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Compensatory mutations and epistasis for protein function

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Adaptive protein evolution may be facilitated by neutral amino acid mutations that confer no benefit when they first arise but which potentiate subsequent function-altering mutations via direct or indirect structural mechanisms. Theoretical and empirical results indicate that such compensatory interactions (intramolecular epistasis) can exert a strong influence on trajectories of protein evolution. For this reason, assessing the form and prevalence of intramolecular epistasis and characterizing biophysical mechanisms of compensatory interaction are important research goals at the nexus of structural biology and molecular evolution. Here I review recent insights derived from protein-engineering studies, and I describe an approach for identifying and characterizing mechanisms of epistasis that integrates experimental data on structure-function relationships with analyses of comparative sequence data.

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Introduction

A number of important questions about mechanisms of protein evolution concern the context-dependence of mutational effects ('epistasis'). Do amino acid mutations produce the same functional effect regardless of sequence context (i.e. regardless of the amino acid states of other sites in the same protein), or do their effects depend on the sequence context in which they occur? Epistatic interactions between mutant sites in the same protein can help explain why evolution follows some pathways rather than others [1–5,6•,7•,8••]. If the sign of a mutation's fitness effect is conditional on genetic background ('sign epistasis'), then pairs of mutations that are individually neutral or beneficial may be deleterious in combination. In such cases, some fraction of all possible mutational pathways connecting ancestral and descendant genotypes will be selectively inaccessible because they include incompatible mutational combinations as intermediate steps [9–15,16•,17,18•]. Conversely, pairs of mutations that are individually deleterious may be neutral or beneficial in combination, thereby opening up new pathways through sequence space that previously would have been off limits.

Intramolecular epistasis has important implications for biochemical adaptation and the evolution of novel protein functions. The evolution of an advantageous change in protein function may be facilitated by neutral mutations that confer no benefit when they first arise but which lay the groundwork for subsequent function-altering mutations. For example, an amino acid mutation at site X may produce a subtle change in protein conformation or stability that — by itself — is functionally inconsequential, but the altered structural context may change the functional effect of subsequent mutations at other sites in the same protein [4,5,7•,15,19–22]. The mutation at site X is neutral when it first arises, but by facilitating the fixation of a beneficial, function-altering mutation at site Y, it then becomes deleterious to revert site X to its ancestral state. Likewise, the mutation at site Y is beneficial on a background in which the mutation at site X has already occurred, but otherwise it would be neutral or deleterious. In principle, the compensatory change at site X could precede the function-altering change at site Y (in which case it is called a 'permissive' substitution), or it could occur afterwards, in which case there would be a transient reduction in fitness. In principle, the two mutations could also be fixed simultaneously if they co-occurred on the same sequence haplotype [23,24]. Evidence for epistatic fitness effects prompts us to view the longstanding 'selectionist/neutralist' debate through a new prism since a given mutation may be neutral, beneficial, or deleterious depending on the genetic background in which it occurs.

Compensatory substitutions are central to questions about the role of historical contingency in shaping pathways and outcomes of protein evolution. If the fitness effects of amino acid mutations are conditional on genetic background, then mutations can have different effects depending on the sequential order in which they occur [2]. Consequently, the accumulated history of substitutions in the past will influence the set of allowable

mutations in the future, and evolutionary outcomes will be historically contingent on ancestral starting points [7•,8••,12,14,25–27].

