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The ups and downs of amino acid co-evolution: evolutionary Stokes and anti-Stokes shifts

Noor Youssef, Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada

Edward Susko, Department of Mathematics and Statistics, Dalhousie University, Halifax, Nova Scotia, Canada

Andrew J. Roger, Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, Canada

Joseph P. Bielawski, Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada
Department of Mathematics and Statistics, Dalhousie University, Halifax, Nova Scotia, Canada

Corresponding Author: Noor Youssef, n.youssef@dal.ca

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Abstract

The most fundamental form of epistasis occurs between residues within a protein. Epistatic interactions can have significant consequences for evolutionary dynamics. For example, a substitution to a deleterious amino acid may be compensated for by replacements at other sites which increase its propensity (a function of its average fitness) over time - this is the evolutionary Stokes shift. We discovered that an opposite trend -the decrease in amino acid propensity with time- can also occur via the same epistatic dynamics. We define this novel and pervasive phenomenon as the evolutionary anti-Stokes shift. Our extensive simulations of three natural proteins show that evolutionary Stokes and anti-Stokes shifts occur with similar frequencies and magnitudes across the protein. This highlights that decreasing amino acid propensities, on their own, are not conclusive evidence of adaptive responses to a changing environment. We find that stabilizing substitutions are often permissive (*i.e.*, expand potential evolutionary paths) whereas destabilizing substitutions are restrictive. We show how these dynamics explain the variations in amino acid propensities associated with both evolutionary shifts in propensities.

Introduction

Amino acid interactions within a single protein are the most fundamental form of epistasis. Epistatic interactions between sites can occur because of functional, structural, or stability constraints (Ortlund *et al.*, 2007; Pollock *et al.*, 2012; Gong *et al.*, 2013). Here we focus on the latter constraints on stability by using a model for protein evolution based on thermodynamic principles. This modeling framework has been shown to reproduce realistic evolutionary dynamics with regards to protein stability values (Goldstein, 2011), evolutionary rates (Youssef *et al.*, 2020), and convergence rates (Goldstein *et al.*, 2015). In this article, we explore the evolutionary dynamics that arise due to nonadaptive stability-

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23 constraints on proteins.

24 Under nonadaptive evolution, a protein evolves on a fixed fitness landscape with no changes in
25 environment or function (Wright, 1932). Natural selection plays an important role in maintaining
26 the protein near a peak on its fitness landscape. Evolutionary dynamics associated with a fitness
27 peak reflect the equilibrium between mutation, drift and selection. At equilibrium, most mutations
28 are deleterious while a small proportion may be beneficial. The higher probability of fixations of the
29 fewer but more advantageous mutations is balanced by the lower probability of fixations of the more
30 frequent yet disadvantageous mutations, resulting in an equal proportion of deleterious and beneficial
31 substitutions (*i.e.*, fixed mutations) at equilibrium (Goldstein, 2013; Cherry, 1998). This stands in
32 sharp contrast to the expected dynamics under adaptive evolution, where a change in protein function
33 or environment (and hence a change in the fitness landscape) renders the current state of the protein
34 suboptimal for the new conditions. The shift in landscape is followed by successive fixations of
35 beneficial substitutions as the protein adapts towards the new fitness peak (dos Reis, 2015; Jones
36 *et al.*, 2017).

37 Since its origin (Kimura, 1968), the strictly neutral model of protein evolution remains the most
38 frequently used null scenario that must first be rejected in order to postulate a history of adaptive
39 evolution (Kimura, 1968, 1991; Duret, 2008). Over the past decade, researchers have shown that
40 equilibrium dynamics under more complex models, informed by the selective constraints for protein-
41 stability, produce equilibrium dynamics that are largely consistent with neutral theory (*e.g.* with
42 regards to the distribution of mutational fitness effects (Goldstein, 2011)). Using these nonadaptive
43 stability-informed models, researchers have observed that various evolutionary phenomena charac-
44 teristic of natural proteins can arise without the need for invoking adaptive evolution. For example,
45 Goldstein (2011) used a thermodynamic model to argue that the marginal stability observed in many
46 natural proteins can emerge from a simple balance between mutation, drift, and selection, challenging

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47 the widely held notion that evolution actively selects for marginal stability (DePristo *et al.*, 2005).

48 Additionally, within the same modeling framework, Pollock *et al.* (2012) observed that the propen-
49 sity for a resident amino acid at a site tends to increase over time due to compensatory substitutions
50 at other sites in the protein. (In this context, propensity is the frequency of an amino acid arising
51 at a site for a fixed background sequence.) They referred to this phenomenon as the evolutionary
52 Stokes shift. Risso *et al.* (2014) found empirical evidence of evolutionary Stokes shifts in the evolu-
53 tion of thioredoxins and β-lactamases; however, those shifts in propensities were minor. Interestingly,
54 Popova *et al.* (2019) recently observed the opposite trend where the fitness of the resident amino acids
55 decreased with time - they termed this phenomenon "senescence". Popova *et al.* (2019) contend that
56 the decrease in preferences must be the result of adaptive protein evolution, and that, unlike the evo-
57 lutionary Stokes shifts, mere epistatic constraints "cannot lead to a systematic reduction in fitness
58 of the incumbent alleles".

59 Do resident amino acid preferences tend to increase, decrease, or remain relatively conserved
60 throughout a protein's evolution? And to what extent are they shaped by adaptive or nonadap-
61 tive processes? Using extensive simulations under a thermodynamic model for protein stability, we
62 show that all three trajectories emerge from the nonadaptive dynamics at mutation-drift-selection
63 equilibrium. Importantly, we describe a novel phenomenon whereby resident amino acid preferences
64 can decrease merely due to epistatic constraints – which we call the evolutionary anti-Stokes shift.
65 We then show that evolutionary anti-Stokes shifts are as common as Stokes shifts, and character-
66 ize the underlying mechanisms that give rise to them. In line with experimental evidence (Gong
67 *et al.*, 2013) we found that stabilizing substitutions are permissive (*i.e.*, expand potential evolution-
68 ary paths) whereas destabilizing substitutions are restrictive. We show how these dynamics explain
69 the variations in amino acid propensities associated with evolutionary Stokes and anti-Stoke shifts.

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Results

We use a thermodynamic model of protein evolution where fitness is equal to the probability of observing an amino acid sequence in a native protein structure at equilibrium (which is a function of its stability, ΔG). We assume no changes in protein structure or function so that the global fitness landscape (*i.e* the mapping between amino acid sequences and fitness values) remains constant. Nonetheless, this modeling framework accounts for stability-mediated epistasis by permitting differences in the marginal site-specific fitness landscapes depending on the residues occupying other sites in the protein (i.e. the background protein sequence). Throughout the simulations, we calculate the site-specific fitness landscape ($f^h(s) = \{f_1^h(s), ..., f_{20}^h(s)\}$ for a given site h and background sequence s), at all sites and given all observed sequences. Amino acids that confer higher fitness values (improve stability) will tend to more frequently occupy the site and will, therefore, have higher expected frequencies at equilibrium for a given background sequence. In this way, the frequency of an amino acid is related to its fitness. As a result, frequency landscapes are similarly site-specific and context-dependent ($\pi^h(s) = \{\pi_1^h(s), ..., \pi_{20}^h(s)\}$). Note, however, that the fittest amino acid may not necessarily be the most frequently observed residue. This can occur when a suboptimal amino acid has many codon aliases - the high number of synonymous codons and/or mutational bias can increase the propensity for the residue despite having slightly lower fitness.

