig. 4. Representative isoelectric focusing (IEF) gel images showing isoHb diversity of highland and lowland deer mice. Each band represents the CO-derivative of a structurally distinct isoHb tetramer. M, pI marker; Bov., bovine Hb; samples with the same letter were pooled for O_2 binding experiments (see Table 1).

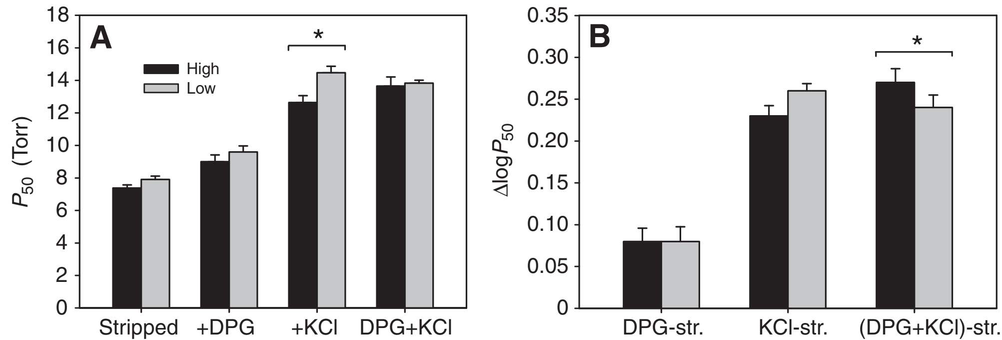

Fig. 5. O_2 binding properties of deer mouse Hbs at pH 7.40 (±0.02) and 37°C in the presence and absence of allosteric cofactors ([Cl^-], 0.10 mol l^{-1}; [NaHepes], 0.1 mol l^{-1}; DPG/Hb tetramer ratio, 2.0; [Heme], 0.10–0.16 mmol l^{-1}). (A) P_{50} (the P_{O_2} at 50% saturation of the heme groups) values (means ± s.e.m.) for stripped hemolysates in the absence of added anions, in the presence of DPG alone, in the presence of Cl^- ions alone (added as KCl), and in the combined presence of both anions. (B) Allosteric effect of DPG and Cl^- ions on Hb–O_2 affinity, expressed as the difference in log-transformed P_{50} values (means ± s.e.m.) for stripped hemolysates (str.) in the presence and absence of added anions. The $\Delta\log P_{50}$ value measures the extent to which Hb–O_2 affinity is reduced in the presence of a given allosteric effector. Asterisks denote statistically significant differences between samples of highland and lowland mice.

must be attributable to amino acid mutations in the α- and/or β-chain subunits of adult Hb. The causal amino acid polymorphisms are most likely those that exhibit the greatest allele-frequency differences between the highland and lowland population samples. According to this criterion, there are a total of 8 candidate sites on the α-chain subunits [50(CD8), 57(E6), 60(E9), 64(E13), 71(E20), 113(GH1), 115(GH3), 116(GH4)] and 4 candidate sites on the β-chain subunits [62(E6), 72(E16), 128(H6), 135(H13)].

DISCUSSION

IsoHb diversity

The results of our survey revealed that deer mice harbor a high level of isoHb diversity that is attributable to allelic polymorphism at two tandemly duplicated α-globin genes (HBA-T1 and HBA-T2) and two tandemly duplicated β-globin genes (HBB-T1 and HBB-T2). This multiplicity of subunit isoform combinations translates into a correspondingly high level of interindividual variation in Hb functional properties. The mass spectrometry analysis did not detect the presence of isoHbs that incorporated the products of globin genes other than the adult HBA and HBB paralogs, indicating that adult deer mice do not co-opt prenatal Hbs for pulmonary-tissue O_2 transport under hypoxia. By contrast, adult alpacas (*Vicugna pacos*) and yaks (*Bos grunniens*) living at high altitude are known to upregulate fetal β-like globin genes, which results in the synthesis of high-affinity isoHbs (Reynafarje et al., 1975; Weber et al., 1988; Sarkar et al., 1999).

