Contribution of a mutational hot spot to hemoglobin adaptation in high-altitude Andean house wrens

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A key question in evolutionary genetics is why certain mutations or certain types of mutation make disproportionate contributions to adaptive phenotypic evolution. In principle, the preferential fixation of particular mutations could stem directly from variation in the underlying rate of mutation to function-altering alleles. However, the influence of mutation bias on the genetic architecture of phenotypic evolution is difficult to evaluate because data on rates of mutation to function-altering alleles are seldom available. Here, we report the discovery that a single point mutation at a highly mutable site in the β^A-globin gene has contributed to an evolutionary change in hemoglobin (Hb) function in high-altitude Andean house wrens (*Troglodytes aedon*). Results of experiments on native Hb variants and engineered, recombinant Hb mutants demonstrate that a nonsynonymous mutation at a CpG dinucleotide in the β^A-globin gene is responsible for an evolved difference in Hb–O_2 affinity between high- and low-altitude house wren populations. Moreover, patterns of genomic differentiation between high- and low-altitude populations suggest that altitudinal differentiation in allele frequencies at the causal amino acid polymorphism reflects a history of spatially varying selection. The experimental results highlight the influence of mutation rate on the genetic basis of phenotypic evolution by demonstrating that a large-effect allele at a highly mutable CpG site has promoted physiological differentiation in blood O_2 transport capacity between house wren populations that are native to different elevations.

biochemical adaptation | hemoglobin | high altitude | hypoxia | mutation bias

An important question in evolutionary genetics is whether certain mutations or certain types of mutation make disproportionate contributions to phenotypic evolution (1–6). Within a given gene, the mutations that contribute to evolutionary changes in phenotype may represent a biased, nonrandom subset of all possible mutations that are capable of producing the same functional effect. The preferential fixation of particular mutations (substitution bias) could have several causes. Most theoretical and empirical attention has focused on causes of fixation bias, i.e., mutations have different probabilities of being fixed once they arise, due to differences in dominance coefficients or the magnitude of deleterious pleiotropy (1, 2, 4, 7–9). In principle, substitution bias can also stem directly from mutation bias (some sites have higher rates of mutation to alleles that produce the change in phenotype) (4, 9–11). However, empirical evidence for the importance of mutation bias is scarce for an obvious reason: even in rare cases where it is possible to document the contributions of individual point mutations to evolutionary changes in phenotype, data on rates of mutation to function-altering alleles are typically lacking. Rare exceptions include cases where loss-of-function deletion mutations can be traced to hot spots of chromosomal instability or highly mutable changes in the copy number of repetitive elements (12). Documenting cases where genetic changes at highly mutable loci contribute to phenotypic divergence is therefore important for elucidating the evolutionary significance of mutation bias. This is especially true for cases where mutations cause fine-tuned modifications of protein activity rather than simple losses of function.

Here, we report the discovery that a single amino acid replacement at a mutational hot spot in the avian β^A-globin gene has contributed to an evolutionary change in hemoglobin (Hb) function that has likely adaptive significance. By conducting experiments on native Hb variants and engineered recombinant Hb mutants, we demonstrate that a nonsynonymous mutation at a CpG dinucleotide in the β^A-globin gene of Andean house wrens (*Troglodytes aedon*) has contributed to an evolved difference in Hb–O_2 affinity between high- and low-altitude populations. In mammalian genomes, point mutations at CpG sites occur at a rate that is over an order of magnitude higher than the average for all other nucleotide sites (13, 14), and available data suggest a similar discrepancy in avian genomes (15, 16).

Andean house wrens are compelling subjects for studies of Hb function because this passerine bird species has an exceptionally broad and continuous elevational distribution, ranging from sea level to elevations >4,500 m (17). At 4,500-m elevation, the standard barometric pressure is ∼450 torr, so O_2 partial pressure (PO_2) is <60% that at sea level (∼96 torr compared to ∼160 torr).

Significance

Within a given gene, there may be many possible mutations that are capable of producing a particular change in phenotype. However, if some sites have especially high rates of mutation to function-altering alleles, then such mutations may make disproportionate contributions to phenotypic evolution. We report the discovery that a point mutation at a highly mutable site in the β-globin gene of Andean house wrens has produced a physiologically important change in the oxygenation properties of hemoglobin (Hb). The mutant allele that confers an increased Hb–O_2 affinity is present at an unusually high frequency at high altitude. These findings suggest that site-specific variation in mutation rate may exert a strong influence on the genetic basis of phenotypic evolution.