An evolutionary Stokes shift is a phenomenon whereby the propensity (π_a^h) for the resident amino acid at that site increases over time due to compensatory substitutions at other positions in the protein (Pollock *et al.*, 2012). The propensity for an amino acid is its equilibrium frequency given a fixed background protein sequence,

$$\pi_a^h(s) = \pi_a^{(0)} e^{2N_e f_a^h(s)} / \sum_x \pi_x^{(0)} e^{2N_e f_x^h(s)} \tag{1}$$

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91 where N_e is the effective population size and $\pi_a^{(0)}$ is the stationary frequency of amino acid a in the
92 absence of selection pressure (dos Reis, 2015). In their formulation, Pollock *et al.* (2012) assume that
93 $\pi_a^{(0)}$ are uniform ($\pi_a^{(0)} = 1/20$). This would be true if all amino acids were specified by the same number
94 of codons and there were no underlying mutational biases. However, our simulations are based on
95 three proteins with DNA mutation parameters estimated from multiple sequence alignments of their
96 natural codon sequences. Importantly, analyses of the three protein genes suggest the presence of
97 mutational biases (with unequal nucleotide frequencies, and transition/transversion rate biases). We
98 account for these mutational biases by estimating protein-specific $\pi_a^{(0)}$ given the estimated mutation
99 parameters for each protein (figure S1). The following results are based on 500 protein-specific
100 simulations for three proteins with PDB structures 1qhw, 2ppn, and 1pek (see Methods). We ran
101 each simulation for 500 substitutions.

102 Both evolutionary Stokes and anti-Stokes shifts emerge from nonadaptive 103 stability-constraints on protein fitness

104 Throughout our simulations, and in real protein evolution (Risso *et al.*, 2014; Gong *et al.*, 2013;
105 Ashenberg *et al.*, 2013), the propensity for certain amino acids relative to other amino acids changes
106 over time. In natural proteins, these variations may be due to global constraints on protein stability,
107 or are related to functional restrictions. By contrast, the fitness model we employ assumes selection
108 acting only on protein stability. Therefore, any variations in sites' fitness and propensity landscapes
109 are solely due to stability-induced epistatic interactions between sites (and not due to external changes
110 in environment or function). Examples of these propensity dynamics from a simulation of the 1pek
111 protein are shown in figure 1. The propensity for aspartic acid (D), the resident amino acid at site
112 232, changes considerably as substitutions occur at other positions in the protein (figure 1A). In this
113 case, the site experiences an evolutionary Stokes shift where its propensity increases over time due to

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114 compensatory substitutions at other positions (figure 1A). In contrast, within the same simulation, 115 the propensity for proline (P), the resident amino acid at site 72, decreases as substitutions occur at 116 other positions (figure 1B). We refer to this phenomenon as the evolutionary anti-Stokes shift.

117 Changes in amino acid propensities are not directly observable in natural proteins. However, 118 Popova *et al.* (2019) recently put forward that sustained increases (or decreases) in propensities may 119 produce a detectable signal in natural protein alignments by causing a change in the rate of amino 120 acid replacement. They suggest that the propensity of an amino acid is inversely related to its rate 121 of replacement: if the propensity for the resident amino acid is high, then the rate of replacement 122 should be low, and vice versa. Therefore, in addition to the amino acid propensities, we calculated 123 the expected rate of replacement (the sum of the substitution rates to all single step neighbouring 124 sequences that differ from the current sequence at the site of interest). Figure 1C and 1D confirms 125 the predicted effect on the change in the rate of leaving the resident amino acids at sites 232 and 126 72. For site 232, the increase in propensity with time (*i.e.*, evolutionary Stokes shift) is accompanied 127 by a decrease in the replacement rate (figure 1A&C). Similarly, the decrease in resident amino acid 128 propensity at site 72 (*i.e.*, evolutionary anti-Stokes shift), is accompanied by an increase in the rate 129 of leaving (figure 1B&D). Importantly, these dynamics arose in a model where no adaptive changes 130 are occurring. In other words, the proteins are evolving at mutation-drift-selection equilibrium with 131 no change in protein structure or function. This shows that neither the evolutionary Stokes *nor* the 132 anti-Stokes shift depends on any external change in selection on protein function or environment.

133 Evolutionary anti-Stokes shifts are common under non-adaptive evolution

134 The previous result shows that the evolutionary anti-Stokes shift can occur without a change in 135 protein function. But, as the anti-Stokes shift is a newly described phenomenon, it is unclear whether 136 it is widespread or rare in natural proteins; Do evolutionary Stokes and anti-Stokes shifts occur with

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137 similar frequencies? To address this, we developed four metrics for quantifying these two phenomena.
138 The metrics are described in detail in the Methods section and illustrated in figure 2. Briefly, the
139 first metric (M1) is the slope of the linear model where x is time (measured in substitutions) and y
140 is the propensity of the resident amino acid a at site h (π_a^h). The slope is calculated over the amino
141 acid residence time (*i.e.*, from $i \leq x \leq j$, where i is the substitution when amino acid a first occupies
142 the site and j is the last substitution). Metric two (M2) is the average change in the propensity
143 of the resident amino acid over its residency time. As such, metrics M1 and M2 measure the *rate*
144 at which propensities change over the time period where an amino acid is resident. In addition, we
145 calculate a third metric (M3) - the difference in the average propensity of an amino acid while it is
146 resident and the propensity of the same amino acid when it was first accepted- which estimates the
147 *magnitude* of the change in propensity. Values of M1-3 greater than zero indicate an evolutionary
148 Stokes shift, while values less than zero indicate an evolutionary anti-Stokes shift. Lastly, we define
149 a more conservative metric, M4, which classifies an evolutionary Stokes shift only when M1-3 are all
150 > 0, and an anti-Stokes shift when M1-3 are all < 0.

151 We found that an evolutionary anti-Stokes shift occurs following approximately half of all sub-
152 stitutions. The estimated proportion of substituted amino acids which experienced an evolutionary
153 anti-Stokes shift ($P_{anti-Stokes}$) were consistent across simulations under different protein structures
154 and mutation models (Table S1). The consistency in $P_{anti-Stokes}$ suggests that protein structures and
155 mutation biases are not major determinants of evolutionary shifts in propensities. The proportions of
156 $P_{anti-Stokes}$ ranged from 0.46 to 0.57 across metrics M1-3 (Figure 3A). However, $P_{anti-Stokes}$ estimated
157 based on metric M4 were significantly less than 0.5 which is expected because of the more conserva-
158 tive requirements under M4. Furthermore, we found that, across all metrics M1-4, the proportion of
159 substitutions that were followed by evolutionary anti-Stokes shifts ($P_{anti-Stokes}$) were approximately
160 equal to the proportion of substitutions that were followed by evolutionary Stokes shifts (P_{Stokes}),

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161 suggesting that both phenomena occur with comparable frequencies (Figure 3B).

162 Importantly, given the current formulations of the metrics, the estimates of both Stokes and anti-
163 Stokes shifts could be comparable while the underlying dynamics may be considerably different. For
164 example, the metrics would estimate similar values for the following scenarios: (1) a rapid increase
165 (or decrease) in amino acid propensity followed by a longer period where the propensity of the amino
166 acid remains high (or low), and (2) a more gradual increase (or decrease) in propensity over time.
167 It may be the case that evolutionary Stokes shifts tend to occur soon after the acceptance of the
168 substitution, while evolutionary anti-Stokes shifts may occur more gradually. To quantify whether
169 propensity changes accelerated or decelerated over time, we compared the value of each metric M1-3
170 calculated over the first half of the amino acid residency (we label this as MX_1) and the estimate over
171 the second half (MX_2), where X is either 1, 2, or 3 representing one of the three metrics. Specifically,
172 we calculated (MX_2 - MX_1) / T_{res} where T_{res} is the amino acid residency time (measured in number
173 of substitutions). We classified amino acids as undergoing an evolutionary Stokes or anti-Stokes shift
174 based on the conservative metric M4. Using Welch's t-test, we found significant differences in the
175 average rates of change between Stokes and anti-Stokes shifts; however, the effect sizes were negligible
176 (differences in means were ≤ 0.001, table S2).