Adaptive significance of population differentiation in Hb function

The relatively subtle differences in Hb function between highland and lowland deer mice prompt the question of whether the observed differences are of adaptive significance. Perutz (Perutz, 1983) considered this same general question with respect to species differences in Hb function: ‘It could be argued that even an amino acid replacement that produces only a very small shift in the O_2 equilibrium curve may give an animal a selective advantage that would prove decisive over thousands of generations; against this it could be held that homeostatic mechanisms allow organisms to compensate efficiently for quite large shifts in the curve.’ Since the cumulative effects of natural selection on genetically based trait variation are expected to leave an imprint on levels and patterns of nucleotide variation at the underlying loci, population genetic analysis of DNA sequence polymorphism provides a means of making indirect, retrospective inferences about the adaptive significance of trait differences between populations. It is therefore noteworthy that the two α-globin genes and the two β-globin genes of deer mice exhibit striking evidence for a history of divergent selection between highland and lowland populations (Storz et al., 2007; Storz and Kelly, 2008; Storz et al., 2009). Moreover, small absolute differences in intrinsic Hb–O_2 affinity may translate into much larger differences under *in vivo* conditions. For example, phosphate-free Hb solutions of the bar-headed goose, *Anser indicus* (a hypoxia-tolerant species renowned for migratory sojourns over

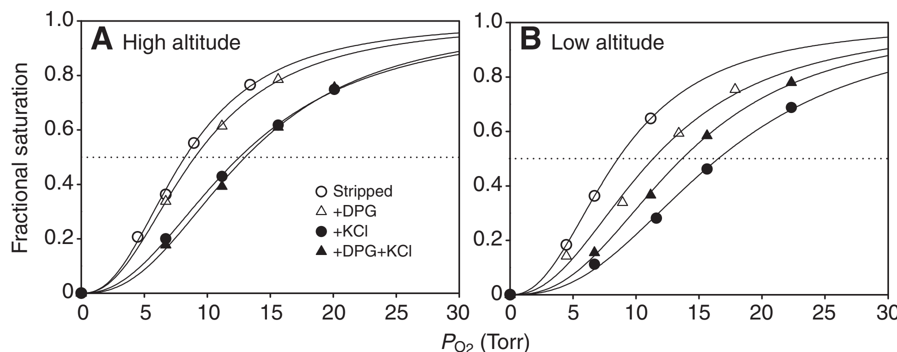

Fig. 6. O_2 equilibrium curves of stripped deer mouse Hbs at pH 7.40 (±0.02) and 37°C, measured under the same experimental conditions as those described in the legend for Fig. 3. Representative curves for pooled hemolysates of highland and lowland mice are shown in A and B, respectively. Highland and lowland mice are represented by samples B and K, respectively (see Table 1 for details). In each panel, the horizontal dotted line denotes 50% O_2 saturation of Hb.

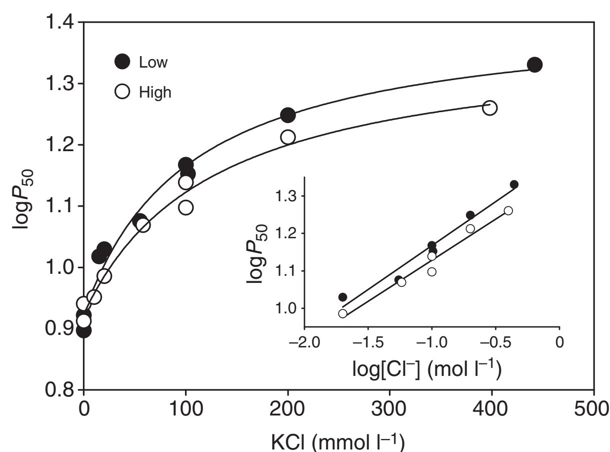

Fig. 7. Variation in Hb–O_2 affinity (as indexed by P_{50}) as a function of Cl^- concentration in hemolysates from high- and low-altitude deer mice. The curves indicate that Hbs from highland mice are characterized by a lower Cl^- affinity than those of lowland mice. The inset figure shows a regression of $\log P_{50}$ *vs* log[Cl^-]. The slope of this regression provides an estimate of the number of allosterically bound Cl^- ions per heme group. The estimated slopes of the regression lines for the Hbs of highland and lowland mice (0.23 and 0.22, respectively) indicate the presence of a single deoxygenation-linked Cl^- binding site per Hb tetramer.