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Data deposition: The newly generated sequences reported in this paper have been deposited in the GenBank database (accession nos. KT759682–KT760400). The parsed Illumina reads reported in this paper have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), www.ncbi.nlm.nih.gov/sra (PRJNA295865).

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Under such conditions, enhancements of pulmonary O_2 uptake and blood O_2 transport capacity are required to sustain O_2 flux to the tissue mitochondria in support of aerobic ATP synthesis (18). To complement changes in the cardiorespiratory system and microcirculation, changes in the O_2-binding affinity and cooperativity of Hb can enhance the O_2 capacitance of the blood (the total amount of O_2 unloaded for a given arteriovenous difference in O_2 tension). Because the optimal Hb–O_2 affinity is expected to vary according to the ambient PO_2, genetic variation in oxygenation properties of Hb may be subject to spatially varying selection between populations that inhabit different elevations. House wrens colonized South America in the late Pliocene or early Pleistocene via the newly formed Panamanian land bridge (19, 20), so the species may have been resident in the Andean highlands for up to ∼3 million years.

The Hb tetramer is composed of two semirigid $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers that undergo a mutual rotation during the oxygenation-linked transition in quaternary structure between the deoxy (low-affinity "T") conformation and the oxy (high-affinity "R") conformation (21). This oxygenation-linked structural transition between the T and R states is the basis for cooperative O_2 binding, and is central to the allosteric function of Hb as an O_2 transport molecule. Our analysis of house wren Hb highlights the influence of mutation rate on the genetic basis of phenotypic divergence by demonstrating that mutation at a CpG dinucleotide produced a large-effect amino acid replacement at an $\alpha_1\beta_1$ intradimer contact (β55Val→Ile)—a replacement that produced a significant increase in Hb–O_2 affinity.

Results

We performed an integrative analysis of Hb polymorphism in Andean house wrens that combined a population genomic analysis of nucleotide variation with mechanistic studies of protein function. Our survey of Hb polymorphism in *T. aedon* was based on a total of 140 museum-vouchered specimens (Table S1) that we collected from a broad range of elevations in the Peruvian Andes (Fig. S1). Andean house wrens are characterized by a high degree of phylogeographic structure. In Peru alone, house wrens are divided into seven highly divergent mtDNA clades that have allopatric or parapatric distributions across the Andes (20). To minimize the confounding effects of population structure in our altitudinal survey of Hb polymorphism, we conducted a detailed population genetic analysis on a sample of 65 specimens from the western slope of the Andes in central Peru that are representatives of the same mtDNA clade (20). Comparisons between highland and lowland population samples were based on specimens collected from >3,000 and <1,000 m, respectively.

Hb Isoform Composition of Red Blood Cells. Most birds express two structurally distinct Hb isoforms during adult life: HbA and HbD (22). HbA is typically the major isoform, constituting ∼60–80% of adult Hb in passerine birds (22, 23). The major HbA isoform incorporates α-chain products of the α^A-globin gene, and the minor HbD isoform incorporates products of the α^D-globin gene; both isoforms incorporate β-chain products of the same β^A-globin gene. To characterize the red cell Hb isoform composition of house wrens, we analyzed blood samples from individual specimens using a combination of isoelectric focusing (IEF) and tandem mass spectrometry (MS/MS). Consistent with data from other passerines, our wild-caught house wrens expressed two distinct isoforms, HbA (pI = 8.7) and HbD (pI = 7.1–7.2). There was no clear difference in relative isoform abundance in house wrens from different elevations: the relative percentages of HbD were 39% and 42% in high- and low-altitude specimens, respectively [n = 14 (7 from >3,900 m and 7 from <395 m)]. MS/MS analysis confirmed that subunits of the two adult Hb isoforms represent products of the α^A-, α^D-, and β^A-globin genes; products of the embryonic α- and β-type globin genes were not detected.

Altitudinal Patterns of Amino Acid Polymorphism. In birds, as in other amniotes, the subfamilies of α- and β-type globin genes are located on different chromosomes (23–26). All oscine passerines examined to date possess three tandemly linked α-type globin genes and four tandemly linked β-type globin genes (22–24, 27). Because the proteomic analyses confirmed that subunits of the two adult-expressed Hb isoforms are exclusively encoded by the α^A-, α^D-, and β^A-globin genes, we surveyed nucleotide polymorphism at each of these three genes in the full panel of high- and low-altitude house wrens (n = 140) to identify amino acid changes that could potentially contribute to intraspecific variation in Hb function.