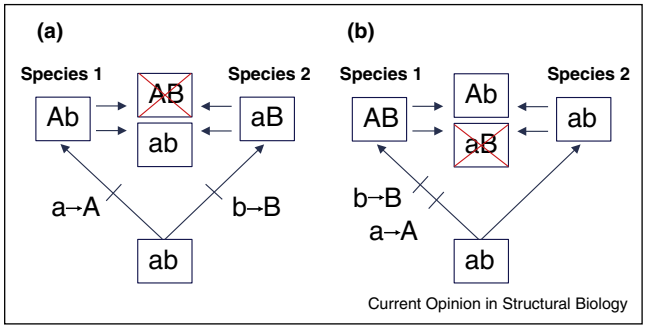
The role of intramolecular epistasis in protein evolution

Insights from protein-engineering experiments

The question of whether mutations have different effects in different genetic backgrounds can be decisively tested with site-directed mutagenesis experiments. Lunzer *et al.* [28] investigated the effects of swapping amino acid states at residue positions that differ between two highly divergent orthologs of isopropymalate dehydrogenase (IMDH). They introduced a total of 168 single mutations into wildtype IMDH of *Escherichia coli* that match the amino acid states at the same sites in the IMDH ortholog of *Pseudomonas aeruginosa*. Of these 168 swapped residues, over 1/3 of the wildtype amino acid states in *P. aeruginosa* compromised enzyme activity on the genetic background of *E. coli*. The fact that identical amino acid states produced different phenotypic effects on the two genetic backgrounds must be attributable to substitutions at other residue positions in one or both lineages that epistatically interact with the focal residues. These could be permissive substitutions that made a given amino acid state acceptable in the native *P. aeruginosa* background, and/or restrictive substitutions that made the same state deleterious in the *E. coli* background. Results of this experiment demonstrate how evolved changes in sequence context can reduce the number of site-specific amino acid states that are unconditionally acceptable in the divergent backgrounds of orthologous proteins (Figure 1). Interestingly, mutagenesis screens revealed that the amino acid replacements that most strongly reduced activity on the *E. coli* background could be compensated by multiple mutations at structurally remote sites; the compensatory mechanisms did not generally involve direct physical interactions [28].

Similar to the 'horizontal' swapping of amino acids between divergent orthologs of extant taxa, Gong *et al.* [15] performed 'vertical' exchanges between ancestral and descendant genotypes of the influenza nucleoprotein that were isolated from different timepoints over the span of several decades. Capitalizing on their ability to infer the temporal order of observed substitutions in a single line of descent, Gong *et al.* individually introduced each of 39 observed amino acid replacements into the genetic background of an extinct viral strain that approximated the ancestral genotype. The experiments revealed that three of the 39 substitutions that occurred as intermediate steps in the pathway had strongly deleterious effects on the ancestral genetic background even though they must have been neutral, nearly neutral, or possibly even beneficial on the derived genetic background in which they actually fixed. The three mutations that had deleterious effects on the ancestral background severely compromised thermal stability of the native protein fold. These same mutations were tolerated at later steps in the pathway because they were preceded by stabilizing substitutions in the same protein. These substitutions provided a stability buffer and therefore permitted the fixation of mutations with destabilizing effects.

Figure 1



Amino acid states that are allowable in one genetic background may be deleterious in other backgrounds. **(a)** Two species diverge from a common ancestor with the two-site genotype, *ab*. The substitution *a*→*A* occurs at the first site in species 1 (yielding *Ab*) and the substitution *b*→*B* occurs at the second site in species 2 (yielding *aB*). A negative epistatic interaction ('Dobzhanksy-Müller imcompatibility') is revealed by moving mutation *A* from species 1 into the orthologous background of species 2, or by moving mutation *B* from species 2 into the orthologous background of species 1. Mutations *A* and *B* are individually neutral on the genetic backgrounds in which they occurred during evolution, but they are deleterious in combination. Swapping mutations *a* or *b* to form genotype *ab* results in a reversion to the ancestral state. **(b)** The same type of incompatibility can arise if both substitutions occur in one lineage, while the other species retains the ancestral states at both sites. In this case, swapping mutations *a* or *B* yields a low-fitness genotype (*aB*). Swapping mutations *A* or *b* yields genotype *Ab*, one of two possible mutational intermediates in the ancestry of species 1. Note that since mutations *a* and *B* are deleterious in combination, substitution *a*→*A* must have preceded substitution *b*→*B* in the ancestry of species 1 because *Ab* is the only viable single-mutant intermediate connecting the ancestral (*ab*) and descendant (*AB*) genotypes.

