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Supplementary Materials

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Materials and Methods
Figs. S1 to S4
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Epistasis Among Adaptive Mutations in Deer Mouse Hemoglobin

Chandrasekhar Natarajan,[1] Noriko Inoguchi,[1] Roy E. Weber,[2] Angela Fago,[2] Hideaki Moriyama,[1] Jay F. Storz[1]*

Epistatic interactions between mutant sites in the same protein can exert a strong influence on pathways of molecular evolution. We performed protein engineering experiments that revealed pervasive epistasis among segregating amino acid variants that contribute to adaptive functional variation in deer mouse hemoglobin (Hb). Amino acid mutations increased or decreased Hb-O_2 affinity depending on the allelic state of other sites. Structural analysis revealed that epistasis for Hb-O_2 affinity and allosteric regulatory control is attributable to indirect interactions between structurally remote sites. The prevalence of sign epistasis for fitness-related biochemical phenotypes has important implications for the evolutionary dynamics of protein polymorphism in natural populations.

Nonadditive interactions between mutations (epistasis) can exert a strong influence on the rate and direction of evolutionary change (*1*, *2*). Insights into mechanisms of epistasis between beneficial mutations can reveal the causes of constraints on adaptive protein evolution (*3*–*10*). Mechanisms of epistasis are often best revealed through detailed examinations of interactions between amino acid mutations in the same protein that contribute to variation in a measurable biochemical phenotype (*7*, *9*–*15*). Such studies are especially relevant to our understanding of evolutionary process when genetically based changes in the measured phenotype contribute to variation in fitness under natural conditions.

We investigated the nature of epistatic interactions between adaptive mutations in the hemoglobin (Hb) of deer mice (*Peromyscus maniculatus*). Deer mice that are native to high altitude have evolved an elevated Hb-O_2 affinity relative to lowland conspecifics (*16*–*18*), and this modification of protein function contributes to an adaptive enhancement of whole-animal physiological performance under hypoxia (*19*, *20*). Comparisons between highland deer mice from the Rocky Mountains and lowland deer mice from the Great Plains revealed genetic differences in Hb-O_2 affinity that are attributable to the independent or joint effects of 12 amino acid polymorphisms: 8 mutations in the α-chain subunits of the $\alpha_2\beta_2$ Hb tetramer, and 4 mutations in the β-chain subunits. These 12 amino acid polymorphisms exhibit pronounced altitudinal shifts in allele frequency, and population genetic analyses of nucleotide variation in the α- and β-globin genes revealed evidence for divergent selection between deer mouse populations that are native to different elevations (*18*, *21*–*23*).

Structural variation in deer mouse Hb has a modular organization that reflects the linkage arrangement of the 12 amino acid polymorphisms. Within the α-chain subunit, five amino acid replacements are located in exon 2 of the underlying gene, and the remaining three replacements are located in exon 3. Polymorphic sites within the same exon are in nearly complete linkage disequilibrium (LD) with one another, but intragenic recombination has produced a partial uncoupling between the two exons (*21*, *23*). The two most common α-globin allele classes are distinguished from each other by eight amino acid replacements at sites 50, 57, 60, 64, 71, 113, 115, and 116 (fig. S1A). The four amino acid polymorphisms in the β-globin gene are also in nearly complete LD with one another (*18*, *22*). The two most common β-globin allele classes are distinguished from each other by four amino acid replacements at sites 62, 72, 128, and 135 (fig. S1B). Thus, in deer mouse populations, most of the naturally occurring variation in Hb

[1]School of Biological Sciences, University of Nebraska, Lincoln, NE 68588, USA. [2]Zoophysiology, Department of Bioscience, Aarhus University, DK-8000 Aarhus, Denmark.

*Corresponding author. E-mail: jstorz2@unl.edu

structure is captured by combinatorial permutations of allelic variants at three loci: α-globin exon 2, α-globin exon 3, and β-globin.