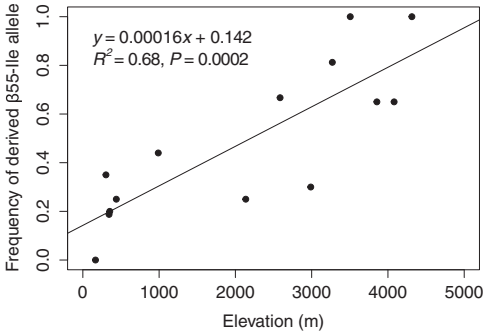

Fig. 1. The β55(Val/Ile) polymorphism exhibits a striking altitudinal pattern of allele frequency variation among 14 natural populations of house wrens from throughout Peru. The derived β55Ile allele predominates at high altitude and the ancestral Val allele predominates at lower altitudes.

This survey revealed a number of low-frequency amino acid polymorphisms, but only one polymorphism, β55(Val/Ile), exhibited a significantly nonrandom pattern of allele frequency variation with respect to altitude (Fig. 1 and Fig. S2). In the central Peru sample, the frequency of the derived Ile variant was 0.72 at high altitude and 0.31 at low altitude. This allele frequency difference of 0.41 was roughly twofold higher than that of any other amino acid polymorphism in the α- or β-globin genes. The three adult-expressed globin genes exhibited silent-site diversities of π = 0.0016–0.0105 in the total sample of Andean house wrens (Table S2).

Recurrent Substitutions at a Mutational Hot Spot. We sequenced the β^A-globin gene in 38 songbird species, including 15 species in the wren family (Troglodytidae) and 23 species representing nine families of oscine passerines. Phylogenetic analysis revealed that repeated nonsynonymous substitutions at the first codon position of β55 were attributable to the recurrent elimination of an ancestral CpG dinucleotide. Specifically, recurrent G→A transition substitutions at the first codon position converted β55Val to Ile in Andean house wrens and seven other passerine lineages, G→C transversion substitutions converted β55Val to Leu in several lineages, and successive A↔C transversions (non-CpG changes) interconverted β55Ile and Leu in thrushes (Fig. 2). Depending on the methylation status of the cytosine, eliminations of CpG dinucleotides via point mutations at either site are expected to occur at a far higher rate than non-CpG mutations at the same sites (16, 17). Consistent with this expectation, the estimated per-path rate for observed substitutions that eliminate the CpG dinucleotide (CpG→CpA and CpG→CpC) was approximately fivefold higher than the rate for other possible substitutions at the same site (0.0196 vs. 0.0043, respectively), and a likelihood ratio test indicated that the two-rate model provided a significantly better fit to the data than a single-rate model ($2\Delta\ln L$ = 4.75, P = 0.029).

Globin Gene Variation in Genome-Wide Context. For the purpose of making comparisons with patterns of variation in the adult-expressed α- and β-type globin genes of Andean house wrens, we surveyed intronic sequence polymorphism in the ρ- and β^H-globin genes, both of which are located immediately upstream of β^A-globin, and we also surveyed intronic sequence of the unlinked

