Repeated elevational transitions in hemoglobin function during the evolution of Andean hummingbirds

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Animals that sustain high levels of aerobic activity under hypoxic conditions (e.g., birds that fly at high altitude) face the physiological challenge of jointly optimizing blood-O_2 affinity for O_2 loading in the pulmonary circulation and O_2 unloading in the systemic circulation. At high altitude, this challenge is especially acute for small endotherms like hummingbirds that have exceedingly high mass-specific metabolic rates. Here we report an experimental analysis of hemoglobin (Hb) function in South American hummingbirds that revealed a positive correlation between Hb-O_2 affinity and native elevation. Protein engineering experiments and ancestral-state reconstructions revealed that this correlation is attributable to derived increases in Hb-O_2 affinity in highland lineages, as well as derived reductions in Hb-O_2 affinity in lowland lineages. Site-directed mutagenesis experiments demonstrated that repeated evolutionary transitions in biochemical phenotype are mainly attributable to repeated amino acid replacements at two epistatically interacting sites that alter the allosteric regulation of Hb-O_2 affinity. These results demonstrate that repeated changes in biochemical phenotype involve parallelism at the molecular level, and that mutations with indirect, second-order effects on Hb allostery play key roles in biochemical adaptation.

high-altitude adaptation | hypoxia | parallel evolution | protein evolution | epistasis

In air-breathing vertebrates, the optimal Hb-O_2 affinity varies according to the partial pressure of atmospheric O_2 (PO_2) because of the trade-off between the need to maximize arterial O_2 saturation under hypoxia while simultaneously ensuring adequate O_2 unloading in the tissue capillaries (1–8). However, in species that are native to high-altitude environments, it is not known how often and to what extent physiological adaptation to hypoxia is mediated by genetically based modifications of Hb-O_2 affinity (9). Such questions can be resolved by conducting systematic comparative studies of Hb function among species with known phylogenetic relationships and contrasting altitudinal distributions.

In cases where multiple species have adapted independently to high-altitude hypoxia, replicated changes in Hb function may be instructive about the relative accessibility of different design solutions to natural selection. If repeated changes in Hb-O_2 affinity involve parallel amino acid substitutions, then this suggests that adaptive protein evolution may be predisposed to follow particular mutational pathways. If, by contrast, myriad different mutational changes can produce the same functional outcome, then particular design solutions may be selectively accessible from a diverse range of ancestral starting points, and pathways of protein evolution may be highly idiosyncratic.

Among vertebrates, hummingbirds have some of the highest basal metabolic rates and the highest metabolic scopes for activity, and are therefore especially compelling subjects for studies of Hb function and blood-O_2 transport under hypoxia (10–13). We conducted an experimental analysis of Hb function in 10 species of Andean hummingbirds that have dramatically different altitudinal distributions. The species included in this study fall into three main clades: the Coquettes, the Brilliants, and the Emeralds + Giant Hummingbird (*Patagona gigas*) (Fig. 1*A*). Each of these three clades contains species that are restricted to low or moderate elevations, as well as independently derived high-elevation species that routinely occur at elevations >4,200 m. We also collected data for one outgroup species from the hermit subfamily (Phaethornithinae), a primarily lowland clade that represents the likely ancestral elevational distribution for hummingbirds (14).

Results and Discussion

Hb Isoform Composition. The Hbs of birds and other jawed vertebrates are heterotetramers, composed of two α-chain and two β-chain subunits (15, 16). During postnatal life, most bird species express two main Hb isoforms in circulating red blood cells: a major isoform, HbA ($\alpha^A{}_2\beta_2$), with α-chain subunits encoded by the α^A-globin gene, and a minor isoform, HbD ($\alpha^D{}_2\beta_2$), with α-chain subunits encoded by the α^D-globin gene (17) (Fig. S1). Given that avian HbD has a consistently higher O_2-affinity relative to HbA (17), changes in the intracellular HbA/HbD ratio

Significance

Hummingbirds have exceedingly high oxygen demands because of their elevated rates of aerobic metabolism, and yet they thrive in high-altitude environments in the Andes where oxygen is scarce. Here we report the finding that when hummingbird species colonized new elevational zones, evolutionary changes in the respiratory properties of hemoglobin were repeatedly mediated by the same amino acid replacements. Specifically, ancestral sequence reconstruction and protein engineering experiments revealed that parallel adaptation of hemoglobin function in multiple species is attributable to repeated amino acid replacements at a single pair of interacting sites. This striking parallelism at the molecular level suggests a surprising degree of reproducibility and predictability in adaptive protein evolution.

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Data deposition: Complete information for all specimens used in this study is archived on the ARCTOS online database (Table S4). The sequences reported in this paper have been deposited in the GenBank database (accession nos. KF222496, KF222499, KF222501, KF222503, KF222506, KF222510–KF222539).