the crest of the Himalayas), were characterized by a P_{50} that was lower than that of the lowland Canada goose, *Branta canadensis*, by 0.7 Torr ($P_{50(\text{pH } 7.2,\, 37°\text{C})}$=4.6 *vs* 5.3 Torr), but this small difference was amplified in the presence of the allosteric effector inositol hexaphosphate (Petschow et al., 1977). The available evolutionary and functional evidence suggests that genetically based differences in Hb–O_2 affinity between highland and lowland deer mice have evolved under the influence of positive directional selection, as suggested originally by L. R. G. Snyder and colleagues (Snyder, 1981; Snyder, 1982; Snyder et al., 1982; Chappell and Snyder, 1984; Snyder, 1985; Chappell et al., 1988; Snyder et al., 1988). The results of the present study and those of Snyder and colleagues suggest that changes in Hb structure and regulatory changes in erythrocytic anion concentrations may both contribute to adaptive variation in blood–O_2 affinity.

Finally, it is worth pointing out that our pair of highland and lowland sampling localities are separated by a relatively short linear distance (347 km), despite the 3189 m difference in elevation, and the resident deer mouse populations are interconnected by gene flow (Storz and Dubach, 2004; Storz and Kelly, 2008). Previous studies of deer mice indicated that differentiation in blood–O_2 affinity was not readily detectable across especially steep elevational gradients, and that the spatial patterning of adaptive physiological differentiation was only discernable at a more macrogeographic scale [for example, in comparisons between highland and lowland populations that are widely separated (Snyder et al., 1988)]. This is presumably due to the homogenizing effects of gene flow, since elevational changes in P_{O_2} and temperature occur over relatively short linear distances relative to the dispersal capabilities of deer mice (Storz and Nachman, 2003; Storz and Dubach, 2004; Storz and Kelly, 2008). Even if a ‘high affinity’ globin allele is unconditionally advantageous at high altitude, the recurrent migration of maladaptive ‘low affinity’ alleles from low altitude populations could constrain adaptive phenotypic differentiation (Snyder et al., 1988). In such cases, strong divergent selection on genetically based trait variation may produce only modest differences in mean phenotype between populations.

Allosteric regulation of Hb–O_2 affinity

The O_2 equilibrium measurements revealed two distinct properties related to the allosteric regulation of Hb function in deer mice. First, Cl^- ions exerted a more pronounced allosteric effect than DPG. Second, high-altitude Hbs showed a lower Cl^- sensitivity (and a higher O_2 affinity) than low-altitude Hbs, but showed similar sensitivities to DPG. Furthermore, in lowland mice, Cl^- ions exerted a more pronounced allosteric effect in isolation than when present in combination with DPG when both effectors occur at physiological concentrations, indicating an inhibitory effect of DPG on the binding of Cl^- ions. This interactive effect has not been previously described in other mammalian Hbs. What structural mechanisms might be responsible for these unusual patterns of allosteric regulation?

DPG binding

DPG carries four negative charges at physiological pH which allow it to bind between the β-chains of deoxyHb *via* charge–charge interactions [in most mammals, this involves the β1(NA1)Val residue of one chain, and the β2(NA2)His, β82(EF6)Lys and β143(H21)His residues of both chains (Perutz, 2001)].