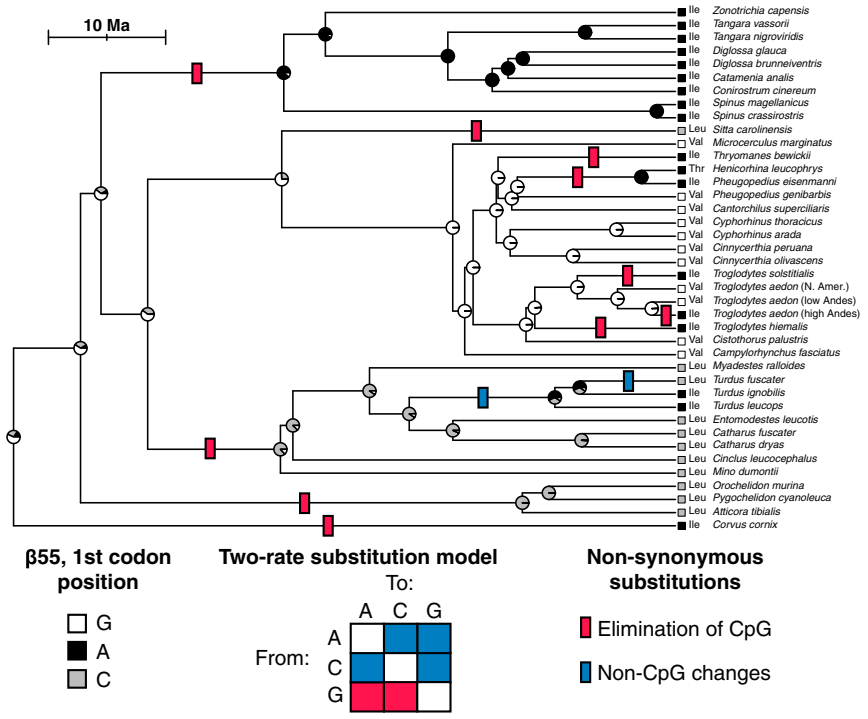

Fig. 2. Phylogeny of the wren family Troglodytidae and representative species from related oscine passerine families showing recurrent nonsynonymous substitutions at the first position of codon 55 in the β^A-globin gene. Blue and red tick marks indicate the minimum number of changes that are consistent with maximum-likelihood estimates of ancestral states at each node (see pie diagrams). An ancestral CpG dinucleotide was eliminated multiple times independently by nonsynonymous G→A transition substitutions (which produced β55Val→Ile replacements in Andean house wrens and several other passerine lineages) and G→C transversion substitutions (which produced β55Val→Leu replacements in multiple lineages). The third position of the codon immediately preceding β55 was cystosine (C) in all examined species except for *Cinclus leucocephalus*, which had thymine (T) and is part of a clade in which the CpG dinucleotide had already been eliminated by a CpG→CpC substitution. Depending on the methylation status of the cytosine, the rate of elimination of the CpG dinucleotide by point mutations at either site is expected to be ~10- to 15-fold higher than the mean mutation rate for non-CpG nucleotide sites. Maximum-likelihood analyses confirmed the expectation that the rate of substitutions that eliminated the CpG dinucleotide (CpG→CpA and CpG→CpC) was significantly higher than the rate of non-CpG substitutions at the same site.

myoglobin (Mb) gene. In comparisons between the high- and low-altitude population samples, the β^A-globin gene exhibited a higher level of nucleotide differentiation than each of the other linked and unlinked globin genes (Table S3).

To complement the multilocus survey of globin variation in the full panel of high- and low-altitude specimens, we used a subset of 28 specimens (14 highland, 14 lowland) in a genome-wide survey of single nucleotide polymorphisms (SNPs) in coding sequence. This allowed us to interpret altitudinal patterns of β55 polymorphism in a genome-wide context. We restricted the genomic analysis to 1,272 SNPs that mapped to putative protein-coding genes in a reference transcriptome (*SI Methods*). In the subset of specimens used in the genomic analysis, the site-specific F_{ST} value for β55 was 0.150, representing the upper 0.084 percentile of the empirical genome-wide distribution for coding SNPs. In the comparison between high- and low-altitude specimens comprising the central Peru sample (n = 108 alleles), the site-specific F_{ST} value for β55 was 0.269, representing the upper 0.013 percentile. The elevational differentiation in allele frequencies at the β55(Val/Ile) polymorphism thus provides suggestive evidence for a history of spatially varying selection.

Oxygenation Properties of Native HbA and HbD Variants. We purified HbA and HbD variants from highland and lowland house wren specimens that had representative globin genotypes. Measured differences in functional properties between the native HbA and HbD variants of highland and lowland house wrens reflect the net effects of naturally occurring allelic variation at two β-chain sites: β55(Val/Ile) and β80(Gly/Ser). Allelic variation at β80 contributes to amino acid heterogeneity in the set of specimens used in our functional experiments, but—unlike the β55(Val/Ile) polymorphism—it represents a low-frequency polymorphism in the global population and it does not exhibit a consistent altitudinal pattern of allele frequency variation (Fig. S3).

We measured O_2 equilibria of purified native Hb solutions under a standardized set of experimental treatments that enabled us to test for physiological differences in O_2 affinity between high- and low-altitude Hb variants while simultaneously providing insights into the molecular mechanism responsible for observed functional differences. We measured O_2 equilibria (*i*) in the absence of allosteric effectors ("stripped"), (*ii*) in the presence of Cl^- ions, added as 0.1 M KCl, (*iii*) in the presence of inositol hexaphosphate (IHP) (a chemical analog of the naturally occurring inositol pentaphosphate), at twofold molar excess over tetrameric Hb, and (*iv*) in the simultaneous presence of Cl^- and IHP. This latter treatment is most relevant to in vivo conditions in avian red blood cells. For each treatment, we estimated P_{50}, the PO_2 at which Hb is 50% saturated, and the Hill coefficient, n_{50}, a measure of cooperativity.

