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-O2 affinity for O2 loading in the pulmonary circulation and O2 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-O2 affinity and native elevation. Protein engineering experiments and ancestral-state reconstructions revealed that this correlation is attributable to derived increases in Hb-O2 affinity in highland lineages, as well as derived reductions in Hb-O2 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-O2 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.

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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.

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.


The authors declare no conflict of interest.

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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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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 expresses 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$\leftrightarrow$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 HbP$_{50}$ values and native elevation (i.e., a positive relationship between Hb-O$_2$ affinity and elevation) (Fig. 1B 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

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**Table 1. Functional properties of hummingbird HbA isoforms**

<table>
<thead>
<tr>
<th>Species</th>
<th>Stripped</th>
<th>+KCl</th>
<th>+IHP</th>
<th>+KCl+IHP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P_{50}$</td>
<td>$n_{50}$</td>
<td>$P_{50}$</td>
<td>$n_{50}$</td>
</tr>
<tr>
<td>Adelomyia melanogaster</td>
<td>2.85 ± 0.01</td>
<td>1.60 ± 0.00</td>
<td>4.60 ± 0.08</td>
<td>1.89 ± 0.08</td>
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<tr>
<td>Oreochothilus estella</td>
<td>2.17 ± 0.12</td>
<td>1.36 ± 0.13</td>
<td>3.39 ± 0.24</td>
<td>1.54 ± 0.13</td>
</tr>
<tr>
<td>Oreochothilus melanogaster</td>
<td>2.10 ± 0.06</td>
<td>1.40 ± 0.01</td>
<td>3.86 ± 0.05</td>
<td>1.75 ± 0.05</td>
</tr>
<tr>
<td>Aglaeactis castelnauddi</td>
<td>2.17 ± 0.06</td>
<td>1.38 ± 0.04</td>
<td>3.23 ± 0.28</td>
<td>1.40 ± 0.02</td>
</tr>
<tr>
<td>Coeligena coeligena</td>
<td>2.49 ± 0.11</td>
<td>1.48 ± 0.06</td>
<td>4.22 ± 0.16</td>
<td>1.67 ± 0.10</td>
</tr>
<tr>
<td>Coeligena violifera</td>
<td>2.12 ± 0.04</td>
<td>1.29 ± 0.03</td>
<td>3.74 ± 0.10</td>
<td>1.65 ± 0.08</td>
</tr>
<tr>
<td>Patagona gigas</td>
<td>2.52 ± 0.06</td>
<td>1.46 ± 0.04</td>
<td>4.14 ± 0.37</td>
<td>1.63 ± 0.13</td>
</tr>
<tr>
<td>Amauzlia viridicauda</td>
<td>2.62 ± 0.03</td>
<td>1.43 ± 0.03</td>
<td>4.47 ± 0.05</td>
<td>1.81 ± 0.05</td>
</tr>
<tr>
<td>Amauzlia amazilia</td>
<td>3.14 ± 0.43</td>
<td>1.38 ± 0.05</td>
<td>5.28 ± 0.25</td>
<td>1.90 ± 0.15</td>
</tr>
<tr>
<td>Phaethornis malipus</td>
<td>2.83 ± 0.10</td>
<td>1.39 ± 0.12</td>
<td>4.70 ± 0.06</td>
<td>1.83 ± 0.06</td>
</tr>
</tbody>
</table>

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 >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-O2 Affinity.** Inspection of the genotypic and phenotypic data suggested that phylogenetically replicated changes in Hb-O2 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-O2 affinities in the presence of allosteric effectors always possessed the two-site genotype β13Ser-β83Ser (Oreothrochilus estella, Oreothrochilus melanogaster, and P. gigas, all of which are predominantly highland species) or β13Gly-β83Ser (Aglaeactis castaneaoides, Coeligena violfer, and Amazilia virdicata, all of which are predominantly highland species), whereas the species with the lowest Hb-O2 affinities always possessed β13Gly-β83Gly (Adelomyia melanogyns, Coeligena coeligena, Amazilia amazilia, and Phaeornis malanis, all of which are predominantly lowland species) (Fig. 2 and Table 1). Comparisons among HbD isofoms are also informative about the effects of these substitutions because HbA and HbD isofoms 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 O2 affinity [P50(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. malanis) had the two lowest affinities [P50(KCl-IHP) = 23.20 ± 1.21 and 24.92 ± 0.52 torr].