As in the Lunzer *et al.* [28] study on IMDH orthologs, the identified compensatory interactions in influenza nucleoprotein involved pairs of structurally remote residues. The permissive mutations did not directly alter the effects of destabilizing mutations; instead, they simply increased overall thermal stability so that the destabilizing mutations did not cause the fraction of folded protein to fall below the critical threshold where the assayed functional property (viral RNA transcription) was compromised. This illustrates how mutations that have additive effects on structural properties can have nonadditive effects on higher-level properties due to nonlinear relationships between structure and function or between function and fitness [18•,19,22,29–31].

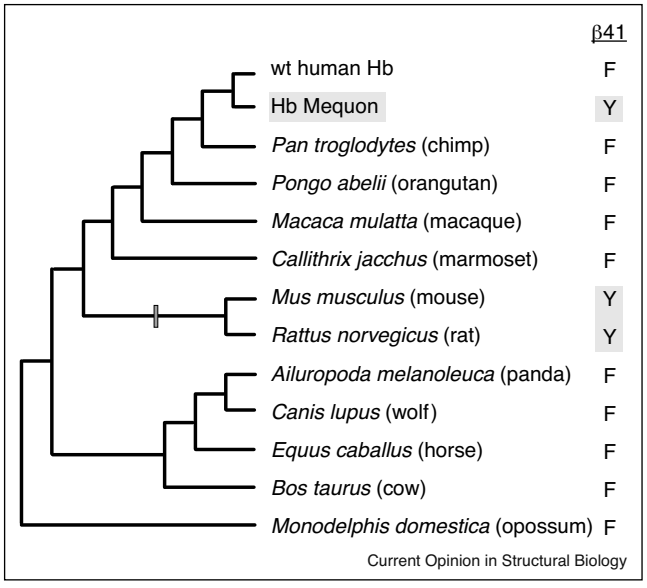
In summary, neutral or nearly neutral mutations that fix in one species may have deleterious effects if they were to

occur on the divergent genetic background of a different species. Likewise, within a single line of descent, substitutions may have different phenotypic effects depending on the sequential order in which they occur. Site-directed mutagenesis experiments that swap residues between orthologs of contemporary species [28,32,33] or between ancestral and descendant sequences [9,17,18•,34–37] have revealed pervasive epistasis for functional properties or fitness proxies. These experimental findings are generally consistent with results of theoretical and computational analyses [38–41,42•,43,44,45•,46] and *in silico* simulations [47,48••]. By contrast, other experimental studies that focused on mutational perturbations of structural stability have suggested a less important role for epistasis during longterm protein evolution [49–51].

Genetic compensation of pathogenic mutations

Additional insights into the prevalence of epistasis and the nature of genetic compensation are provided by cases where a pathogenic amino acid mutation in a human protein appears as the wildtype residue at the same site in the orthologous protein of one or more nonhuman species [52] (Figure 2). In such cases, the pathogenic variant is invariably present at low frequency in the human gene pool, but the same amino acid is fixed (present at a frequency of 1.0) in the nonhuman species. In order for the disease-associated residue (DAR) to become fixed in the nonhuman species, its deleterious effects must have been compensated by one or more substitutions at other sites in the same protein or in an interacting protein. This permits an indirect inference of sign epistasis for fitness: the DAR produces a deleterious effect in the human protein, but is neutral in the nonhuman ortholog due to genetic compensation [52–58,59•].

Figure 2



Indirect evidence for genetic compensation is provided by cases where a pathogenic amino acid mutation in a human protein appears as the wildtype residue at the same site in the orthologous protein of one or more nonhuman species. The pathogenic hemoglobin (Hb) mutant, Hb Mequon (β41Phe→Tyr), provides an illustrative example. Although the Hb Mequon mutation is associated with severe hemolytic anemia in humans, the disease-associated β41Tyr is wildtype in *Mus* and *Rattus*. In order for the Tyr variant to become fixed in the common ancestor of these rodent taxa, the deleterious effects that are manifest in human Hb must have been compensated by one or more rodent-specific substitutions at other sites in the same protein.