177 Goldstein and Pollock (2017) observed that when a site experiences an evolutionary Stokes shift,
178 not only does the propensity for the resident amino acid increase, but so does the propensity for
179 physicochemically similar residues. For example, if V becomes newly resident at a site, then, over
180 time, the propensity for similar amino acids (e.g. L) will likewise increase. This leads us to the
181 question: does the decrease in propensity for the resident amino acid (an evolutionary anti-Stokes
182 shift) imply a decrease in the propensities for similar amino acids? To address this, we grouped amino
183 acids into bins of residues that tend to interchange rapidly and that tend to have similar chemical
184 properties: [AST], [C], [DE], [FY], [GN], [HQ], [IV], [KR], [LM], [P], [W] (Susko and Roger, 2007).

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185 Rather than evaluating how the propensity for an individual resident amino acid changed over time,
186 we tracked how the propensity of a group of amino acids changed (by summing the propensities of
187 all amino acids within the group). We then applied metrics M1-4 to the summed group propensity.
188 If evolutionary anti-Stokes shifts tend to affect individual amino acids, while Stokes shifts tend
189 to occur for similar amino acids, then we would expect $P_{anti-Stokes} \neq P_{Stokes}$ based on the group
190 analysis. However, we found that even when considering how the propensities for groups of similar
191 amino acids changed $P_{anti-Stokes}$ remained approximately equal to P_{Stokes} (figure S2). Overall, these
192 results suggest that evolutionary anti-Stokes shifts are as common as Stokes shifts, and have similar
193 dynamics.

194 Evolutionary Stokes and anti-Stokes shifts occur at exposed and buried 195 sites

196 It has long been observed that a site's location in the protein influences its evolutionary dynamics. For
197 globular proteins, surface residues are usually involved with protein function (*e.g.*, binding affinity,
198 enzymatic activity) with a preference for hydrophilic residues, while buried sites are usually occupied
199 by hydrophobic residues and tend to evolve much slower (Yeh *et al.*, 2013; Shahmoradi *et al.*, 2014;
200 Marcos and Echave, 2015; Echave *et al.*, 2016). Popova *et al.* (2019) recently suggested that buried
201 sites are more likely to undergo evolutionary Stokes shifts because of stability-constraints, while
202 exposed sites are prone to senescence (decreases in amino acid favorability with time) due to changes
203 in the environment external to the protein. We have shown that evolutionary anti-Stokes shifts can
204 occur due to epistatic stability constraints without any external environmental or functional changes.
205 Motivated by the results of Popova *et al.* (2019), we assessed whether some positions in the protein are
206 more susceptible to evolutionary Stokes shifts, whereas others are more likely to undergo evolutionary
207 anti-Stokes shifts. To do this, we examined the relationship between the metrics and two measures of

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208 the site's location in the protein: relative solvent accessibility (RSA) and weighted contact number
209 (WCN), both of which correlate significantly with substitutions rates in natural (Yeh *et al.*, 2013;
210 Shahmoradi *et al.*, 2014; Marcos and Echave, 2015) and simulated proteins (Youssef *et al.*, 2020).
211 Specifically, exposed sites have higher substitution rates, higher RSA, and lower WCN as compared
212 to buried sites. In line with these observations, we found a negative correlation between RSA and
213 average residency time, and a positive correlation between WCN and average residency time (figure
214 S3). However, we found no correlation between any of our metrics and RSA or WCN (figure S4 and
215 S5). In this context (c.f. Popova *et al.* (2019)), the lack of correlations suggests there is no innate
216 tendency for evolutionary Stokes or anti-Stokes shifts to be specifically associated with the location
217 of sites in a protein.

218 While all sites are equally susceptible to undergoing evolutionary Stokes or anti-Stokes shifts,
219 it remained unclear if the entailed dynamics were comparable. We were interested in assessing
220 whether the site's location in the protein might influence the rate of propensity changes. For example,
221 if a deleterious substitution at a surface residue is easily compensated for (by adjustments at a
222 small number of interacting sites), then we might expect a rapid increase in the resident amino
223 acid propensity, and therefore a rapid evolutionary Stokes shift. Alternatively, if the site is highly
224 connected, then the fixation of a deleterious amino acid at that site might require more adjustments
225 at other positions, and, therefore, the Stokes shift might occur over a longer period of time. Across
226 all metrics, we found no correlation between RSA and rate of propensity changes (figure S6) and,
227 similarly, no correlation between WCN and rate of propensity changes (figure S7). This suggests that
228 both exposed and buried sites undergo evolutionary Stokes and anti-Stokes shifts with comparable
229 dynamics.

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230 # Propensity shifts are consistent with random fluctuations

231 In the absence of selection, all mutations are neutral and are fixed (or lost) by the action of genetic
232 drift, resulting in propensities that vary randomly over time (Wright, 1929). In contrast, our simula-
233 tions take into account the action of selection with mutations conferring different fitness effects and,
234 therefore, different fixation probabilities. Nonetheless, consistent with Goldstein (2011), we observed
235 that approximately 90% of fixed nonsynonymous substitutions in our simulations were effectively
236 neutral (i.e. where the selection coefficient from a current state i to a mutation j is $|s_{ij}| < 1/2N_e$;
237 table S3). The high proportion of neutral fixations suggests that substitutions, and hence propensity
238 fluctuations, are mainly driven by random genetic drift. Furthermore, the smooth symmetric M1-3
239 distributions centered at zero (figure 3C) are suggestive of random fluctuations in propensities. We
240 were therefore interested in assessing if resident amino acid propensities tend to change randomly
241 or whether they undergo phases of systematic increase and decrease over time. To address this, we
242 conducted a mixed model analysis with a null model assuming random changes in propensities (see
243 Methods). We did not find any evidence to reject the null random walk model. Interestingly, however,
244 *all* p-values were > 0.95. This result is surprising since if propensities were varying randomly over
245 time, then some sites should have rejected the null model just by chance. A potential explanation
246 for the lack of fit (excess of high p-values), is if propensities changes were autocorrelated. Indeed, we
247 observed a substantial negative autocorrelation in the differences in $\pi_a^h(s_x)$ and $\pi_a^h(s_{x+1})$ (table S4),
248 implying that an increase in propensity tends to be followed by a decrease (and vice versa). This is
249 perhaps not surprising since if the resident amino acid propensity decreases, then the site will either
250 substitute away from the current amino acid or replacements will occur in other parts of the proteins
251 increasing the propensity for that amino acid. Alternatively, as the propensity for a resident amino
252 acid increases, there will be fewer ways for it to increase further than for it to decrease (for example,
253 consider the dynamics when propensity is equal to one).