Because HbA and HbD share the same β-type subunit, functional effects of β-chain mutations should be manifest in comparisons between high- and low-altitude variants of both isoforms. Thus, data from both HbA and HbD provide replicate measurements of the mutations at β55 and β80 on two different α-chain backgrounds. The O_2 equilibrium measurements revealed pronounced differences in O_2 affinity between high- and low-altitude variants of both HbA and HbD (Table S4 and Fig. 3). $P_{50(KCl+IHP)}$ for the high-altitude HbA variant was 34% lower (O_2 affinity was higher) than that of the low-altitude variant (17.07 vs. 25.88 torr). Similarly, $P_{50(KCl+IHP)}$ for the high-altitude HbD variant was 17% lower than that of the low-altitude variant (13.45 vs. 16.29 torr). In high- and low-altitude samples, O_2 affinity differences between the two isoforms were consistent, as $P_{50(KCl+IHP)}$ was considerably higher for HbA relative to HbD (Table S4). Both isoforms exhibited cooperative O_2 binding, as estimated Hill coefficients (n_{50} values) in the KCl-plus-IHP treatment were 1.36–2.11 for HbA, and 2.28–2.36 for HbD (Table S4).

In the case of the high- and low-altitude HbA variants, the slight difference in intrinsic O_2 affinity [$P_{50(stripped)}$ = 2.47 vs. 2.80 torr, respectively] was greatly augmented in the presence of IHP and in the simultaneous presence of Cl^- and IHP (Table S4 and Fig. 3*A*). In the case of the HbD isoforms, there was no discernible difference in intrinsic O_2 affinity between the high- and low-altitude variants [$P_{50(stripped)}$ = 1.59 vs. 1.58 torr, respectively], but—as with the HbA variants—there was a highly significant affinity difference in the presence of Cl^- and IHP (Table S4 and Fig. 3*B*). Results for high- and low-altitude variants of HbA and HbD indicate that allelic differences in Hb function stem from changes in both intrinsic O_2 affinity and anion sensitivity. In both isoforms, these changes are clearly attributable to the independent or joint effect of shared amino acid mutations at β55 and β80.

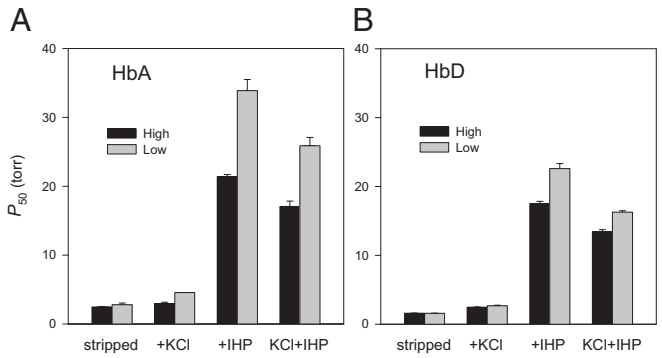

Fig. 3. O_2 affinities of HbA and HbD isoforms from high- and low-altitude populations of house wrens. (*A*) P_{50} values (mean ± SEM) for purified HbA variants of highland and lowland house wrens measured in 0.1 M Hepes buffer at pH 7.4 and 37 °C in the absence (stripped) and presence of allosteric effectors ([Cl^-], 0.1 M; IHP/Hb tetramer ratio, 2.0; [heme], 0.3 mM). (*B*) P_{50} values for HbD variants of highland and lowland wrens (experimental conditions as in *A*).

Functional Effects of Individual Mutations. To measure the relative contributions of the mutations β55Val→Ile and β80Gly→Ser, we used site-directed mutagenesis to engineer four recombinant Hb (rHb) mutants representing each possible genotypic combination of allelic variation at the two sites. The measured O_2 affinities of the ancestral genotype (55Val-80Ser) and the β55 single-mutant (55Ile-80Ser) recapitulated the measured difference between the native HbA variants from high- and low-altitude populations (Tables S4 and S5). The β55Val→Ile mutation produced a 25% reduction in $P_{50(KCl+IHP)}$ (an increase in O_2 affinity) on the ancestral 55Val-80Ser background (difference in P_{50} = 5.79 torr, 95% confidence limits of ΔP_{50} = 11.06, 0.52), and a 9% reduction on the background with the derived Gly at β80 (Fig. 4 and Table S5) (difference in P_{50} = 1.65 torr, 95% confidence limits of ΔP_{50} = 5.22, −1.92). The β80Ser→Gly mutation also had a substantial affinity-enhancing effect on the ancestral background (Fig. 4 and Table S5).