The pathogenicity of amino acid mutations often stems from their negative effects on protein structural stability [60], and evidence suggests that the destabilizing effects of such DARs are sometimes partly or wholly compensated by substitutions at structurally proximal residue positions [52,55,56,58,59•,61,62]. For example, Xu and Zhang [58] demonstrated that uncompensated DARs in human proteins are associated with lower average structural stabilities than the corresponding wildtype DARs in cases where the latter are accompanied by one or more lineage-specific substitutions at residue positions within a 4 Å radius of the DAR. This finding suggests that one or more of the observed substitutions in the nonhuman background compensate for the destabilizing effect of the DAR.

In cases where compensatory substitutions have not been identified, there are other possible explanations for the existence of wild-type DARs that do not invoke sign epistasis for fitness [54]. For example, the observation that a DAR is wildtype in a given species does not rule out the possibility that it has a mildly deleterious effect, as such mutations can fix due to drift, especially in small or bottlenecked populations [63]. This is unlikely to be a general explanation, however, especially in cases where the wildtype DAR is shared by multiple species within the same clade. A number of wildtype DARs have been documented in laboratory mice (*Mus domesticus*)[53], but the majority of these are shared by multiple species of *Mus* that diverged over one million years ago, so they clearly do not represent deleterious variants that were fixed as a result of founder effects during the history of mouse breeding and domestication [54].

Rather than using the identification of wildtype DARs to indirectly infer sign epistasis for fitness, a similar approach can be used to infer sign epistasis for biochemical phenotypes without making assumptions about fitness effects. This is possible in cases where we have detailed information about structure–function relationships. For example, in the case of human hemoglobin (Hb), crystallographic and NMR studies of mutant Hbs have provided exquisitely detailed insights into the structural mechanisms responsible for observed functional effects of specific amino acid replacements. In cases where a particular mutation in human Hb is known to alter a particular functional property, it is often possible to

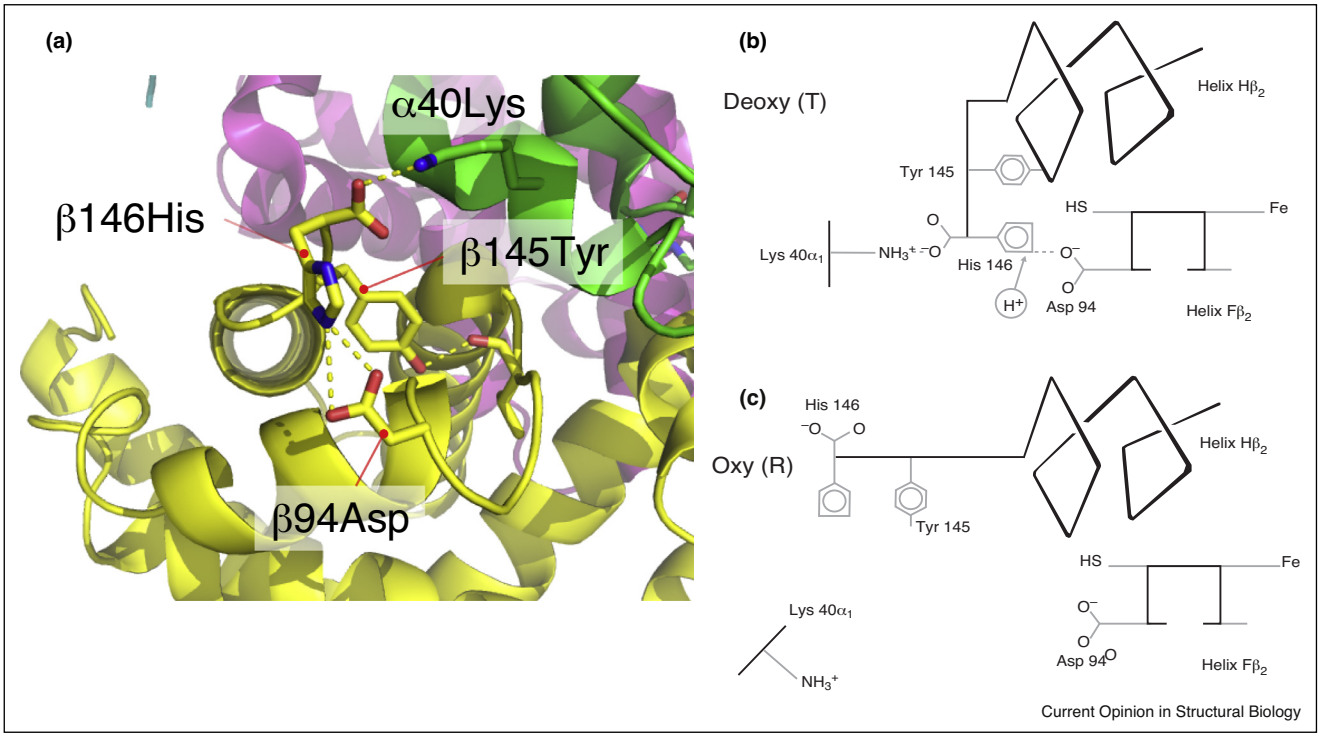
identify Hbs from nonhuman species in which the same amino acid is wildtype and yet the property of interest is not altered in the same way. In such cases the connection between genotype and biochemical phenotype can be experimentally tested. By contrast, associations with disease states do not generally permit direct insights into the mapping functions that relate genotype to phenotype or phenotype to fitness.