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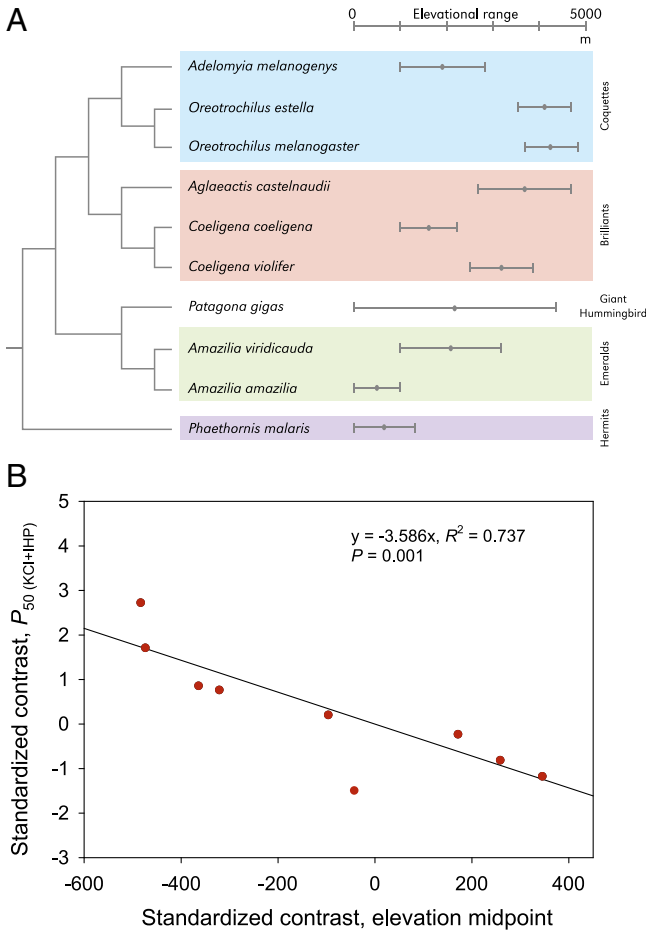

Fig. 1. Phylogenetically independent contrasts reveal a positive association between Hb-O_2 affinity and native elevation in Andean hummingbirds. (*A*) Phylogenetic relationships (25) and elevational distributions (ranges and midpoints) of 10 hummingbird species included in the analysis of Hb function. (*B*) Least-squares regression of phylogenetically independent contrasts revealed a significant negative relationship between $P_{50(KCl+IHP)}$ and native elevation (i.e., a positive relationship between Hb-O_2 affinity and elevation). See Table S2 for full results.

could substantially alter blood-O_2 affinity, and it has been suggested that regulatory changes in Hb isoform composition may contribute to adaptive changes in blood-O_2 transport in high-altitude species (18–20). To test this hypothesis we conducted a proteomic analysis of red cell lysates from each of the 10 hummingbird study species. Results of this analysis revealed that each of the hummingbird species express both HbA and HbD isoforms, and the relative concentration of the minor HbD isoform ranged from 1.6 to 24.2% (mean ± SD = 13.3 ± 6.2%). However, phylogenetically independent contrasts revealed no clear association between HbA/HbD isoform ratio and native elevation ($R^2 = 0.026$, $P = 0.657$).

Altitudinal Variation in Hb-O_2 Affinity. Evolutionary adjustments in Hb-O_2 affinity can be achieved via changes in intrinsic O_2 affinity or changes in the sensitivity of Hb to the modulating effects of physiological allosteric cofactors, such as Cl^- ions and organic phosphates (8, 15). The allosteric regulation of Hb-O_2 affinity involves the oxygenation-linked binding of nonheme ligands that indirectly modulate heme reactivity by shifting the equilibrium between a low-affinity "T-state" and a high-affinity "R-state." Allosteric cofactor molecules typically reduce Hb-O_2 affinity by preferentially binding and stabilizing deoxygenated Hb, thereby displacing the R↔T equilibrium in favor of the low-affinity T-state conformation (Fig. S2). After isolating and purifying the HbA and HbD isoforms from each hummingbird species, we measured oxygenation properties in the presence and absence of the two main allosteric effectors that regulate Hb-O_2 affinity: inositol hexaphosphate (IHP, a chemical analog of the naturally occurring inositol pentaphosphate in avian red cells, at twofold molar excess over tetrameric Hb) and Cl^- ions (added as KCl; 0.1 mol·L^{-1}). Hb-O_2 affinity was indexed by P_{50}, the PO_2 at which Hb is half-saturated. O_2-equilibrium measurements revealed that the major HbA isoforms of the high-altitude hummingbird species were generally characterized by elevated O_2-affinities in the absence of allosteric effectors ("stripped" Hb) and the difference in P_{50} values between highland and lowland species was amplified in the presence of IHP alone and in the simultaneous presence of both IHP and Cl^- ions (Table 1 and Fig. S3).