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254 Lastly, we were interested in assessing whether random fluctuations in propensities could result
255 in P_{Stokes} and $P_{anti-Stokes}$ comparable to those observed in our simulations. To do this, we simulated
256 500 bounded random walks (between 0 and 1) of amino acid propensities with step sizes drawn from a
257 normal distribution with mean (μ=0) and standard deviation (σ= 0.1) estimated from the step sizes
258 observed in the stability-constrained simulations (figure S8). Then, we applied metrics M1-4 to the
259 random walk simulations in order to estimate the proportion of evolutionary Stokes and anti-Stokes
260 expected when propensities vary randomly. We found that the proportion of evolutionary Stokes
261 and anti-Stokes shifts from stability-constrained simulations were consistent with the proportions
262 estimated under a bounded random walk model (figure S9). Overall, these results suggest that the
263 dynamics of propensities are likely not too different from a random walk at equilibrium, and that
264 random fluctuations in resident amino acid propensities will occasionally lead to increases in propen-
265 sities over time (evolutionary Stokes shifts) and decreases in propensities over time (evolutionary
266 anti-Stokes shifts).

267 Most stabilizing substitutions are permissive and most destabilizing sub-268 stitutions are restrictive

269 We have shown that the evolutionary Stokes and anti-Stokes shifts are both common within a system
270 evolving under selection for stability with no adaptive changes. We next turn to the underlying
271 mechanisms that give rise to these dynamics. Important questions about how substitutions impact
272 resident amino acids propensities remain unanswered: Do substitutions tend to favourably impact
273 some sites (by increasing their resident amino acid propensities) while simultaneously disadvantaging
274 other sites (by decreasing their resident amino acid propensities)? Or does a substitution tend to
275 impact the propensity of resident amino acids similarly across the protein? If so, what explains the
276 observed balance between P_{Stokes} and $P_{anti-Stokes}$?

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277 A consequence of stability-mediated epistasis is that any change in the protein sequence will cause
278 shifts in resident amino acid propensities at other sites. For example, when a substitution occurs,
279 so that the sequence changes from $s_x \rightarrow s_{x+1}$, the fitness and propensity landscapes at most other
280 sites in the protein will subsequently change. In figure 4A, the grey dots represent the change in the
281 propensity of the resident amino acid at each site following a substitution ($\Delta\pi_a^h = \pi_a^h(s_{x+1}) - \pi_a^h(s_x)$).
282 The red dots represent the change in the propensity of the resident amino acid at the substitution
283 site, and therefore a change in the resident amino acid from $a \rightarrow b$ ($\Delta\pi^h = \pi_b^h(s_{x+1}) - \pi_a^h(s_x)$). We
284 found that the effect of a substitution on resident amino acid propensities is usually unbalanced. In
285 other words, substitutions either favourably impact most sites (so that the proportion of sites with
286 negative $\Delta\pi_a^h$ is less than 0.5), or decrease the propensity of the resident amino acid at most sites
287 (so that the proportion of sites for with negative $\Delta\pi_a^h$ is greater than 0.5). Surprisingly, stabilizing
288 substitutions ($\Delta\Delta G < 0$) were associated with decreases in propensities of resident amino acids at
289 most sites, while destabilizing substitutions ($\Delta\Delta G > 0$) rendered the resident amino acids at most
290 sites more favorable (figure 4B).

291 This result initially appears counter-intuitive. To clarify, let us consider examples of the dynamics
292 following the fixation of a stabilizing and a destabilizing substitution observed within our simulations
293 (figure 5). First, consider the dynamics following a stabilizing substitution. Let s_1 be the initial
294 protein sequence with fitness = 0.999 and stability (-$\Delta G = 4.041$; figure 5A). A substitution occurred
295 so that the sequence changed from $s_1 \rightarrow s_2$, that increased the overall stability of the protein with
296 a minor improvement in fitness. As a result of this substitution, the fitness landscapes at most sites
297 changed. We focus on the fitness landscape at site 145 before (s_1) and after (s_2) the change in the
298 background sequence. Given that sequence s_2 is more stable, a destabilizing mutation at site 145 has
299 relatively lower impact on the fraction of correctly folded proteins at equilibrium (i.e., fitness). Thus,
300 the effect of the background sequence having higher stability is that the fitness landscape at site 145

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301 becomes more uniform (figure 5B). How does the change in the fitness landscape induce a change in
302 the propensity of the resident amino acid? Since a larger number of amino acids can now occupy
303 the site with little or no detriment to protein fitness, the propensity landscape will similarly become
304 more uniform (figure 5C). Amino acids like R, N, and P that had low propensity in the context
305 of background sequence s_1, are more likely to be observed given the "stability-buffered" sequence
306 s_2 (figure 5C). Propensities are the expected equilibrium frequencies of each amino acid given the
307 current background sequence; they must, therefore, sum to one. The increase in the propensity of
308 some amino acids (*e.g.*, R, N, and P) will cause a decrease in the propensity of the resident amino
309 acid (K in this example; figure 5). The opposite relationship was observed following the fixation
310 of a destabilizing mutation (figure 5D): The fitness and propensity landscapes became less uniform
311 (figure 5E&F), with fewer amino acids having non-zero propensities. This resulted in an increase in
312 the propensity for the resident amino acid following the destabilizing substitution (figure 5F).

313 To quantify the uniformity of a landscape, we calculated its Shannon entropy $\mathrm{H}^h(s)$ (see Methods
314 section for detail). Entropy is maximized when the landscape is uniform (*i.e.*, all amino acids have
315 equal frequencies), and is at a minimum (= 0) when only one amino acid is observed at the site.
316 Note that entropy of fitness and propensity landscapes are highly correlated (figure S10). The fitness
317 landscape describes fitnesses of nearby sequences, whereas the propensity landscape considers how
318 frequently nearby sequences are explored via mutation and substitution. We, therefore, report the
319 entropy of the propensity landscapes, although similar results are expected based on the fitness
320 landscapes. We found that at higher stability values (lower ΔG) the propensity landscapes at most
321 sites tend to be more uniform compared to at lower stability values (figure 6A). This suggests that as
322 the protein becomes more stable, most amino acids tend to have similar impacts on fitness and are,
323 therefore, equally likely to be observed at a site. Next, we were interested in assessing the impact of a
324 substitution on the uniformity of the landscapes at other sites in the protein. To do this, we calculated

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325 the change in landscape entropy following the acceptance of a substitution, $\Delta H^h = H^h(s_{x+1})$ - $H^h(s_x)$.
326 A change from a more uniform to a more rugged landscape (where a smaller number of amino acids
327 have non-zero propensities) will result in a negative ΔH^h. In contrast, a positive ΔH^h indicates a
328 change towards a more uniform landscape. We considered a substitution as permissive if it causes
329 a flattening in the propensity landscape at most sites (*i.e.*, a positive average ΔH). A restrictive
330 substitution is one where following its acceptance, the landscapes at most sites permit fewer amino
331 acids (*i.e.*, a negative average ΔH). We found a strong positive correlation between the stability effect
332 of a substitution ($\Delta\Delta G$) and its influence on the fitness landscapes at other sites (figure 6B). Figure
333 6C reports the average proportions of the different types of substitutions estimated based on three
334 protein-specific simulations. Consistent with the results reported in figure 5, stabilizing substitutions
335 - by increasing the overall stability of the protein - provide a "stability-buffered" background so
336 that slightly destabilizing substitutions are more likely to be fixed, expanding the space of potential
337 evolutionary paths. In contrast, destabilizing substitutions restrict potential evolutionary paths.
338 These results are in line with experimental work by Gong *et al.* (2013), which found that stabilizing
339 substitutions permit otherwise inaccessible mutations.