The Structural Mechanism Responsible for Changes in Hemoglobin–O_2 Affinity. Results of homology modeling suggest that the β55Val→Ile mutation increases intrinsic Hb–O_2 affinity through indirect effects on the β-chain distal heme pocket (the site of heme–ligand binding). The β55Val→Ile mutation results in the insertion of an additional carbonyl group in the $\alpha_1\beta_1$ intradimer gap between β55 and α119Pro, thereby forming a van der Waals contact between the two residues (Fig. 5). In the deoxy state, this added atomic contact at the $\alpha_1\beta_1$ interface induces strain on the adjacent D helix of the β-subunit, as indicated by a 1.3-fold increase in the single-residue frustration index for the derived β55Ile relative to Val on the ancestral background (Table S6). This effect propagates to the adjacent E helix, resulting in a subtle repositioning of key residues at the solvent interface that function as a gate for ligand entry/exit in the distal heme pocket and that directly or indirectly stabilize the heme–ligand complex (28).

Discussion

Possible Adaptive Significance of Altitudinal Differences in Hb–O_2 Affinity. The evolved difference in Hb–O_2 affinity between the highland and lowland house wrens is consistent with theoretical and empirical results demonstrating that the optimal blood–O_2 affinity varies according to the ambient PO_2, reflecting an unavoidable trade-off between the need to preserve arterial O_2 saturation under hypoxia while simultaneously ensuring adequate O_2 unloading in the peripheral circulation (29–31).

Patterns of convergence in Hb function among different high-altitude vertebrates also provide insights into the possible adaptive significance of changes in Hb–O_2 affinity. Comparative studies of Andean hummingbirds revealed that species with extraordinarily high elevational range limits consistently have higher Hb–O_2 affinities than closely related lowland species (32). Studies of birds and mammals have documented altitude-related differences in Hb–O_2 affinity in some cases (32–38), but not in others (39–42). Andean house wrens provide the first example (to our knowledge) of a continuously distributed bird species in which high-altitude natives have evolved a derived increase in Hb–O_2 affinity relative to lowland conspecifics. In contrast to recently documented cases where changes in Hb function between populations or closely related species evolved via multiple mutational changes that had individually minor effects (37, 38, 41), the increased Hb–O_2 affinity in high-altitude house wrens is clearly attributable to a single, large-effect mutation.

Insights into Structural Mechanism. Results of the protein-engineering experiments clearly demonstrate the affinity-enhancing effect of the β55Val→Ile mutation. A different amino acid substitution at this same site (β55Leu→Ser) has been implicated in the evolution of an increased Hb–O_2 affinity in the Andean goose (33, 34), although phylogenetic surveys of β^A-globin sequence variation have revealed that the β55Ser character state is not uniquely derived in the Andean goose—it is actually a shared, ancestral character state in South American sheldgeese, and most species in this group are lowland natives (43). The β55Leu→Ser substitution eliminates a van der Waals interaction between β55Leu and α119Pro at the $\alpha_1\beta_1$ intradimer interface, thereby destabilizing the T-state conformation and shifting the allosteric equilibrium in favor of the high-affinity R state, resulting in an increase in overall O_2 affinity. Engineering the same β55Leu→Ser substitution into recombinant human Hb produced the predicted increase in O_2 affinity, corroborating the hypothesized structural mechanism (33, 34).

In house wren Hb, an amino acid replacement at the same site also produces a dramatic change in O_2 affinity, but the structural mechanism is completely different. The affinity-enhancing β55Leu→Ser replacement in Andean goose Hb eliminates an atomic contact between α119 and β55, thereby destabilizing the low-affinity T-state conformation. By contrast, the affinity-enhancing β55Val→Ile replacement in house wren Hb adds an intradimer atomic contact in the same $\alpha_1\beta_1$ interface (Fig. 5), which indirectly affects deoxy β-heme reactivity by reorienting the E helix. This illustrates how substitutions involving different pairs of amino acid residues at the same site can alter protein function via different structural and functional mechanisms.