Regressions based on phylogenetically independent contrasts (PICs) revealed a significantly negative relationship between HbA P_{50} values and native elevation (i.e., a positive relationship between Hb-O_2 affinity and elevation) (Fig. 1*B* and Table S1). Similarly, for five species that expressed the HbD isoform at levels sufficient for experimental analysis, regressions based on PICs revealed significantly negative relationships between P_{50} values and native elevation, both for HbD alone and for the weighted average of HbA and HbD in their naturally occurring relative concentrations (Table S1). From this point onward, we

Table 1. Functional properties of hummingbird HbA isoforms

Species	Stripped		+KCl		+IHP		+KCl+IHP	
	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}
Adelomyia melanogenys	2.85 ± 0.01	1.60 ± 0.00	4.60 ± 0.08	1.89 ± 0.08	28.83 ± 1.54	2.16 ± 0.14	32.02 ± 3.84	2.31 ± 0.07
Oreotrochilus estella	2.17 ± 0.12	1.36 ± 0.13	3.39 ± 0.24	1.54 ± 0.13	21.82 ± 1.09	1.98 ± 0.11	20.20 ± 0.28	2.00 ± 0.03
Oreotrochilus melanogaster	2.10 ± 0.06	1.40 ± 0.01	3.86 ± 0.05	1.75 ± 0.05	26.65 ± 0.71	2.25 ± 0.09	19.88 ± 0.27	2.08 ± 0.05
Aglaeactis castelnaudii	2.17 ± 0.06	1.38 ± 0.04	3.23 ± 0.28	1.40 ± 0.02	22.45 ± 0.93	1.51 ± 0.18	17.23 ± 0.66	1.61 ± 0.14
Coeligena coeligena	2.49 ± 0.11	1.48 ± 0.06	4.22 ± 0.16	1.67 ± 0.10	27.83 ± 0.37	1.91 ± 0.08	22.90 ± 3.16	2.19 ± 0.11
Coeligena violifer	2.12 ± 0.04	1.29 ± 0.03	3.74 ± 0.10	1.65 ± 0.08	23.55 ± 0.74	1.96 ± 0.04	19.12 ± 1.27	1.70 ± 0.19
Patagona gigas	2.52 ± 0.06	1.46 ± 0.04	4.14 ± 0.37	1.63 ± 0.13	29.97 ± 1.00	2.28 ± 0.11	25.86 ± 1.66	2.49 ± 0.42
Amazilia viridicauda	2.62 ± 0.03	1.43 ± 0.03	4.47 ± 0.05	1.81 ± 0.05	28.49 ± 1.20	2.13 ± 0.08	24.24 ± 0.87	2.07 ± 0.11
Amazilia amazilia	3.14 ± 0.43	1.38 ± 0.05	5.28 ± 0.25	1.90 ± 0.15	36.77 ± 0.85	2.16 ± 0.08	29.84 ± 0.32	2.42 ± 0.01
Phaethornis malaris	2.83 ± 0.10	1.39 ± 0.12	4.70 ± 0.06	1.83 ± 0.06	37.00 ± 0.44	2.27 ± 0.13	28.13 ± 0.60	2.04 ± 0.11

O_2-affinities (P_{50}, torr) and cooperativity coefficients (n_{50}; mean ± SEM) of purified HbA isoforms measured in 0.1 M Hepes buffer at pH 7.40 (± 0.01), 37 °C, in the absence of allosteric effectors (stripped), in the presence of KCl (0.1 M) or IHP (IHP/Hb tetramer ratio = 2.0), and in the presence of both allosteric effectors. [Heme], 0.3 mM. As explained in the text, P_{50} is an inverse measure of Hb-O_2 affinity. High-altitude species with maximum elevational ranges of >3,000 m are denoted by gray shading.

primarily focus on oxygenation properties of Hb in the presence of IHP and Cl^- ions, the experimental treatment that is most relevant to in vivo conditions in avian red cells.