Molecular basis of the Bohr effect: a case study of genetic compensation

The Hbs of jawed vertebrates are heterotetramers, composed of paired αβ dimers ($\alpha_2\beta_2$) that undergo a symmetrical rotation during oxygenation-linked transitions in quaternary structure [64]. This allosteric transition is mediated by a conformational equilibrium between the low-affinity, deoxygenated 'T state' and the high-affinity, oxygenated 'R state'. Hb-O_2 affinity is reduced at low pH because protons preferentially bind and stabilize deoxyHb, thereby shifting the allosteric equilibrium in favor of the low-affinity T conformation [65]. Because Hb-O_2 affinity decreases with reductions in pH over the physiological range (6.6–7.6), the metabolic acidosis of capillary blood induces Hb to release O_2 to the tissues that need it most. This pH-sensitivity of Hb-O_2 affinity is known as the Bohr effect.

At physiological pH and temperature, the Bohr effect of human Hb is mainly attributable to the oxygenation-linked deprotonation of surface histidines because their imidazole side chains typically have acid dissociation constants, pK_a's, in the physiological pH range [66]. The C-terminal histidine of the β-chain, β146His, makes an outsized contribution, accounting for ~60% of the Bohr effect in the presence of 0.1 M chloride [67]. In deoxy (T state) Hb, the positive charge on the imidazole sidechain of β146His is stabilized by formation of a salt bridge with the carbonyl group of β94Asp in the same β-chain subunit (Figure 3). This ionization of the β146His side chain substantially raises its pK_a in the deoxy T state. Consequently, mutational replacements of either β146His or β94Asp result in a severely diminished Bohr effect because the β94Asp-β146His salt-bridge in the T state is replaced by an unionizable hydrogen bond; thus, no protons are released in the allosteric T→R transition in quaternary structure. Surprisingly, substitutions at these highly conserved residue positions have been identified in the Hbs of several