Given that these residues are conserved in deer mouse Hbs, the attenuated effect of DPG on Hb–O_2 affinity could be due to steric hindrance of DPG binding in the central cavity, or it could be attributable to the neutralization of positively charged DPG binding sites by nearby residues. Site-directed mutagenesis studies of recombinant Hbs and crystallographic analyses will be required to determine the structural basis of the observed variation in allosteric effects.

Previous experiments had suggested that the magnitude of DPG sensitivity was one of the most salient differences between the Hbs of highland and lowland deer mice, and also suggested that the effect was attributable to the independent or joint effects of 4 amino acid mutations that distinguish products of the two main β-globin alleles (represented by the β^{I} and β^{III} sequence variants in Fig. 3B) (Storz et al., 2009). This prior study involved comparisons between matched pairs of highland and lowland mice that possessed Hbs with the same α-chains but different β-chains. This permitted an assessment of functional differences between the products of alternative β-globin variants against uniform genotypic backgrounds at the α-globin genes. The results of the present study – which were based on a larger sample of mice that carried a more diverse array of α- and β-globin genotypes – revealed no significant difference in average DPG sensitivity (Fig. 5B). This does not rule out an important effect of the alternative β-globin alleles on Hb–O_2 affinity, but it does suggest that there may not be any completely uniform or consistent differences in DPG sensitivity between the particular pair of highland and lowland deer mouse populations considered in this study. Our measurements (obtained at a DPG:Hb ratio of 2.0) also indicate that the small differences in intracellular DPG concentration observed in deer mice that were experimentally acclimated to high- and low-altitudes (DPG:Hb ratios of 1.3–1.8) (Snyder, 1982) are not likely to exert a significant effect on Hb–O_2 affinity.

Cl^- binding

In contrast to the Hbs of most mammals where DPG is the most important allosteric effector, the O_2 affinity of deer mouse Hb was reduced to a greater extent by Cl^- ions than by DPG (Fig. 5B). The

Hbs of highland mice exhibited a significantly lower Cl^- sensitivity than the Hbs from lowland mice, which constituted the most pronounced difference in Hb function between the highland and lowland population samples (Fig. 5). Differences in Cl^- sensitivity are associated with differences in Cl^- binding constants: 0.127±0.027 $mol\,l^{-1}$ and 0.104±0.025 $mol\,l^{-1}$ for highland and lowland mice, respectively. These values are similar to those for other vertebrate Hbs (Antonini and Brunori, 1971; Weber, 1992; Hofmann et al., 1995) and are close to the Cl^- concentration normally present in the vertebrate red blood cell (0.1 $mol\,l^{-1}$). A relatively greater allosteric effect of Cl^- ions than of DPG on Hb–O_2 affinity has also been reported for house mice *Mus musculus* (Runck et al., 2010), and may be a feature shared by other rodents. Larger allosteric effects of Cl^- ions than of DPG have also been reported in select lineages of artiodactyls, carnivores, and prosimian primates which, unlike deer mice, possess Hbs with intrinsically low O_2 affinities (Bunn, 1971; Bunn et al., 1974).

It has been suggested that Cl^- ions modulate Hb–O_2 affinity through delocalized electrostatic effects that do not involve binding at specific residues (Perutz et al., 1993; Shih et al., 1993; Bonaventura et al., 1994; Perutz et al., 1994). According to this view, Cl^- partially neutralizes the excess positive charge in the central cavity between the β-chains of deoxyHb, thereby stabilizing the tense-state conformation. However, the Hbs of highland and lowland deer mice are not distinguished by any charge-changing amino acid substitutions at known phosphate or Cl^- binding sites, nor are they distinguished by differences in the net electropositivity of the central cavity, so the structural mechanism responsible for the observed differences in anion sensitivity is not obvious.