Causative Substitutions and the Structural Mechanisms Underlying Evolutionary Transitions in Hb-O_2 Affinity. Inspection of the genotypic and phenotypic data suggested that phylogenetically replicated changes in Hb-O_2 affinity were largely attributable to repeated amino acid replacements at two sites: β13 (position 10 in the A helix) and β83 (position 7 in the EF interhelical segment) (Fig. 2 and Fig. S4). Within each clade, the species with the highest Hb-O_2 affinities in the presence of allosteric effectors always possessed the two-site genotype β13Ser-β83Ser (*Oreotrochilus estella*, *Oreotrochilus melanogaster*, and *P. gigas*, all of which are predominantly highland species) or β13Gly-β83Ser (*Aglaeactis castelnaudii*, *Coeligena violifer*, and *Amazilia viridicauda*, all of which are predominantly highland species), whereas the species with the lowest Hb-O_2 affinities always possessed β13Gly-β83Gly (*Adelomyia melanogenys*, *Coeligena coeligena*, *Amazilia amazilia*, and *Phaethornis malaris*, all of which are predominantly lowland species) (Fig. 2 and Table 1). Comparisons among HbD isoforms are also informative about the effects of these substitutions because HbA and HbD isoforms of the same species share identical β-chain subunits. Among the five species in which HbD was examined, the only species with the β13Ser-β83Ser genotype (the predominantly highland *P. gigas*) had the highest HbD O_2 affinity [$P_{50(KCl+IHP)}$ = 16.56 ± 0.56 torr], and the two species that shared the alternative β13Gly-β83Gly genotype (the exclusively lowland *A. amazilia* and *P. malaris*) had the two lowest affinities [$P_{50(KCl+IHP)}$ = 23.20 ± 1.21 and 24.92 ± 0.52 torr].

To identify the structural basis of variation in Hb-O_2 affinity, a comparison between the HbA isoforms of *A. melanogenys* (an exclusively lowland species) and *O. estella* (a high-altitude specialist) is especially informative because they exhibit pronounced differences in O_2-affinity [$P_{50(KCl+IHP)}$ = 32.02 ± 3.84 vs. 20.20 ± 0.28 torr, respectively] (Table 1), and yet they differ by just one conservative α-chain substitution (α8Thr→Ser) and two polarity-changing β-chain substitutions (β13Gly→Ser and β83Gly→Ser) (Fig. 2). To isolate the functional effects of the two β-chain substitutions, we used a recombinant expression vector (21) and site-directed mutagenesis to synthesize the reconstructed ancestral Coquette HbA (β13Gly-β83Gly, identical to wild-type *A. melanogenys* β-globin), the derived double-mutant genotype that is identical to wild-type *O. estella* β-globin (β13Ser-β83Ser), and each of the alternative single-mutant intermediates (β13Ser-β83Gly and β13Gly-β83Ser). Consistent with measurements of

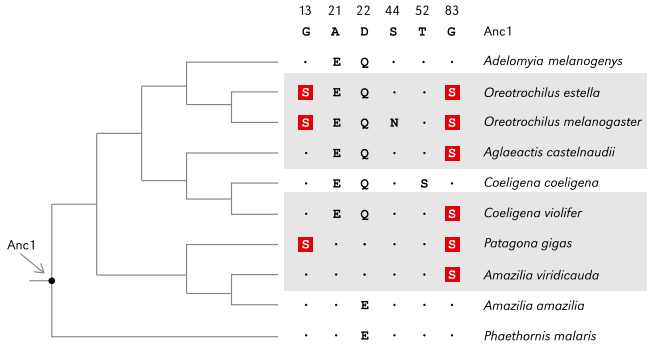

Fig. 2. Variable residue positions in a multiple alignment of hummingbird β^A-globin sequences. The estimated sequence for the common ancestor of hummingbirds (Anc1) is included for comparison, and derived Ser residues at β13 and β83 are shown in red boxes. High-altitude species with maximum elevational ranges of >3,000 m are denoted by shading. Sequences represent the most common haplotypes for each species.

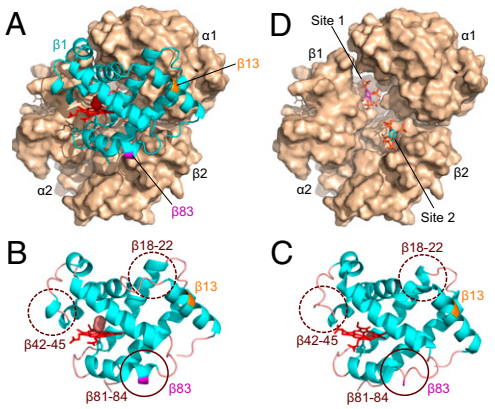

Fig. 3. Amino acid replacements at β13 and β83 produce second-order perturbations of tertiary structure that affect the allosteric regulation of O_2-binding by IHP. (*A*) Homology-based structural model of hummingbird Hb showing the locations of amino acid replacements at sites 13 and 83 in the β1 subunit (shown in cyan). β-Chain residues 18–22, 42–45, and 81–84 (including the variable site 83, the penultimate C-terminal residue in the EF interhelical loop) alternate between helical and nonhelical secondary structure in the allosteric transition between the oxy (*B*) and deoxy (*C*) states. (*D*) Structural model of hummingbird Hb (with the β1 subunit removed) showing alternative IHP-binding sites in the central cavity. Polarity-changing amino acid replacements at β13 and β83 produce nonadditive changes in the favorability of alternative conformation states for polyphosphate-binding: IHP preferentially binds at "site 1" in the β13Gly-β83Gly and β13Ser-β83Ser mutants (representing wild-type β-globin genotypes for *A. melanogenys* and *O. estella*, respectively) and at "site 2" in the β13Ser-β83Gly and β13Gly-β83Ser mutants.

the native Hbs in the presence of allosteric effectors, $P_{50(KCl+IHP)}$ for the recombinant *O. estella* Hb was significantly lower (i.e., O_2-affinity was higher) than that of *A. melanogenys* (Table S2), confirming the affinity-enhancing effect of the β13Gly→Ser and β83Gly→Ser substitutions in combination.