We used site-directed mutagenesis to engineer all eight combinations of the α- and β-chain variants in recombinant Hb (rHb), and we measured O_2-binding properties of the purified proteins (*24*). In addition to the chimeric multipoint mutants, we also engineered 10 additional single- and double-mutant rHbs to measure the functional effects of specific point mutations individually and in pairwise combination. We synthesized rHbs representing the two most common variants from high- and low-altitude populations, designated "HH-H" and "LL-L," respectively (the first two letters denote the separate α-chain subdomains encoded by exons 2 and 3, and the third letter denotes the β-chain subunit). To test for epistasis, we also synthesized rHbs representing the remaining six combinations of H- and L-type alleles at each of the three loci (Fig. 1A). To examine variation in the allosteric regulation of Hb-O_2 affinity, we measured O_2-binding properties of each rHb mutant in the presence and absence of the two principal allosteric effectors present in mammalian red blood cells: Cl^- ions and 2,3-diphosphoglycerate (DPG). These effectors reduce Hb-O_2 affinity by preferentially binding and stabilizing deoxyHb, thereby shifting the allosteric equilibrium in favor of the low-affinity T-state quaternary structure. By using standardized concentrations of Cl^- and DPG in the physiological range, we ensured that in vitro measurements were relevant to in vivo conditions (*24*).

A

	α-globin (exon 2)					α-globin (exon 3)			β-globin			
	50	57	60	64	71	113	115	116	62	72	128	135
HH-H	Pro	Gly	Ala	Gly	Ser	His	Ala	Glu	Gly	Gly	Ala	Ala
HH-L	Pro	Gly	Ala	Gly	Ser	His	Ala	Glu	Ala	Ser	Ser	Ser
HL-H	Pro	Gly	Ala	Gly	Ser	Leu	Ser	Asp	Gly	Gly	Ala	Ala
HL-L	Pro	Gly	Ala	Gly	Ser	Leu	Ser	Asp	Ala	Ser	Ser	Ser
LH-H	His	Ala	Gly	Asp	Gly	His	Ala	Glu	Gly	Gly	Ala	Ala
LH-L	His	Ala	Gly	Asp	Gly	His	Ala	Glu	Ala	Ser	Ser	Ser
LL-H	His	Ala	Gly	Asp	Gly	Leu	Ger	Asp	Gly	Gly	Ala	Ala
LL-L	His	Ala	Gly	Asp	Gly	Leu	Ser	Asp	Ala	Ser	Ser	Ser

B

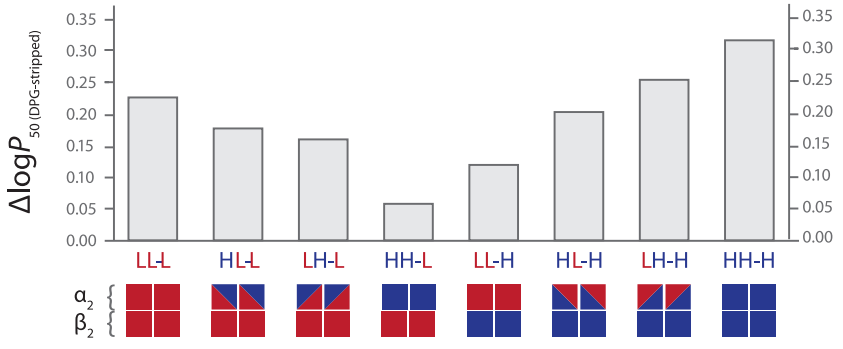

Fig. 1. Structural and functional variation among recombinant deer mouse Hbs (rHbs). (**A**) rHbs representing all combinatorial permutations of allelic variants at α-globin exon 2, α-globin exon 3, and β-globin. Shaded regions represent the products of low-altitude L-type alleles (red), and unshaded regions represent products of high-altitude H-type alleles (blue). (**B**) Variation in the allosteric regulation of Hb-O_2 affinity by DPG. Sensitivity to DPG is indexed by the difference in log-transformed P_{50} values between stripped Hb in the presence and absence of DPG.