340 These results are also consistent with previous theoretical predictions by Cherry (1998), and
341 Goldstein (2011). Cherry (1998) observed that, given a saturating fitness function, the distribution
342 of potential mutational fitness effects ($s_{ij} = f_j - f_i$ where i and j are the wildtype and mutant alleles
343 respectively) will be related to the current fitness of the organism (or protein): the distribution of
344 fitness effects broadens as fitness decreases. In the model used here, fitness (the probability of folding)
345 is a saturating function of protein stability (ΔG, equation 4). We find that at higher stability values
346 (lower ΔG) the propensity landscapes tend to be more uniform (figure 6A). This suggests that the
347 magnitude of the fitness effects for potential mutations are smaller at higher stability values compared
348 to the distribution when the background sequence is less stable. Second is the prediction that at equi-

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349 librium the proportions of deleterious substitutions will be balanced by the proportion of beneficial
350 substitutions. We found that the proportion of stabilizing (beneficial) substitutions balanced those
351 that were destabilizing (deleterious) in our simulations (figure 6B). Taken together, these predictions
352 shed light onto the observed balance between P_{Stokes} and $P_{anti-Stokes}$. At mutation-drift-selection
353 equilibrium we expect that $P_{stabilizing} = P_{destabilizing}$ (figure 6B; Cherry (1998); Goldstein (2011)).
354 We have shown that stabilizing substitutions result in a decrease in the propensity of the resident
355 amino acids at most sites ($\Delta\pi_a^h < 0$, because of a flattening in the site-specific fitness landscapes),
356 while destabilizing substitutions increase resident amino acids propensities at most sites ($\Delta\pi_a^h > 0$,
357 figure 5 & 6A). This suggests that the proportion of $\Delta\pi_a^h < 0$ should be equal to the proportion of
358 $\Delta\pi_a^h > 0$ (figure S8). Since evolutionary Stokes and anti-Stokes shifts are the result of such changes
359 in propensities, we would expect that at equilibrium $P_{Stokes} \approx P_{anti-Stokes}$.

360 Discussion

361 We have examined evolutionary dynamics under a nonadaptive stability-constrained model of protein
362 evolution. Consistent with previous observations, we found that as proteins become more stable
363 (lower ΔG values), the fitness effects of most mutations diminished compared to the fitness effects
364 of the same mutation at lower stability (higher ΔG values). This suggests that substitutions which
365 increase stability will make more mutations accessible to the protein, thereby expanding the space
366 of potential evolutionary paths. In contrast, destabilizing substitutions will tend to limit potential
367 evolutionary trajectories.

368 Previous studies observed that functionally important residues are often destabilizing (Schreiber
369 *et al.*, 1994; Nagatani *et al.*, 2007; Wang *et al.*, 2002; DePristo *et al.*, 2005), suggesting a trade-off
370 between function and stability. This leads to the hypothesis that highly stable proteins are more

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371 readily adaptable to new functions compared to less stable proteins since they are more likely to
372 accept destabilizing yet functionally beneficial substitutions. We suggest that more stable proteins,
373 all other things being equal, may be more adaptable, not only because they can tolerate destabilizing
374 yet functionally beneficial substitutions, but also because they are more apt to explore neighboring
375 regions of sequence space. At low protein stability fewer mutational paths are permissible (since
376 most changes are deleterious), resulting in longer waiting times between substitutions and hence
377 fewer opportunities to explore sequence space and adapt to other functions. However, it is important
378 to note that selection on other properties of proteins, such as their expression level and the cost of
379 translation error (Drummond *et al.*, 2005), can also influence their rate of evolution. Therefore, the
380 relationship between evolvability and stability of proteins in nature is likely to reflect the complex
381 interplay of multiple factors.

382 As more (or fewer) mutations become accessible, the propensity (i.e., the equilibrium frequen-
383 cies given a particular background sequence) for the currently resident amino acid at a site will
384 consequently change. By expanding accessible paths, stabilizing substitutions tended to result in a
385 decrease in the propensity for the resident amino acids at most sites, while destabilizing substitutions
386 frequently increased propensities (figure 4B & 6A). Shifts in resident amino acid propensities were
387 occasionally consistent with an evolutionary Stokes shifts (Pollock *et al.*, 2012), where the propensity
388 for an amino acid increases over time due to compensatory adjustments at other sites in the protein.
389 In other instances, we observed the opposite trend where the propensity of a resident amino decreased
390 with time, an evolutionary anti-Stokes shift. In our simulations, both evolutionary Stokes and anti-
391 Stokes shifts were caused entirely by stability-mediated epistasis and not by any external changes
392 in protein function or environment. Alternatively, Popova *et al.* (2019) recently observed that the
393 fitness of the resident amino acid at a site may decrease with time since substitution. They attributed
394 the decrease in amino acid fitnesses to external changes in the protein's environment (*e.g.*, host im-

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395 mune response) and termed this trend senescence. This highlights the main difference between the
396 notion of an evolutionary anti-Stokes shift and the concept of senescence: evolutionary anti-Stokes
397 shifts are a result of non-adaptive processes mediated by the emergent property of marginal stability,
398 whereas senescence is a consequence of an adaptive response to some change in the protein's external
399 environment.

400 Alternatively, propensity shifts may be viewed as dynamics that arise due to the protein adapting
401 to internal, rather than external, changes. In this sense, neighbouring sites may "compensate for"
402 or "adapt to" a deleterious substitution that occurred at an interacting site. However, our results
403 challenge even this more localized interpretation of adaptation. Evolutionary dynamics at equilibrium
404 are predominantly driven by random drift where the vast majority of substitutions (approximately
405 90%, table S3) are nearly neutral with $|s_{ij}| < 1/2N_e$. If drift were the only evolutionary force acting on
406 a gene (e.g. pseudogene), then allele propensities are expected to vary randomly over time. We found
407 that propensity changes are largely consistent with random fluctuations in propensities that may
408 occasionally favour or disfavour a resident amino acid at a site. Furthermore, if evolutionary Stokes
409 shifts are the result of local co-adaptation, then we would expect a higher fraction of substitutions at
410 neighbouring sites compared to the proportion when an anti-Stokes shift occurs. However, we found
411 no evidence in support of this; the fraction of substitutions at neighbouring sites were comparable
412 for both evolutionary Stokes and anti-Stokes shifts (figure S11).

413 We see that a major advantage of the thermodynamic stability model used here, and previously
414 (Goldstein, 2011; Pollock *et al.*, 2012; Goldstein and Pollock, 2017), is that it provides a plausible
415 nonadaptive null model for protein evolution. Such stability-informed models of protein evolution
416 have previously been used to discredit adaptationist claims about the necessary trade-offs between
417 protein function and stability (Taverna and Goldstein, 2002; Goldstein, 2011), and protein function
418 and foldability (Govindarajan and Goldstein, 1996). "Despite the seduction of adaptive rationaliza-

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419 tions", to quote one of the original authors of this model, "neutral evolutionary dynamics remains the
420 null model that must first be rejected" (Goldstein, 2011). Our observation that amino acid propen-
421 sities may decrease over time in the absence of external environmental changes does not preclude
422 that environmental shifts could render resident amino acid less favourable. Rather our simulations
423 demonstrate that decreases in propensities can occur in the absence of environmental changes, and
424 therefore that their mere occurrence should not, on their own, be taken as conclusive evidence of
425 adaptations to external environmental changes.