Figure 3

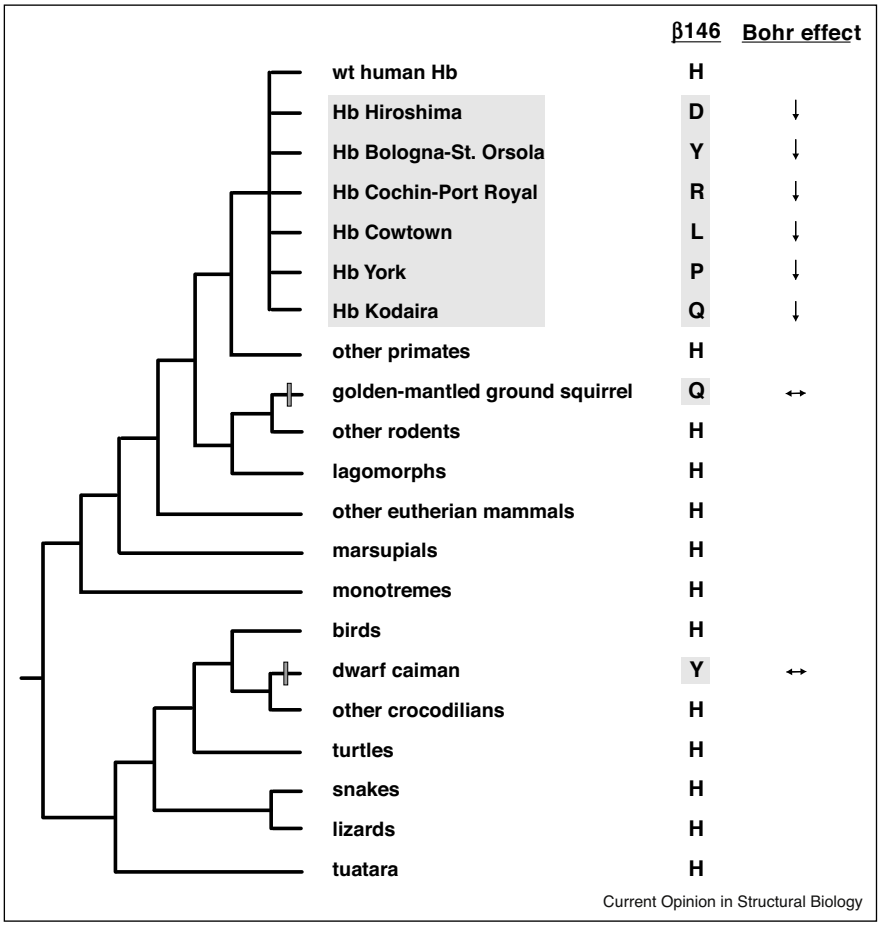


The structural basis for the Bohr effect in tetrameric ($\alpha_2\beta_2$) human Hb. **(a, b)** The C-terminal His of each β-chain subunit (β146His) participates in two electrostatic interactions in the deoxy (T) state. The positively charged imidazole side chain of β146His forms an intrasubunit salt bridge with β94Asp (which increases its pK_a) and its negatively charged carboxyl group forms an intradimer salt bridge with α40Lys. **(c)** When Hb is oxygenated, the allosteric transition in quaternary structure shifts the triad of residues apart from one another, outside the range of electrostatic interaction. The consequent rupturing of the β146His-β94Asp salt bridge results in the deprotonation of the His side chain (two protons are released per tetramer), which makes a major contribution to the Bohr effect.

vertebrate species that do not have reduced Bohr effects [68–70]. For example, human Hb mutants such as Hb Bologna-St. Orsola (β146His→Tyr) and Hb Kodaira (β146His→Gln) exhibit increased O_2-affinities (due to destabilization of the T-state) and severe reductions in the Bohr effect [71,72]. Remarkably, the same amino acid states are observed as wildtype in the adult Hbs of the dwarf caiman (*Paleosuchus palpebrosus*) (β146Tyr) and the golden-mantled ground squirrel (*Callospermophilus lateralis*) (β146Gln), and yet the Hbs of both species exhibit Bohr effects that are undiminished relative to normal human Hb [68,69] (Figure 4). In the case of both caiman and ground-squirrel Hb, the loss of a single key residue with a major effect on pH-sensitivity, β146His, appears to be compensated by the lineage-specific gain of multiple solvent-exposed, titratable histidines with individually minor effects [68,69].