Our experiments revealed substantial variation in intrinsic Hb-O_2 affinity, as P_{50} values (the O_2 tension at 50% heme saturation) for stripped, cofactor-free rHbs ranged from 4.55 to 7.09 torr (Table 1). The high-altitude HH-H variant exhibited a 26% lower stripped P_{50} (i.e., higher intrinsic O_2 affinity) relative to the low-altitude LL-L variant (Table 1). Hb-O_2 affinity was reduced in the presence of Cl^- ions (added as 0.1 M KCl), in the presence of DPG at a twofold molar excess over tetrameric Hb, and in the simultaneous presence of both effectors (Table 1). All rHbs exhibited cooperative O_2 binding, and Hill coefficients ranged from 1.36 to 2.28 in the presence of Cl^- and DPG.

Contrary to the expectations of an additive null model, the phenotypic effects of allelic substitutions (L→H and H→L) at α-globin exon 2, α-globin exon 3, and β-globin were highly dependent on genetic background, as pairwise epistasis accounted for 40% of the variance in P_{50} values in the absence of allosteric effectors and 90% in the simultaneous presence of Cl^- and DPG (Table 1). In the presence of both allosteric effectors, the HH α-globin allele conferred an increased affinity on the βL background and a decreased affinity on the βH background. Similarly, the H-type β-globin allele conferred an increased affinity on the αLL background and a decreased affinity on the αHH background. These are examples of sign epistasis (*2*), where the sign of the phenotypic effect of an allele is conditional on genetic background.

Because mammalian Hb is a heterotetramer ($\alpha_2\beta_2$), epistatic interactions could involve closely linked sites in the same gene or sites in unlinked genes that encode different subunits of the protein. Intragenic (within-subunit) epistasis could stem from localized modifications of secondary or tertiary structure, whereas intergenic (between-subunit) epistasis could stem from allosteric transitions in quaternary structure between different oxygenation states of the Hb tetramer. Epistasis for Hb-O_2 affinity is mainly attributable to the suppressed DPG sensitivity of chimeric rHb variants that incorporate the products of α- and β-globin alleles of unlike type (αHH combined with βL, and vice versa; Table 1 and Fig. 1B). Although DPG sensitivity was suppressed in

Table 1. O_2 affinities (P_{50}, torr; mean ± SEM) and allosteric properties of purified rHbs. Sensitivities to allosteric effectors are measured as the difference in log-transformed P_{50} values in the presence and absence of each effector, individually and in combination. V_A and V_E are estimated components of additive and epistatic variance, respectively (*24*).

	LL-L	HL-L	LH-L	HH-L	LL-H	HL-H	LH-H	HH-H	V_A	V_E
P_{50} (torr)										
stripped	5.72 ± 0.23	6.37 ± 0.05	7.09 ± 0.55	6.59 ± 0.11	5.25 ± 0.09	5.49 ± 0.04	6.32 ± 0.15	4.55 ± 0.08	0.597	0.403
+KCl	10.20 ± 0.03	12.55 ± 0.41	10.83 ± 0.78	12.88 ± 0.27	12.15 ± 0.31	11.18 ± 0.30	12.01 ± 0.20	9.70 ± 0.47	0.051	0.949
+DPG	9.72 ± 0.17	9.64 ± 0.12	10.33 ± 0.20	7.54 ± 0.25	6.93 ± 0.19	8.84 ± 0.26	11.44 ± 0.30	9.53 ± 0.27	0.189	0.811
+KCl + DPG	10.46 ± 0.30	11.21 ± 0.33	12.51 ± 0.59	8.63 ± 0.21	7.65 ± 0.22	11.36 ± 0.34	12.83 ± 0.34	10.13 ± 0.35	0.096	0.904
Δlog P_{50}										
KCl − stripped	0.251	0.295	0.184	0.291	0.364	0.309	0.279	0.329	0.671	0.329
DPG − stripped	0.230	0.180	0.163	0.058	0.121	0.206	0.258	0.321	0.726	0.274
(KCl + DPG) − stripped	0.262	0.246	0.247	0.117	0.164	0.316	0.308	0.348	0.214	0.786

HH-L and LL-H, allosteric regulatory capacities of the chimeric rHbs were partially restored by reciprocally converting either of the two α-chain subdomains to the type that matched the associated β-chain subunit: DPG sensitivity of the chimeric LL-H was partially restored by L→H substitutions at α-globin exon 2 or exon 3, and reciprocally, DPG sensitivity of the chimeric HH-L was partially restored by H→L substitutions at these same loci (Fig. 1B). In principle, a suppressed DPG sensitivity (and hence, increased Hb-O_2 affinity) could be produced by charge-changing amino acid replacements that eliminate phosphate-binding sites in the β-chain subunits. Because the positively charged phosphate-binding sites are invariant in deer mouse β chains (*17*, *18*), allelic variation in DPG sensitivity must stem from indirect, second-order perturbations.