426 Methods

427 Protein description

428 We simulated the evolution of three proteins with PDB codes 1qhw, 2ppn, and 1pek. These protein
429 structures are described in detail in Youssef *et al.* (2020). The proteins differ in structure, function,
430 length, and contact density. The 1qhw protein is a phosphatase, the 1pek protein is a proteinase,
431 and the 2ppn protein is an isomerase. The 1qhw protein is 300 amino acid residues long, 1pek is
432 made of 297 amino acids, and the 2ppn protein comprises 107 residues. The 1pek protein was the
433 most densely packed with an average number of contacts per site of 8.4 compared to 7.5 for the 1qhw
434 protein and 6.9 for the 2ppn protein. During the simulations, we used the nucleotide frequencies (π_n)
435 and transition/transversion rate (κ) parameters estimated from multiple sequence alignments for the
436 corresponding protein used in Youssef *et al.* (2020). The mutation parameters ($\kappa, \pi_A, \pi_C, \pi_G, \pi_T$)
437 were set equal to (4.37, 0.21, 0.32, 0.28, 0.20) for the 1qhw protein; (0.90, 0.19, 0.35, 0.56, 0.21) for
438 the 1pek protein; and (2.50, 0.27, 0.24, 0.29, 0.19) for the 2ppn protein.

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439 # Evolutionary model

440 The evolutionary process is based on the mutation-selection (MutSel) framework (Halpern and Bruno,
441 1998). We assume a Wright-Fisher population with fixed effective population size (N_e) evolving under
442 a weak mutation, strong selection regime so that only a single variant exists in the population at
443 any time point. The probability of a particular mutation b going to fixation in a diploid population
444 currently fixed at variant a is calculated as

$$P_{fix} = \frac{1 - \exp(-2s_{ab})}{1 - \exp(-4N_e s_{ab})} \tag{2}$$

445 where $s_{ab} = f_b - f_a$ is the relative fitness effect of mutant b (Kimura, 1962). We model the substitution
446 process as a continuous-time Markov chain which is specified by the instantaneous rate matrix Q
447 with elements

$$q_{ab} \propto 2N_e \mu_{ab} P_{fix} \tag{3}$$

448 where q_{ab} is the substitution rate from a to b which depends on the mutation rate (μ_{ab}) and the
449 fixation probability (P_{fix}). Mutations arise at the DNA-level following the HKY model (Hasegawa
450 *et al.*, 1985) allowing only single nucleotide changes. Selection is assumed to act on the final protein
451 product, and therefore all synonymous codons have the same fitness. We assumed a fixed $N_e = 100$.

452 We initiated each simulation at a randomly generated amino acid sequence. Then, we used
453 the algorithm outlined in table S5 to obtain protein sequences with fitness values ≥ 0.99 given the
454 corresponding structure. Following this equilibration phase, we evolve the equilibrated sequence for
455 500 substitutions while keeping track of the site-specific fitness landscapes at all sites. The reported
456 results are based on the post-equilibration phase. We generated 500 protein-specific replicates.

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457 # Stability model

458 We use the same stability model outlined in Goldstein (2011), Pollock *et al.* (2012), Goldstein and
459 Pollock (2017), and Youssef *et al.* (2020). For a detailed description of the model derivation see
460 section "The protein model" in Goldstein (2011). Briefly, we assume that the fitness is equal to
461 the probability of an amino acid sequence being in the native (folded) structure at thermodynamic
462 equilibrium, which is a function of the stability (ΔG) of the sequence.

$$\text{fitness} = \frac{e^{-\beta \Delta G(s)}}{e^{-\beta \Delta G(s)} + 1} \tag{4}$$

463 where $\beta = \frac{1}{kT}$, k is the Boltzmann constant, T is the absolute temperature, and $\Delta G(s)$ is stability
464 of sequence s measured as the difference in free energy between the folded and the unfolded states.
465 The free energy of a sequence in a given structure is approximated as the sum of pairwise potentials
466 (from Miyazawa and Jernigan (1985)) for amino acids in contact. Residues are considered to be in
467 contact if the C_β atoms are within 7Å of each other. If the amino acid present is glycine, distance is
468 considered with reference to the C_α atom.

469 # Amino acid propensities

470 Suppose that for a simulation trial we observed $s_1 \rightarrow s_2 \rightarrow ... \rightarrow s_{500}$ where the s_x's are the codon
471 sequences realized during the simulations, and s_x and s_{x+1} differ by a single nucleotide substitution
472 (synonymous or nonsynonymous). Given each s_x, we can calculate the fitness landscape at site h,
473 given the rest of the sequence is held constant, as $f_a^h(s_x) = \{f_1^h(s_x), ..., f_{20}^h(s_x)\}$. We use the fitness
474 values to calculate the amino acid stationary frequencies using equation (1). We calculate $\pi_a^{(0)}$ as the
475 sum over the neutral stationary frequencies for synonymous codons for each amino acid. The neutral

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476 frequency for a codon made up of nucleotide triplet ijk will be proportional to $\pi_i\pi_j\pi_k$.

477 Description of metrics used to quantify evolutionary Stokes and anti-478 Stokes shifts

479 We define four metrics to quantify shifts in propensities. First, let the residence time of an amino 480 acid (T_{res}) be the time period between i and j, where i is the substitution when amino acid a first 481 occupies the site and j is the last substitution. The first metric (M1) is the slope of the linear model 482 over T_{res} where x is time (measured in substitutions) and y is the propensity of the resident amino 483 acid a at site h (π_a^h).

484 Metric two (M2) is the average change in the propensity of the resident amino acid over its 485 residency time. Following each substitution we calculate the change in propensity as

$$\Delta_x \pi_a^h = \pi_{a|res}^h(s_{x+1}) - \pi_{a|res}^h(s_x) \tag{5}$$

486 where $\pi_{a|res}^h(s_x)$ is the propensity of the resident amino acid given the background sequence present 487 at substitution x. M2 is then the average calculated as

$$avg[\Delta\pi_{a|res}^h] = 1/N \sum_{x=i}^{j-1} \Delta_x \pi_a^h \tag{6}$$

488 where $N = j - i$.

489 Pollock *et al.* (2012) observed that following an evolutionary Stokes shift "the inherent propensity 490 for [an] amino acid at that position will be, on average, higher than it was when the substitution 491 occurred". We, therefore, define a third metric, which is perhaps the most consistent with the 492 definition provided by Pollock *et al.* (2012). Metric three (M3) is the difference in the average

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493 propensity of an amino acid while it is resident (avg[$\pi^h_{a|res}$]) and the propensity of the same amino
494 acid when it was first accepted ($\pi^h_{a|new}$).

495 For M1, M2, and M3, values greater than 0 are suggestive of an evolutionary Stokes shift and values
496 less than 0 are indicative of evolutionary anti-Stokes shifts. Lastly, we define a more conservative
497 metric, M4, which classifies an evolutionary Stokes shift only when all three metrics indicate a Stokes
498 shift (all M1-3 values are >0), and an anti-Stokes shift when M1-3 are all < 0. Figure 2 provides a
499 visual representation of the metrics.

500 Quantifying the uniformity of a landscape

501 We use the entropy of a propensity landscape as a measure of its uniformity. We calculate entropy
502 as

$$\mathrm{H}^h(s) = -\sum_a \pi^h_a(s) \ln \pi^h_a(s) \tag{7}$$

503 where $\pi^h_a(s)$ is the propensity of amino acid a at site h given background sequence s. The entropy is
504 maximized when all amino acids are equally likely, and is minimized ($= 0$) when only a single amino
505 acid is observed. To determine how the landscapes change in response to changes in the background
506 protein sequence, we compared the entropy before and after the substitution

$$\Delta\mathrm{H}^h = \mathrm{H}^h(s_{x+1}) - \mathrm{H}^h(s_x) \tag{8}$$

507 We classified a substitution as permissive if the average ΔH across all sites was positive, and restrictive
508 if the average ΔH was negative.