To identify the structural basis of variation in Hb-O2 affinity, a comparison between the HbA isofoms of A. melanogyns (an exclusively lowland species) and O. estella (a high-altitude specialist) is especially informative because they exhibit pronounced differences in O2 affinity [P50(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 (a8Thr→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. melanogyns 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 the native Hbs in the presence of allosteric effectors, P50(KCl-IHP) for the recombinant O. estella Hb was significantly lower (i.e., O2-affinity was higher) than that of A. melanogyns (Table S2), confirming the affinity-enhancing effect of the β13Gly→Ser and β83Gly→Ser substitutions in combination.

**Epistasis for Hb-O2 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; P50(KCl-IHP) values exhibited a significant epistatic deviation from expectations of an additive model (λ = 12.94, 95% confidence interval = 10.05–15.82). In the presence of allosteric effectors, the β13Gly→Ser substitution increased O2-affinity on the ancestral Coquette background (in the presence of β83Gly) and reduced O2-affinity in the presence of the derived β83Ser. Similarly, the β83Gly→Ser substitution increased O2-affinity on the ancestral Coquette background (in the presence of β13Gly) and reduced O2-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-O2 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 (B) and deoxy (C) 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. castanacauda, C. violifer, P. gigas, and A. viridicauda) (Fig. 2), the effect on Hb allostericity 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. 3D), and the resultant changes in the location of IHP-binding account for the observed epistasis for Hb-O 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 O₂-affinity in the presence of organic phosphates (24).

**Parallelism of β-Chain Substitutions Among Species.** We sequenced β₄-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 affinity) occurred at least 17 times independently (≥4 and ≥3 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 P₅₀ and native elevation (Fig. 1B) is attributable to derived increases in Hb-O₂ affinity in highland lineages, as well as derived reductions in Hb-O₂ affinity in lowland lineages. For example, the common ancestor of the highland genus Oreotrochilus (β13Ser-β83Ser) evolved a derived increase in Hb-O₂ affinity relative to the likely ancestral state of the Coquette clade (β13Gly-β83Gly). In contrast, in the Brilliant clade the lowland C. coeligena (β13Gly-β83Gly) evolved a derived reduction in Hb-O₂ 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 α₂-α₂- or β₄-β₄-globin genes exhibited any obvious association with species differences in P₅₀ values for HbA or HbD, although it is likely that particular lineage-specific substitutions (Fig. S4) account for residual variation in Hb-O₂ affinity among species.

**Possible Adaptive Significance of Altitudinal Differences in Hb-O₂ Affinity.** The evolution of divergent Hb-O₂ affinities between highland and lowland hummingbirds is consistent with theoretical predictions (1–6). At low altitude, a low Hb-O₂ affinity is expected to be physiologically advantageous for hummingbirds and other animals with high mass-specific metabolic rates because O₂ unloading in the peripheral circulation can occur at relatively high PPO₂, thereby optimizing tissue oxygenation by increasing the O₂ diffusion gradient between capillary blood and tissue mitochondria. At low altitude, the trade-off with pulmonary O₂ loading is alleviated because arterial O₂ saturation will still be near-maximal. However, under conditions of severe environmental hypoxia at very high altitudes, an increased Hb-O₂ affinity becomes advantageous because tissue O₂ 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). β83Ala was observed in a single species, Doryfera ludoviciæ, 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, Philognophilus 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.
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-O2 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-O2 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-O2 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 O2 binding, but such mutations typically compromise structural stability or increase the susceptibility to heme autodestruction (the spontaneous oxidation of the heme iron from the ferrous Fe2+ state to the ferric Fe3+ state, which renders Hb functionally inert as an O2-transport molecule) (29). In contrast, mutations remote from the active site—like those at β13 and β83—can potentially produce fine-tuned changes in O2-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-O2 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-O2 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 independently and thus may be especially likely to evolve in parallel (34).

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 species. 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-globin sequences (41, 42–44), including αA, αB, and βA-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-O2 Equilibria. The HbAs 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. O2 equilibrium 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-O2 affinity, we measured O2-equilibrium curves in the absence of allosteric effectors (stripped), in the presence of KH2PO4 (pH 7.4), and in the presence of 1 μM ATP (Hb tetramer ratio = 2.0), and in the simultaneous presence of both effectors. For details of the purification protocol and the measurement of Hb-O2 equilibrium curves, see SI Methods.

Vector Construction, Site-Directed Mutagenesis, and Synthesis of rhBs. To produce rhBs for the protein engineering experiments, the αA and βA-globin genes of A. melanogenys were synthesized by Genscript after optimizing nucleotide sequences with respect to Escherichia coli codon preferences. Gene cassettes for the αA- and βA-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 βA-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 βA-βD genotypes using maximum-likelihood and parsimony with the APE package in R (46). Two of the observed genotypes included rare variants at βA G13Gly/β83Asn
and β13Gly-β83Ala) that differed by a single codon change from physico-chemically 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 (13±13) 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 Hb 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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