Analysis of the crystal structure of deer mouse Hb at 1.8 Å resolution (*24*, *25*) revealed that each of the eight rHb mutants is characterized by a unique constellation of hydrogen bonds within and between subunits (Table 2 and fig. S2). Additional hydrogen bonds between subunits of the same αβ dimer are formed in the presence of β128Ser (an L-type residue; fig. S2), which contributes to the observed epistasis between allelic α- and β-chain variants. Structural analysis also revealed that in Hbs with L-type α-globin, the imidazole ring of α50His forms a hydrogen bond with α30Glu in the same subunit. The replacement of α50His with Pro (the H-type residue) eliminates this hydrogen bond and causes a subtle reorientation of the E helix and CD loop (Fig. 2), an effect that propagates to the α1β2 intersubunit contact and shifts the allosteric equilibrium in favor of the high-affinity oxyHb (R-state) quaternary structure.

To test the effects of charge-changing α-chain mutations in the CD loop (α50His/Pro) and the adjacent E helix (α64Asp/Gly), we synthesized each of the alternative single and double mutants on both HH-H and LL-L backgrounds. The experiments revealed that, on the LL-L background, substitutions of H-type residues (α50His→Pro and α64Asp→Gly) did not produce a significant increase in Hb-O_2 affinity individually or in combination; however, on the HH-H background, single-step reversions to L-type residues at both sites produced significant reductions in Hb-O_2 affinity in the presence of allosteric effectors (fig. S3). We also measured the individual effects of all four amino acid mutations in the β-chain subunit. On the LL-L background, the substitution β128Ser→Ala, which removes an α1β1 hydrogen bond (fig. S2), produced an increased anion sensitivity (and hence, a decreased Hb-O_2 affinity in the presence of Cl^- and DPG; fig. S4). However, on this same background, introducing all four H-type β-chain mutations in combination produced a highly significant increase in Hb-O_2 affinity in the presence of allosteric effectors (fig. S4).

In summary, results of our mutagenesis experiments revealed pervasive epistasis among segregating amino acid variants in deer mouse Hb (Table 1). The individual and joint effects of α- and β-chain point mutations contribute to the elevated Hb-O_2 affinity of highland deer mice, but the effects of these mutations are highly dependent on the allelic state of other residue positions.

Directed mutagenesis studies have unveiled "cryptic" epistasis between amino acid substitutions that distinguish deeply diverged orthologous proteins (*7*, *11*, *13*, *14*). Similarly, experimental studies of microbial systems have revealed intragenic epistasis between sites that underwent successive allelic substitutions but that were never simultaneously polymorphic (*6*, *10*). By contrast, the interacting mutations in deer mouse Hb are segregating in natural populations and, given the extensive intragenic and intergenic LD, the epistasis contributes to additive genetic variance in Hb function, providing an explanation for the previously documented variation in anion sensitivity of deer mouse Hbs (*17*, *18*). Given the evidence for spatially varying selection on Hb polymorphism in relation to altitude, the pervasiveness of sign epistasis for Hb-O_2 affinity suggests that the selection coefficient for a given allele will often be highly dependent on the allelic composition of the local population. Thus, sign epistasis among segregating amino acid variants may exert a strong influence on allele frequency dynamics and mutational pathways of protein evolution.

Table 2. Allelic variation in the network of atomic contacts within and between subunits of deer mouse Hb. Plus signs denote the presence of hydrogen bonds within subunits (α50His-α30Glu and α113His-α24Tyr) or between subunits of unlike type (α34Cys-β128Ser). Polymorphic sites are shown in bold.