In human Hb, Cl^- and other monovalent anions are considered to bind at two sites: a β-chain cationic site within the central cavity that involves β1(NA1)Val and β82(EF6)Lys, and an α-chain site between α1(NA1)Val and α131(H14)Ser (Riggs, 1988). In accordance with this view, double-logarithmic 'dose–response' plots of the Cl^- sensitivity of P_{50} recorded for human Hb in several studies show slopes of 0.4–0.6 (Weber, 1992), indicating that each tetrameric Hb molecule binds 2 Cl^- ions upon deoxygenation. The markedly lower slopes recorded for the highland and lowland deer mice (Fig. 7) indicate approximately one oxygenation-linked Cl^- binding site per Hb tetramer. This raises questions about the molecular basis of the reduced oxygenation-linked Cl^- binding of deer mouse Hb. In human embryonic Hbs (Hb Portland and Hb Gower I) (Zheng et al., 1999) and in the major isoHb of the Andean frog *Telmatobius peruvianus* (Weber et al., 2002) drastic reductions in Cl^- sensitivity correlate with substitutions of non-polar residues for α131(H14)Ser, which may reflect the elimination of a specific oxygenation-linked Cl^- binding site. The low number of oxygenation-linked Cl^- ions in deer mouse Hb is unexpected, given the presence of α1(NA1)Val, α131(H14)Ser and β82(EF6)Lys. Moreover, as with the Hbs of ungulates and some other mammalian species, deer mouse Hbs possess a triad of positively charged, solvent-exposed residues [β8(A5)Lys, β76(E20)Lys and β77(EF1)His] (De Rosa et al., 2004) that may serve as a discrete Cl^- binding site (Fronticelli, 1990). Together these findings indicate a novel mechanism governing the Cl^- sensitivity of deer mouse Hbs. The mass spectrometry data indicate that highland specimens in sample B possess the β^{I} sequence variant (Fig. 3), whereas the lowland specimens in sample I possess the β^{III} variant. Thus, the observed differences in Cl^- sensitivity (Fig. 7) may be due to structural changes stemming from one or more of the 4 amino acid substitutions that distinguish these two allelic β-globin variants (Fig. 3B). However, both highland and lowland samples are characterized by extensive amino acid variation in the α-chain subunits, which prevents conclusive inferences about structure–function relationships at this stage.

DPG and Cl^- binding

Experimental evidence from a number of vertebrate species supports the idea that organic phosphates and Cl^- ions compete for common binding sites within the central cavity, as phosphates alone often exert a more pronounced allosteric effect than when present in combination with Cl^- ions (Piccinini et al., 1991; Weber et al., 1993; Weber and White, 1994; Weber et al., 2000; Weber et al., 2002). Remarkably, a number of the low-altitude deer mouse specimens showed the reverse pattern of competitive inhibition, as Cl^- ions alone exerted a more pronounced allosteric effect than when present in combination with DPG at physiologically relevant concentrations (Table 1, Fig. 5), suggesting that putative Cl^- binding sites may also bind DPG. This pattern of anion interaction has not been documented in any other vertebrate Hbs and suggests that detailed structural studies of deer mouse Hbs may provide important mechanistic insights into the anion-dependent allosteric regulation of Hb–O_2 affinity.

CONCLUSIONS

Consistent with previous evidence for genetically based differences in blood–O_2 affinity between highland and lowland deer mice, our functional analysis of stripped hemolysates revealed that, on average, the Hbs of highland mice exhibited slightly higher intrinsic O_2 affinities and significantly lower Cl^- sensitivities relative to the Hbs of lowland mice. In light of results from previous studies (Snyder, 1982; Snyder et al., 1982; Snyder, 1985), it appears that modifications of Hb structure may play a more important role than regulatory adjustments of erythrocytic anion concentrations in the adaptive fine-tuning of blood–O_2 affinity.

LIST OF ABBREVIATIONS

DPG	2,3-diphosphoglycerate
Hb	hemoglobin
IEF	isoelectric focusing
isoHb	hemoglobin isoform
n_{50}	Hill's cooperativity coefficient at 50% saturation
P_{O_2}	partial pressure of oxygen
P_{50}	partial pressure of oxygen at which Hb is 50% saturated

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