Effects of Mutation Bias on Propensities of Molecular Adaptation. Results of our molecular evolution analysis demonstrated a quantitative asymmetry in rates of CpG and non-CpG substitution in the first codon position of β55, and results of our protein-engineering experiments demonstrated that the mutationally favored

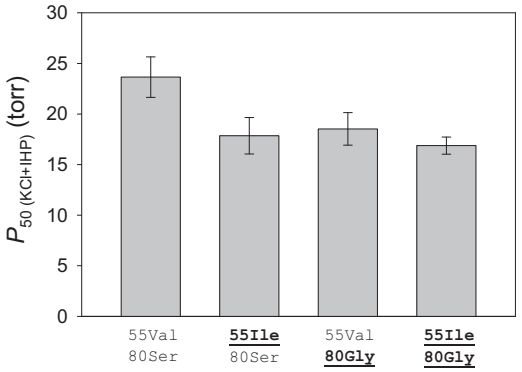

Fig. 4. O_2 affinities [$P_{50(KCl+IHP)}$, torr; mean ± SEM] of purified house wren rHb mutants measured in the presence of physiological concentrations of Cl^- ions (0.1 M KCl) and IHP (at twofold molar excess over tetrameric Hb). O_2 equilibrium curves for each rHb mutant were measured in 0.1 M Hepes buffer at pH 7.40, 37 °C, and [heme], 0.3 mM. Numbers refer to residue positions in the β-chain subunit. "55Ile-80Ser" and "55Val-80Ser" are the two-site genotypes that predominate in high- and low-altitude house wrens, respectively. At each site, the derived (nonancestral) amino acid residues are underlined.

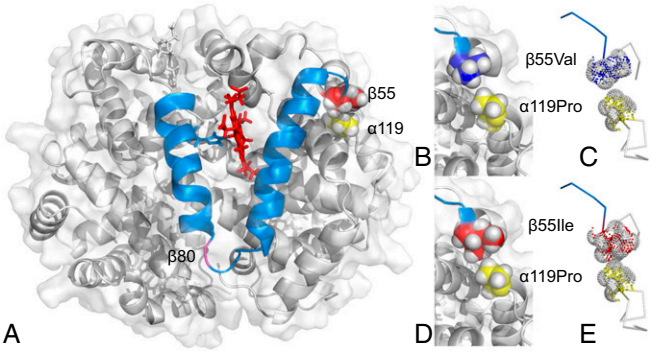

Fig. 5. Homology model of house wren HbA showing the location of amino acid replacements that distinguish high- and low-altitude variants. (*A*) The E and F helices of the β-chain subunit are shown in blue. The side chain of the proximal histidine, β92 (which covalently binds the fifth coordination site of the heme iron) is also shown in blue, and the residues forming the $\alpha_1\beta_1$ intradimer contact between β55 and α119 are shown in red and yellow, respectively, with space-filling representation of van der Waals radii. The site of the β80Ser→Gly mutation in the EF interhelical loop is shown in pink. (*B* and *C*) There is no interchain atomic contact between β55Val and α119Pro at the $\alpha_1\beta_1$ contact surface. (*D* and *E*) Because Ile has an additional carbon atom relative to Val, a van der Waals interaction is formed between β55Ile and α119Pro.

Val→Ile replacement at this site produces a significant increase in Hb–O_2 affinity on the ancestral genetic background (Fig. 4 and Table S5). The direction of character state change is consistent with the expectation that an increased Hb–O_2 affinity is adaptive at high altitude. This inference is bolstered by results of the population genomic analysis, which suggest that the altitudinal shift in frequency of the derived β55-Ile variant is attributable to a history of spatially varying selection. Our results therefore demonstrate how a mutationally favored amino acid change produced a large phenotypic effect that has likely adaptive significance. The important question is whether the increased rate of mutation to the function-altering allele made the observed evolutionary outcome especially likely to occur. This is relevant to the more general question of whether propensities of mutational change cause propensities in pathways of adaptive molecular evolution (10, 11, 44, 45).

Studies of naturally occurring mutations in human Hb and engineered mutations in recombinant Hbs, as well as comparative studies of Hbs from different animal species, demonstrate that there are numerous possible amino acid changes that are capable of producing fine-tuned increases in Hb–O_2 affinity (46–49). As an adaptive solution to the respiratory challenges of O_2 transport at high altitude, there is no reason to think that the β55Val→Ile replacement was a forced option; any number of amino acid mutations in the same protein presumably could have produced a quantitatively similar phenotypic effect. Assuming that an increased Hb–O_2 affinity confers a fitness benefit in birds living at high altitude, there seems little reason to suppose that the observed β55Val→Ile mutation would have had a higher fixation probability than any number of other possible affinity-enhancing mutations. However, if the rate of β55Val→Ile mutation is 10-fold higher than the rate of mutation to any other affinity-enhancing amino acid at any other site in the protein, then—in the absence of contributions from standing variation—this would bias evolutionary outcomes in the same way as a 10-fold higher probability of fixation. When adaptive evolution is mutation-limited, an increase in the rate of mutation to a particular allele and a commensurate increase in the mutant allele's probability of fixation have the same effect on the odds that the allele will be the next to fix (4, 10, 11, 50). The extent to which adaptation in natural populations approximates the mutation-limited scenario envisioned by origin-fixation models remains an open question in evolutionary genetics (50). Our findings suggest that variation in the mutation rate to function-altering alleles may be an important factor influencing the preferential fixation of mutations during phenotypic evolution.