	LL-L	HL-L	LH-L	HH-L	LL-H	HL-H	LH-H	HH-H
H-bonds								
α50His-α30Glu	+		+		+		+	
α113His-α24Tyr			+	+			+	+
α34Cys-**β128Ser**	+	+	+	+				

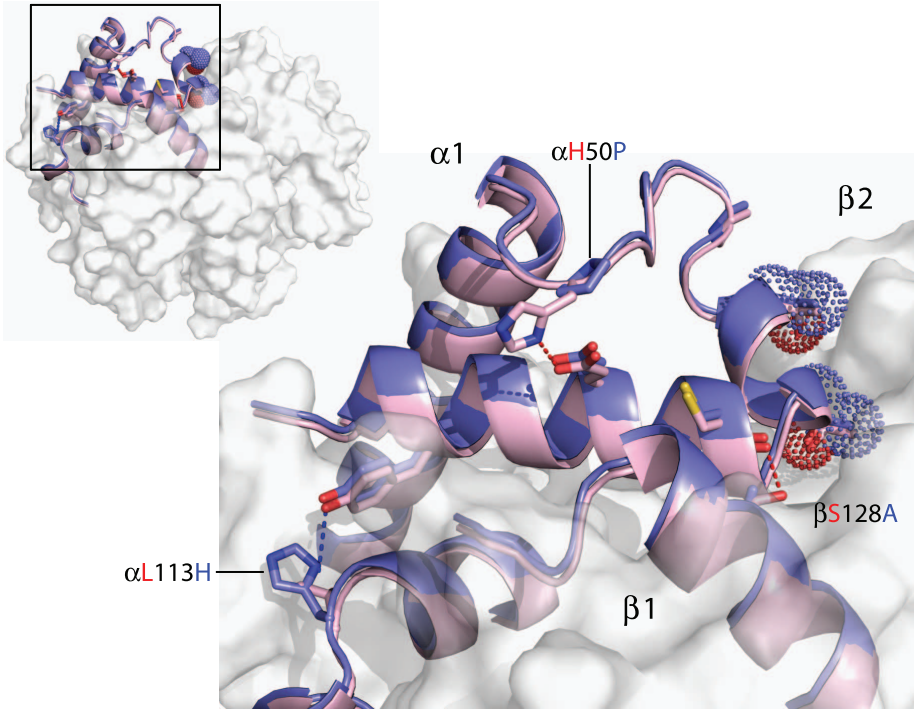

Fig. 2. Difference in the network of hydrogen bonds between high- and low-altitude Hb variants, HH-H and LL-L, respectively. The α1 and β1 subunits of HH-H (light blue) and LL-L (light red) are superimposed, and van der Waals radii are shown for α-chain residues that are in atomic contact with β-chain residues of the opposing α2β2 dimer.

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Supplementary Materials

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Figs. S1 to S5
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Root Effect Hemoglobin May Have Evolved to Enhance General Tissue Oxygen Delivery

Jodie L. Rummer,[1,2]* David J. McKenzie,[3] Alessio Innocenti,[4] Claudiu T. Supuran,[4] Colin J. Brauner[1]

The Root effect is a pH-dependent reduction in hemoglobin-O_2 carrying capacity. Specific to ray-finned fishes, the Root effect has been ascribed specialized roles in retinal oxygenation and swimbladder inflation. We report that when rainbow trout are exposed to elevated water carbon dioxide (CO_2), red muscle partial pressure of oxygen (PO_2) increases by 65%—evidence that Root hemoglobins enhance general tissue O_2 delivery during acidotic stress. Inhibiting carbonic anhydrase (CA) in the plasma abolished this effect. We argue that CA activity in muscle capillaries short-circuits red blood cell (RBC) pH regulation. This acidifies RBCs, unloads O_2 from hemoglobin, and elevates tissue PO_2, which could double O_2 delivery with no change in perfusion. This previously undescribed mechanism to enhance O_2 delivery during stress may represent the incipient function of Root hemoglobins in fishes.