509 For all results described in this study, we only considered the dynamics when a residue was
510 accepted and subsequently replaced within the time-frame of the simulation. However, we repeated
511 the analyses with the inclusion of partial windows (where for example an amino acid is accepted

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512 during the simulation but the simulation ends prior to its replacement) which revealed similar results
513 with respect to the proportion of evolutionary Stokes and anti-Stokes shifts (figure S12).

514 The rate of amino acid replacement

515 Popova *et al.* (2019) recently observed that changes in amino acid propensities are accompanied by
516 changes in the relative rates of leaving the resident amino acid. Amino acids that have high fitness
517 values, are more likely to occupy the site (have high π_a^h), and will have a low rate of being replaced.
518 Conversely, sites with low fitness benefit are less likely to be present at the site (low π_a^h), and will
519 have a high rate of being replaced. Therefore, in addition to amino acid propensities, we looked at
520 the replacement rates over time. We calculate the rate of leaving the resident amino acid at a site h
521 as the sum of the transition rates (using equation (3)) over all sequences that differ from the current
522 sequence by a single nucleotide and have a different amino acid at site h.

523 Mixed linear model analysis

524 In order to assess if amino acid propensities shifts were consistent with random fluctuations we fitted
525 the data to a mixed linear model of the form

$$\pi_a^h(s_x) = \pi_{a|new}^h + \beta x + \epsilon_x \tag{9}$$

526 where $\epsilon_x \sim \mathrm{N}(0,\sigma^2)$ and $\beta \sim \mathrm{N}(0,\sigma_\beta^2)$. We tested a null model assuming random shifts in propensities
527 where $\sigma_\beta^2 = 0$ against an alternative model where $\sigma_\beta^2 > 0$.

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528 # Code availability

529 All code used to simulate, analyze, and plot data has been uploaded and is freely available from
530 https://github.com/noory3/antiStokes_shifts.

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531 # Figures

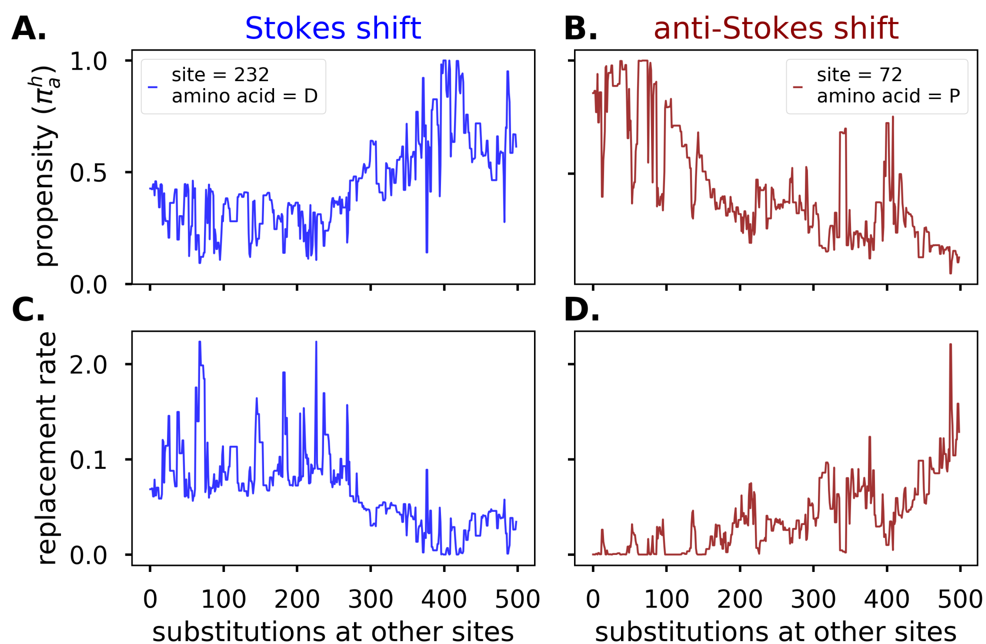

Figure 1: Neutral non-adaptive evolution can result in evolutionary Stokes and anti-Stokes shifts. Results are based on evolutionary simulation of the 1pek protein for 500 substitutions. Site 232 (A,C) undergoes an evolutionary Stokes shift whereas site 72 (B,D) undergoes an anti-Stokes shift. No substitutions occurred at either site, and the resident amino acids were aspartic acid (one letter code D) and proline (one letter code P) for sites 232 and 72, respectively. (A) and (B) plot the propensity of the resident amino acids as replacements occur at other positions in the protein. (C) and (D) show the expected rate of leaving the resident amino acid.

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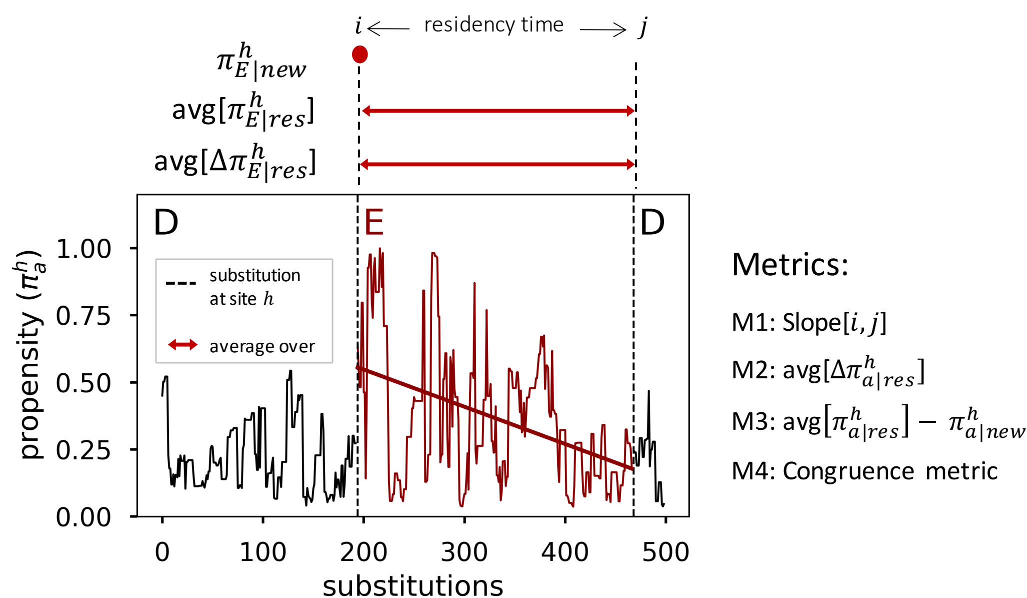

Figure 2: Description of metrics used to quantify evolutionary Stokes and anti-Stokes shifts. The example trajectory is based on site 82 of the 1pek protein. The site accepts two substitutions (vertical dotted lines) and the resident amino acid changes from D→E→D. For clarity, we focus on the dynamics following the acceptance of amino acid E. In general, metric 1 (M1) is the slope of the linear regression where x is the number of substitutions and y is the propensity of the resident amino acid a at site h (π^h_a) calculated over $i \leq x \leq j$; i is the substitution where amino acid a first occupies the site and j is the last substitution. Metric 2 (M2) is the average change in the propensity of the resident amino acid over its residency time (from i to j). Metric 3 (M3) is the difference between the average propensity of an amino acid while it is resident ($\text{avg}[\pi^h_{a|res}]$) and the propensity of the same amino acid when it was first accepted ($\pi^h_{a|new}$). For M1-3, values > 0 indicate evolutionary Stokes shifts and values < 0 indicate evolutionary anti-Stokes shifts. Metric 4 (M4) is a congruence metric which classifies an evolutionary Stokes (or anti-Stokes) shift when all other metric values are > 0 (or < 0).