Epistasis for Hb-O_2 Affinity. Analysis of the alternative single- and double-mutant recombinant Hbs (rHbs) revealed that phenotypic effects of mutations at β13 and β83 are highly context-dependent; $P_{50(KCl+IHP)}$ values exhibited a significant epistatic deviation from expectations of an additive model (ε = 12.94, 95% confidence interval = 10.05–15.82). In the presence of allosteric effectors, the β13Gly→Ser substitution increased O_2-affinity on the ancestral Coquette background (in the presence of β83Gly) and reduced O_2-affinity in the presence of the derived β83Ser. Similarly, the β83Gly→Ser substitution increased O_2-affinity on the ancestral Coquette background (in the presence of β13Gly) and reduced O_2-affinity in the presence of the derived β13Ser (Table S2). This is an example of sign epistasis (22, 23), where the sign of the phenotypic effect of a mutation is conditional on the genetic background in which it occurs.

Structural Basis of Species Differences in Hb Function. To determine the structural mechanisms responsible for the additive and epistatic effects of substitutions at β13 and β83, we conducted homology-based modeling analyses of hummingbird Hb (*Methods*). These analyses revealed that Gly→Ser replacements at β13 and β83 produce localized changes in secondary structure of the A and F helices, respectively (Table S3), which impinge indirectly on the allosteric regulatory control of Hb-O_2 affinity. Site β83 is located within a segment of the β-subunit main chain (residues 81–84) that alternates between helical and nonhelical secondary structure in the allosteric transition between the oxy and deoxy states, respectively (Fig. 3 *A*–*C*). At β83, either Gly or Ser can donate a helix-capping, amide H-bond to the carbonyl oxygen of

β85Phe (the N-terminal residue of the F-helix), but the polar -OH side-chain of β83Ser forms additional intermolecular H-bonds that alter the torsion angle of the F-helix, thereby constraining allosteric movement. When nonpolar Gly is replaced by polar, hydrophilic Ser at β83 (as in the predominantly highland *Oreotrochilus*, *A. castelnaudii*, *C. violifer*, *P. gigas*, and *A. viridicauda*) (Fig. 2), the effect on Hb allostery is contingent on the presence of Gly or Ser at β13. Changes in the network of atomic contacts involving β13 and β83 (Table S3) alter the favorability of alternative conformation states for IHP-binding in the central cavity (Fig. 3*D*), and the resultant changes in the location of IHP-binding account for the observed epistasis for Hb-O_2 affinity in the presence of IHP (Table S2). Our experimental results for the hummingbird rHb mutants are consistent with functional studies of a naturally occurring human Hb mutant, Hb Pyrgos (β83Gly→Asp), which is also characterized by an increased O_2-affinity in the presence of organic phosphates (24).

Parallelism of β-Chain Substitutions Among Species. We sequenced β^A-globin in 63 hummingbird species and we then used maximum-likelihood and parsimony to map the β13 and β83 replacements onto an independently derived and well-resolved phylogeny (25). This analysis revealed that the substitutions (and, by implication, the associated changes in Hb-O_2 affinity) occurred at least 17 times independently (≥4 and ≥13 transitions between Gly and Ser at β13 and β83, respectively). Maximum-likelihood ancestral-state estimates for native elevation indicated that hummingbird species have shifted upwards and downward during the evolution of the group, in conjunction with repeated substitutions and back-substitutions at β13 and β83 (Fig. 4 and Fig. S5). Hence, the negative correlation between P_{50} and native elevation (Fig. 1*B*) is attributable to derived increases in Hb-O_2 affinity in highland lineages, as well as derived reductions in Hb-O_2 affinity in lowland lineages. For example, the common ancestor of the highland genus *Oreotrochilus* (β13Ser-β83Ser) evolved a derived increase in Hb-O_2 affinity relative to the likely ancestral state of the Coquette clade (β13Gly-β83Gly). In contrast, in the Brilliants the lowland *C. coeligena* (β13Gly-β83Gly) evolved a derived reduction in Hb-O_2 affinity relative to the likely ancestral state for that clade (β13Gly-β83Ser) (Fig. 4). Species' maximum elevation was strongly associated with β13-β83 genotype in a phylogenetic general linear model ($R^2 = 0.53$; $P < 10^{-11}$) (Fig. 4).