Methods

Sample Collection. We collected 140 house wren specimens from a range of elevations (120–4,454 m above sea level) in the Peruvian Andes and adjacent lowlands. All specimens were preserved as vouchers in the ornithological collection of the Museum of Southwestern Biology of the University of New Mexico and the Centro de Ornitología y Biodiversidad (CORBIDI) (Lima, Peru). Birds were handled in accordance with protocols approved by the University of New Mexico Institutional Care and Use Committee (Protocol 08UNM033-TR-100117; Animal Welfare Assurance number A4023-01). Complete specimen data are available via the ARCTOS online database (Table S1). Details regarding specimen collection and permits are provided in *SI Methods*.

Characterization of Hb Isoform Composition. We characterized Hb isoform composition in the mature erythrocytes of 14 house wren specimens (7 highland and 7 lowland). Native Hb components were separated by means of IEF, gel bands were excised and digested with trypsin, and MS/MS was used to identify the resultant peptides, as described in *SI Methods*.

Molecular Cloning and Sequencing. Details regarding cloning and sequencing protocols are provided in *SI Methods*. All sequences were deposited in GenBank under accession numbers KT759682–KT760400.

Phylogenetic Survey of β^A-Globin Sequence Variation in Oscine Passerines. We sequenced the β^A-globin gene in representative wren species and species from related oscine passerine families. We used a time-scaled supertree (51) and the "ace" function of the R package *ape* (52) to test alternative maximum-likelihood models of character state change and to estimate ancestral character states for the first codon position of β55. Our model was based on a 3 × 3 rate matrix representing all possible interconversions among observed character states at the focal site: A, C, and G (T was not an observed character state). The null model used a single rate parameter for all six substitution types. The alternative model included a second rate parameter for substitutions that eliminated the CpG dinucleotide (CpG→CpA and CpG→CpC). We used a likelihood ratio test to compare the one-rate and two-rate models.

Population Genetic Analysis. For each of the adult-expressed globin genes (α^A-, α^D-, and β^A-globin), we computed summary statistics of nucleotide polymorphism, as described in *SI Methods*.

Survey of Genomic Differentiation. We used a genotyping-by-sequencing approach to survey genome-wide patterns of nucleotide differentiation in coding sequence. Briefly, we used genomic DNA samples from 28 house wren specimens to produce multiplexed, reduced-representation Illumina libraries. Details of library preparation, library sequencing, and quality control filtering are provided in *SI Methods*. Parsed Illumina reads have been deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) (PRJNA295865).

Protein Purification and in Vitro Analysis of Hb Function. The experimental analysis of native HbA and HbD variants was based on pooled hemolysates from seven highland specimens and seven lowland specimens that had representative genotypes. For each of the pooled hemolysates, we isolated and purified the HbA and HbD isoforms by means of anion-exchange fast-protein liquid chromatography, using a HiTrap QHP column (GE Healthcare). Details regarding sample preparation and the measurement of O_2 equilibrium curves are provided in *SI Methods*.

Vector Construction and Site-Directed Mutagenesis. The α^A- and β^A-globin sequences were synthesized by Eurofins MWG Operon after optimizing the nucleotide sequences in accordance with *Escherichia coli* codon preferences. The synthesized α^A-β^A globin gene cassette was cloned into a custom pGM vector system along with the *methionine aminopeptidase* (MAP) gene, as described previously (37, 53). We engineered each of the β-chain codon substitutions using the QuikChange II XL Site-Directed Mutagenesis kit from Stratagene. Each engineered codon change was verified by DNA sequencing.

Expression and Purification of Recombinant Hbs. Recombinant Hb expression was carried out in the *E. coli* JM109 (DE3) strain as described previously (53). Additional details are provided in *SI Methods*.

Structural Modeling. Homology-based structural modeling was performed on the SWISS-MODEL server (54), using human deoxyHb (Protein Data Bank ID 2hhb) as template. To predict mutational effects on conformational stress, we computed an index of energetic frustration using the Frustratometer program (55). Graphics were produced by the PyMol (Schrödinger).

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