In the case of β94Asp, human Hb mutants such as Hb Barcelona (β94Asp→His) and Hb Bunbury (β94Asp→Asn) also exhibit marked increases in O_2-affinity and concomitant reductions in the Bohr effect due to the disruption of the β94Asp-β146His intrachain salt bridge. In two different species of high-altitude Andean waterfowl (crested duck [*Lophonetta specularioides*] and Puna teal [*Anas puna*]), β94Asp→Glu mutations have contributed to adaptive increases in Hb-O_2 affinity, but the Bohr effect is not compromised relative

Figure 4



In the Hbs of amniote vertebrates, comparative sequence analysis and experimental data on structure–function relationships reveal intramolecular epistasis for the Bohr effect. In the β-chain subunit of vertebrate Hb, the highly conserved β146His generally accounts for a major fraction of the Bohr effect [66,67,71,72]. This is well-documented by experimental studies of naturally occurring human Hb mutants which demonstrate that mutational replacements of β146His (H) with Asp (D), Tyr (Y), Arg (R), Leu (L), Pro (P), or Gln (Q) invariably result in a severely diminished Bohr effect. Surprisingly, however, two of these amino acid states, Q (Hb Kodaira) and Y (Hb Bologna-St. Orsola), occur as wildtype in the Hbs of two nonhuman vertebrates, golden-mantled ground squirrel (*Callospermophilus lateralis*) and dwarf caiman (*Paleosuchus palpebrosus*), respectively, and yet the Hbs of both species exhibit Bohr effects that are undiminished relative to normal human Hb [68,69]. In both species, the aggregate effect of other lineage-specific substitutions (e.g. gains of solvent-exposed histidines at other sites in the α-chains and/or β-chains of the Hb tetramer) may have rendered β146His redundant with respect to oxygenation-linked proton binding, so it could therefore be replaced without unduly compromising the Bohr effect.

to wildtype Hbs with the ancestral β94Asp [70]. A consideration of the crystal structure of avian Hb provides a clear explanation for this result [67], as the β94Asp-β146His salt bridge is not formed in the deoxy T conformation, so the amino acid state of β94 does not affect the pK_a of β146His. This may also explain why the β146His→Tyr substitution in dwarf caiman Hb is not associated with a diminished Bohr effect relative to the Hbs of other crocodilians, and demonstrates how 'major effect' Bohr groups in human Hb may have minor or nonexistent effects in the Hbs of other species. These examples also illustrate how subtle changes in the three-dimensional orientation of highly conserved amino acids (caused by substitutions at other sites) can alter the functional effects of substitutions at those conserved sites [73].

Conclusions and future directions

Efforts to elucidate mechanisms of epistasis represent a nexus between the fields of structural biology and molecular evolution [1,5,74]. An especially important question is whether permissive/compensatory mutational effects are typically localized and specific, or whether they typically involve generalized effects on global properties such as structural stability. If function-altering mutations can only be compensated by mutations with localized and specific effects (e.g. via direct steric or electrostatic side-chain interactions between structurally proximal residues), then accessible mutational pathways to novel functions may be fortuitously contingent on the acquisition of exceedingly rare mutations [7•,25]. By contrast, there is a much larger mutational target size for perturbations of overall structural stability, so destabilizing, function-altering mutations may be effectively compensated by stabilizing mutations at many possible residue positions in the same protein, and the compensatory effect would not require direct site–site interaction. If deleterious pleiotropic effects of adaptive, function-altering mutations can be effectively compensated by such global suppressor mutations, then the optimizing power of selection should be considerably less constrained and evolutionary outcomes will be less strongly contingent on ancestral starting points.

Conflict of interest statement

The author declares no conflict of interest.

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- •• of outstanding interest

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