Among distantly related species, parallel substitutions at sites β13 and β83 are likely attributable to the repeated fixation of identical-by-state alleles that had independent mutational origins. Among some of the more closely related species, the sorting of ancestral polymorphism may produce the same pattern of parallelism because of the repeated fixation of identical-by-descent alleles in recently diverged lineages (26). Further work is needed to elucidate the mutational origins of the β13 and β83 variants, but it is clear that repeated changes at both sites have contributed to the repeated elevational shifts in Hb function among different lineages. Aside from the variation at sites β13 and β83, no other substitutions in the α^A-, α^D-, or β^A-globin genes exhibited any obvious association with species differences in P_{50} values for HbA or HbD, although it is likely that particular lineage-specific substitutions (Fig. S4) account for residual variation in Hb-O_2 affinity among species.

Possible Adaptive Significance of Altitudinal Differences in Hb-O_2 Affinity. The evolution of divergent Hb-O_2 affinities between highland and lowland hummingbirds is consistent with theoretical predictions (1–6). At low altitude, a low Hb-O_2 affinity is expected to be physiologically advantageous for hummingbirds and other animals with high mass-specific metabolic rates because O_2 unloading in the peripheral circulation can occur at relatively high PO_2, thereby optimizing tissue oxygenation by increasing the O_2 diffusion gradient between capillary blood and tissue mitochondria. At low altitude, the trade-off with pulmonary O_2 loading is alleviated because arterial O_2 saturation will still be near-maximal. However, under conditions of severe environmental hypoxia at very high altitudes, an increased Hb-O_2 affinity becomes advantageous because tissue O_2 delivery can be

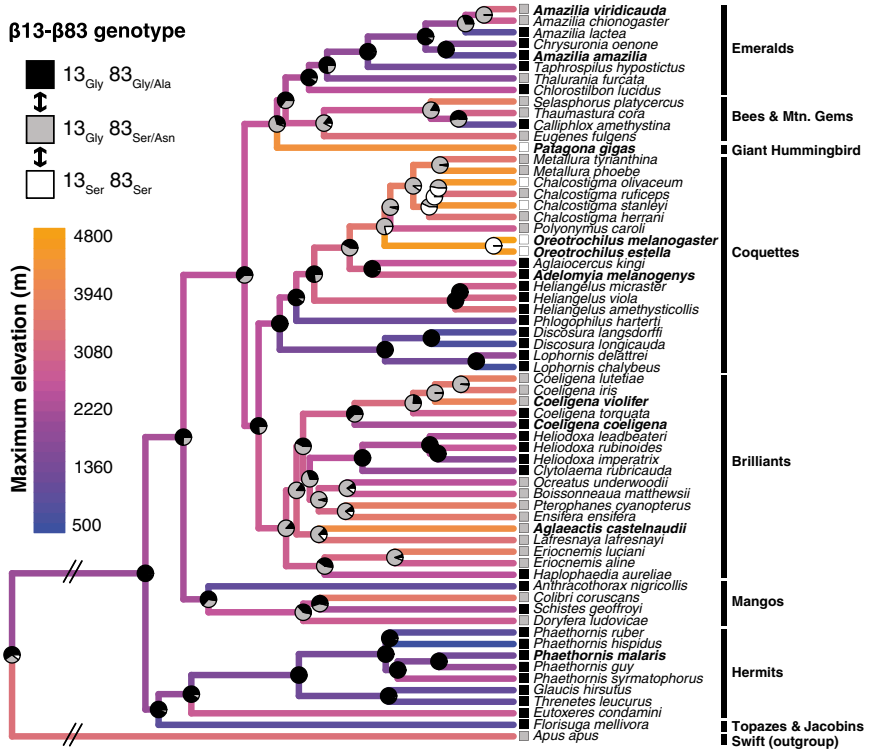

Fig. 4. Ancestral state estimates for β13 and β83 in hummingbirds. Pie diagrams at the nodes indicate the probability of each genotype based on a stepwise, single-rate maximum-likelihood model with two reversible transitions, as indicated in the inset diagram. Terminal branches of the phylogenetic tree are color-coded according to the upper limit of the species' elevational range, and internal branches are color-coded based on maximum-likelihood estimates of the ancestral states. The phylogenetically corrected association between β13-β83 genotype and native elevation was highly significant (see text for details). Parsimony analysis revealed a minimum of 17 changes in genotype across the tree (Fig. S5). β83Asn was observed in a single species, *Doryfera ludoviciae*, and was therefore binned with the β83Ser character state because side-chains of the two residues have the same polarity and the underlying codons are connected by a single mutational step. Similarly, β83Ala was observed in a single species, *Phlogophilus harterti*, and was binned with the β83Gly character state in this analysis. Branch lengths are proportional to relative time, except where indicated. Species names in bold are those that were included in the experimental analysis of Hb function.

preserved more effectively by safeguarding arterial O_2 saturation than by maximizing O_2 unloading from partially desaturated blood (1–6, 8, 9).