In vertebrates, hemoglobin (Hb) plays a crucial role in optimizing tissue oxygen (O_2) delivery by increasing blood O_2-carrying capacity and regulating the partial pressure (PO_2) at which O_2 is delivered. Within tissues (such as muscle), metabolically produced carbon dioxide (CO_2) reduces blood pH and thus Hb-O_2 affinity, elevating blood PO_2 and enhancing O_2 delivery, which is collectively termed the Bohr effect (*1*). In mammals, this may elicit an increase in blood PO_2 of up to 2 mmHg (*2*, *3*) in vivo, providing some 5% increase in O_2 delivery. Teleost fishes often have much more pH-sensitive Hbs, and a recent in vitro study indicates that this effect may be an order of magnitude greater (*4*). The current study confirms this in vivo. Enhanced O_2 delivery may represent an important step in the extraordinary adaptive radiation of the teleost fishes (*5*), which make up almost half of all vertebrate species.

In teleosts, a reduction in blood pH reduces both Hb-O_2 affinity and O_2 carrying capacity, which is known as the Root effect (*6*) and has a well-studied role in securing O_2 delivery to the retina and swimbladder (*7*, *8*). These tissues possess specialized acid-producing cells in conjunction with a dense counter-current capillary network (*rete*) that localizes and magnifies a large acidosis, thus promoting O_2-offloading via the Root effect (*7*, *8*). This system is effective enough to generate O_2 tensions exceeding 50 atm (~38,000 mmHg) within the gas-filled swimbladder (*7*).

[1]Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada. [2]Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland 4811, Australia. [3]Equipe Diversité et Ecologie des Poissons, UMR 5119 Ecologie des Systèmes Marins Côtiers, Université Montpellier II, Place Eugène Bataillon, Case 093, F-34095 Montpellier cedex 5, France. [4]Università degli Studi di Firenze, Neurofarba Department, Sezione di Scienze Farmaceutiche, Via Ugo Schiff 6, 50019 Sesto Fiorentino (Florence), Italy.

*Corresponding author. E-mail: jodie.rummer@jcu.edu.au

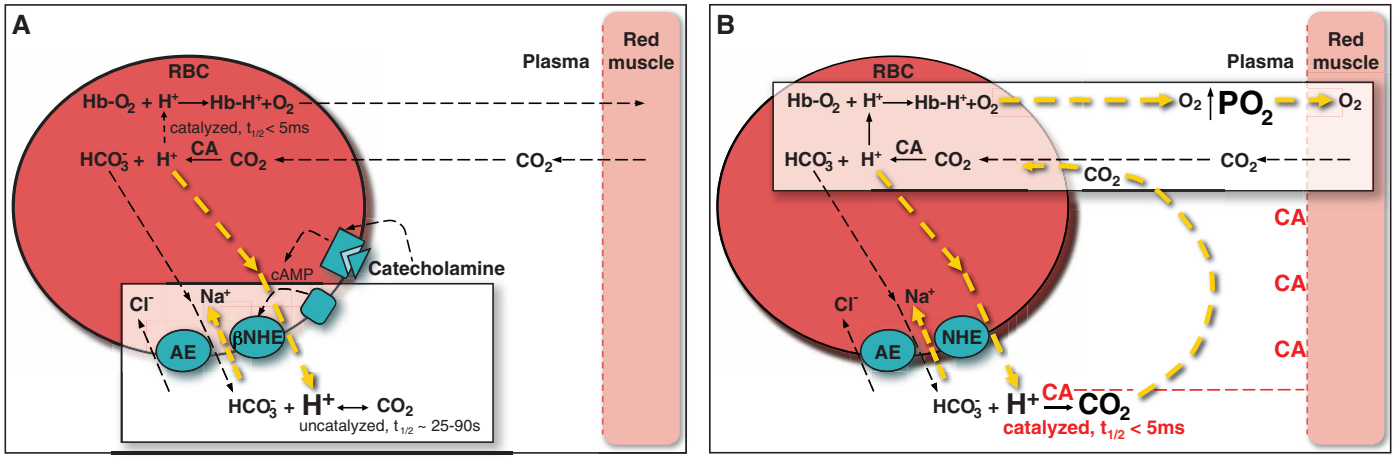

Fig. 1. (A and B) Schematic representation of a catecholamine-activated RBC pH disequilibrium (A) short-circuited by plasma-accessible CA to elevate tissue PO_2 (B). AE, anion exchange; cAMP, adenylate cyclase and 3′,5′-cyclic monophosphate.