Mechanisms of Hb Adaptation and Causes of Parallelism at the Sequence Level. Comparative studies of Hb function in different animal species and experimental studies of naturally occurring or recombinant human Hb mutants have demonstrated that genetically based changes in Hb-O_2 affinity can be produced by numerous possible structural changes (27–29). In Andean hummingbirds, amino acid replacements at β13 and β83 contribute to species differences in Hb-O_2 affinity, but it is certainly not because they represent the only possible mutational changes that are capable of producing the observed changes in protein function. Although there may be numerous possible mutations that can produce identical changes in Hb-O_2 affinity, many of those changes are known to have deleterious pleiotropic effects. For example, active site mutations that alter the polarity or hydrophobicity of the distal heme pocket can produce direct changes in the association constant for O_2 binding, but such mutations typically compromise structural stability or increase the susceptibility to heme autoxidation (the spontaneous oxidation of the heme iron from the ferrous Fe^{2+} state to the ferric Fe^{3+} state, which renders Hb functionally inert as an O_2-transport molecule) (29). In contrast, mutations remote from the active site—like those at β13 and β83—can potentially produce fine-tuned changes in O_2-affinity with minimal pleiotropic effects through subtle displacements of the allosteric equilibrium (28–30). Within the set of all possible mutations that produce functionally equivalent effects on Hb-O_2 affinity, those that incur a lesser magnitude of deleterious pleiotropy are predicted to have a higher fixation probability, and such mutations may therefore contribute disproportionately to biochemical adaptation (31–33). When such changes are driven by positive directional selection, theory predicts that they are especially likely to evolve in parallel (34).

The parallel β13 and β83 substitutions that we have documented in hummingbirds have not been implicated in the adaptation of Hb function in other high-altitude birds or mammals (35–39), although a survey of sequence variation in the globin genes of Andean waterfowl documented a shared β13Gly/Ser polymorphism in speckled teals (*Anas flavirostris*) and yellow-billed pintails (*Anas georgica*), and in both species the derived Ser variant was present at high frequency in high-altitude populations (40). The phenotypic effects of the β13Gly/Ser variants in these waterfowl species have not yet been investigated, but the similar altitudinal patterns in Andean ducks and hummingbirds suggest parallel mechanisms of Hb evolution. At β13 and β83 in Andean hummingbirds, it may be that recurrent mutation and retention of ancestral polymorphism both contributed to variation in Hb function—variation that was then recruited when selection favored fine-tuned adjustments in blood-O_2 transport (e.g., during elevational range shifts). When closely related species independently adapt to a shared environmental challenge, natural selection may be predisposed to hit upon the same design solution in different lineages if one particularly accessible (and minimally pleiotropic) solution happens to be located within striking distance from the same ancestral starting point.

Methods

Specimen Collection. We preserved blood and tissue samples from voucher specimens of hummingbirds that were collected from numerous Andean localities spanning an elevational range of ~4,500 m (Table S4). Our analysis of Hb function was based on blood samples from 70 hummingbird specimens (n = 3–8 individuals per species). All hummingbirds were live-trapped in mistnets and were bled and killed in accordance with guidelines of the Ornithological Council (41), and protocols approved by the University of New Mexico Institutional Animal Care and Use Committee (Protocol number 08UNM033-TR-100117; Animal Welfare Assurance number A4023-01). All fieldwork was carried out under permits issued by the management authorities of Peru (76-2006-INRENA-IFFS-DCB, 087–2007-INRENA-IFFS-DCB, and 135–2009-AG-DGFFS-DGEFFS).

For each individual bird, we collected 0.03–0.20 mL of whole blood from the brachial or ulnar vein using heparinized microcapillary tubes. Red blood cells were separated from the plasma fraction by centrifugation, and the packed red cells were then snap-frozen in liquid nitrogen and were stored at −80 °C before use as a source of Hb for experimental studies. We collected liver and pectoral muscle from each specimen as sources of genomic DNA and globin mRNA, respectively. Muscle samples were snap-frozen or preserved using RNAlater and were subsequently stored at −80 °C before RNA isolation. Voucher specimens were preserved along with ancillary data and were deposited in the collections of the Museum of Southwestern Biology of the University of New Mexico and the Centro de Ornitología y Biodiversidad (CORBIDI), Lima, Peru. Complete specimen data are available via the ARCTOS online database (Table S4).

Molecular Cloning and Sequencing. We cloned and sequenced the adult globin genes (α^A-, α^D-, and β^A-globin) from at least two specimens per species. We used the RNeasy Mini Kit (Qiagen) to isolate RNA, and we used 5′ and 3′ RACE (Invitrogen Life Technologies) to obtain cDNA sequence for the 5′ and 3′ UTRs of each adult-expressed globin gene. After designing paralog-specific PCR primers with annealing sites in the 5′ and 3′ UTRs, complete cDNAs were synthesized for each gene by reverse transcription using the OneStep RT-PCR kit (Qiagen). We cloned gel-purified RT-PCR products into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen Life Technologies). All new sequences were deposited in GenBank under accession nos. KF222496, KF222499, KF222501, KF222503, KF222506, and KF222510–KF222539.

Characterization of Hb Isoform Composition. We used isoelectric focusing (IEF; PhastSystem, GE Healthcare Bio-Sciences) to characterize Hb isoform composition in red cell lysates from each of the 70 hummingbird specimens. After separating native Hbs by means of IEF, gel bands were excised and digested with trypsin. The resultant peptides were then identified by means of tandem mass spectrometry (MS/MS). Database searches of the resultant MS/MS spectra were performed using Mascot (Matrix Science, v1.9.0), whereby peptide mass fingerprints were used to query a custom database of avian α- and β-chain sequences (17, 42–44), including α^A-, α^D-, and β^A-globin sequences from each of the surveyed hummingbird species. After separating the HbA and HbD isoforms by native gel IEF and identifying each of the constituent subunits by MS/MS, the relative abundance of the different isoforms in the hemolysates of each individual was quantified densitometrically using ImageJ (45).

Protein Purification and Measurement of Hb-O_2 Equilibria. The HbA and HbD isoforms (isoelectric points = 8.9–9.1 and 6.8–7.3, respectively) were separated and stripped of organic phosphates by means of ion-exchange chromatography. O_2 equilibria of purified Hb solutions [3 μL thin-layer samples, (heme) 0.3 mM] were measured at 37 °C in the presence of 0.1 M Hepes buffer (pH 7.4). To characterize the allosteric regulation of Hb-O_2 affinity, we measured O_2-equilibrium curves in the absence of allosteric effectors (stripped), in the presence of Cl^- ions (0.1 M KCl), in the presence of IHP (IHP/Hb tetramer ratio = 2.0), and in the simultaneous presence of both effectors. For details of the purification protocol and the measurement of Hb-O_2 equilibrium curves, see *SI Methods*.

Vector Construction, Site-Directed Mutagenesis, and Synthesis of rHbs. To produce rHbs for the protein engineering experiments, the α^A- and β^A-globin genes of *A. melanogenys* were synthesized by Genscript after optimizing nucleotide sequences with respect to *Escherichia coli* codon preferences. Gene cassettes for the α^A- and β^A-globin genes and the *methionine aminopeptidase* (*MAP*) gene were tandemly cloned into the custom pGM expression plasmid described by Natarajan et al. (21). All rHbs were expressed in the JM109 (DE3) *E. coli* strain. See *SI Methods* for details regarding the site-directed mutagenesis experiments, the expression and purification of the hummingbird rHb mutants, the measurement of rHb oxygenation properties, and the measurement of epistasis.

Ancestral State Estimates. To infer the polarity of character-state changes at β13 and β83, we sequenced the β^A-globin gene of 63 hummingbird species with known phylogenetic relationships. Orthologous sequence from the common swift (*Apus apus*) was used as an outgroup. Fifty-six of the 63 nodes in the independently derived phylogeny were resolved with >95% posterior probability (Dataset S1). We estimated ancestral states of the β13-β83 genotypes using maximum-likelihood and parsimony with the APE package in R (46). Two of the observed genotypes included rare variants at β83 (β13Gly-β83Asn

and β13Gly-β83Ala) that differed by a single codon change from physiochemically similar alternative states (β83Ser and β83Gly, respectively). We binned each of these singleton changes with the related codon state, resulting in three classes of two-site β13-β83 genotypes. In the maximum-likelihood model, we allowed only the two reversible transitions that each comprised a single nucleotide change. We applied a model with one rate for all transitions because likelihood ratio tests indicated that models with two to four rate parameters were not justified (46) (Fig. 4). For details regarding the phylogenetic topology and phylogenetic comparative methods, see *SI Methods*.

Structural Modeling and Molecular Docking. Homology-models of hummingbird Hb were built by the SWISS-MODEL server in the automated model (47), using *Anas platyrhynchos* Hb (PDB ID code 3EOK) as template. For each of the four rHb mutants, the root-mean-square-deviation was 0.74 Å between model and template and the QMEAN value remained between 0.70 and 0.78 for all models. Molecular docking of IHP in the Hb central cavity was performed using AutoDock Vina (48). Internal molecular contacts were identified by the Frustratometer program (49).

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