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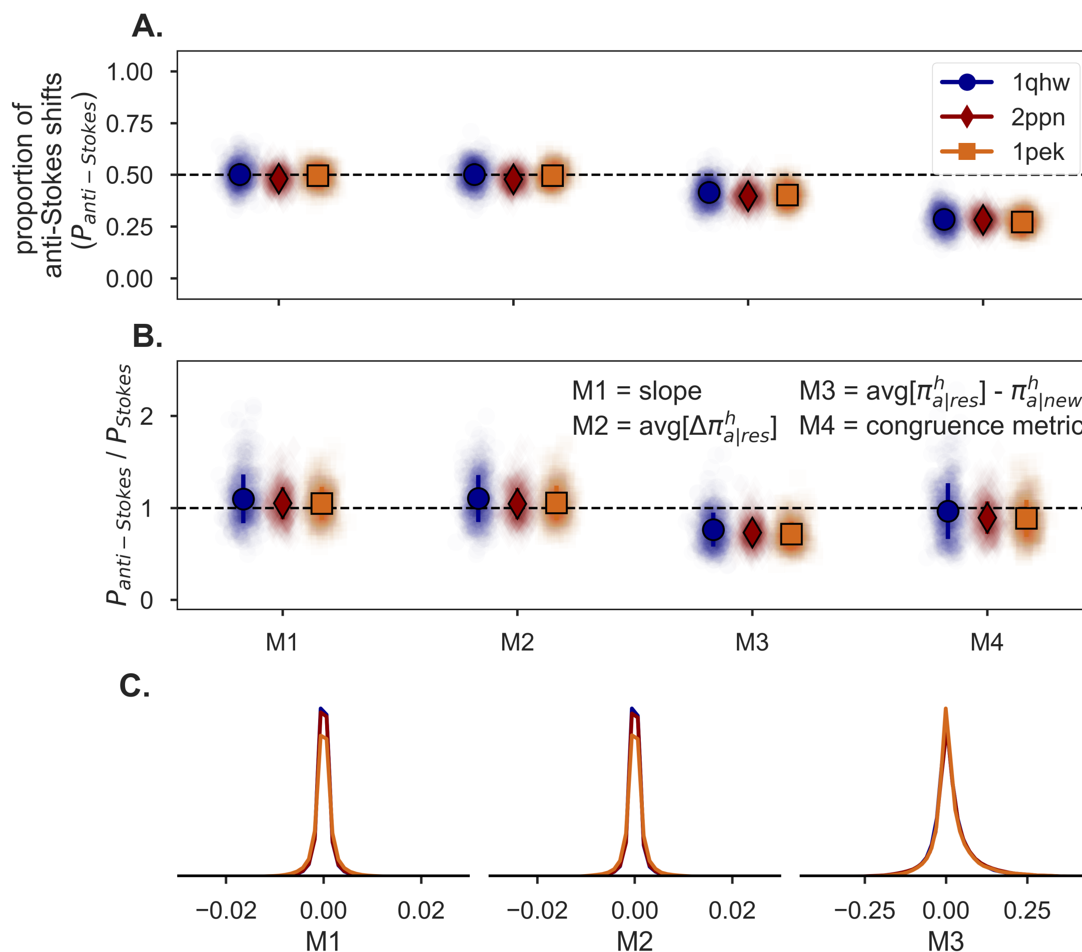

Figure 3: Evolutionary anti-Stokes shifts are common under non-adaptive evolution. (A) Approximately half of substitutions are followed by evolutionary anti-Stokes shifts based on metrics M1-3. The more conservative metric M4 estimates a slightly lower proportion (approx. 0.3). (B) Across all metrics M1-4, evolutionary Stokes and anti-Stokes shifts occur at similar frequencies ($P_{anti-Stokes}/P_{Stokes} \approx 1$). (C) The values of M1-3 are normally distributed and centered at zero which suggests that both the magnitude and frequencies of Stokes and anti-Stokes shifts are balanced.

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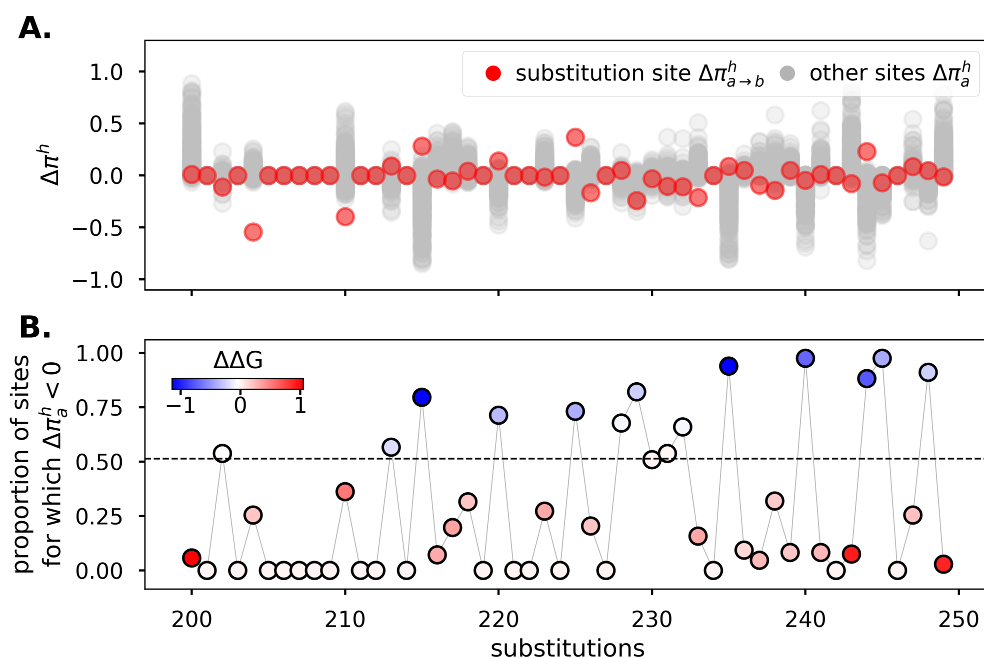

Figure 4: Stability-mediated epistasis between sites results in changes in resident amino acid propensities as substitutions occur in the protein. (A) Following an amino acid replacement at one position in the protein, so that the sequence changes from $s_x \to s_{+1}$, the propensity of the resident amino acids at all sites will subsequently change. The grey dots are the changes in the propensities of the resident amino acids at each site following a substitution, $\Delta\pi^h_a = \pi^h_a(s_{x+1}) - \pi^h_a(s_x)$. The red dots are the change in the propensity of the resident amino acid at the substitution site, and therefore a change in the amino acid from $a \to b$ ($\Delta\pi^h_{a\to b} = \pi^h_b(s_{x+1}) - \pi^h_a(s_x)$). (B) Stabilizing substitutions ($\Delta\Delta G < 0$) result in higher proportions of $\Delta\pi^h_a < 0$. In other words, they result in a decrease in the propensity of amino acids at most other sites. In contrast, destabilizing substitutions ($\Delta\Delta G > 0$) result in lower proportions of $\Delta\pi^h_a < 0$. Dotted line is the average proportion of $\Delta\pi^h_a < 0$ over all substitutions (average = 0.51).

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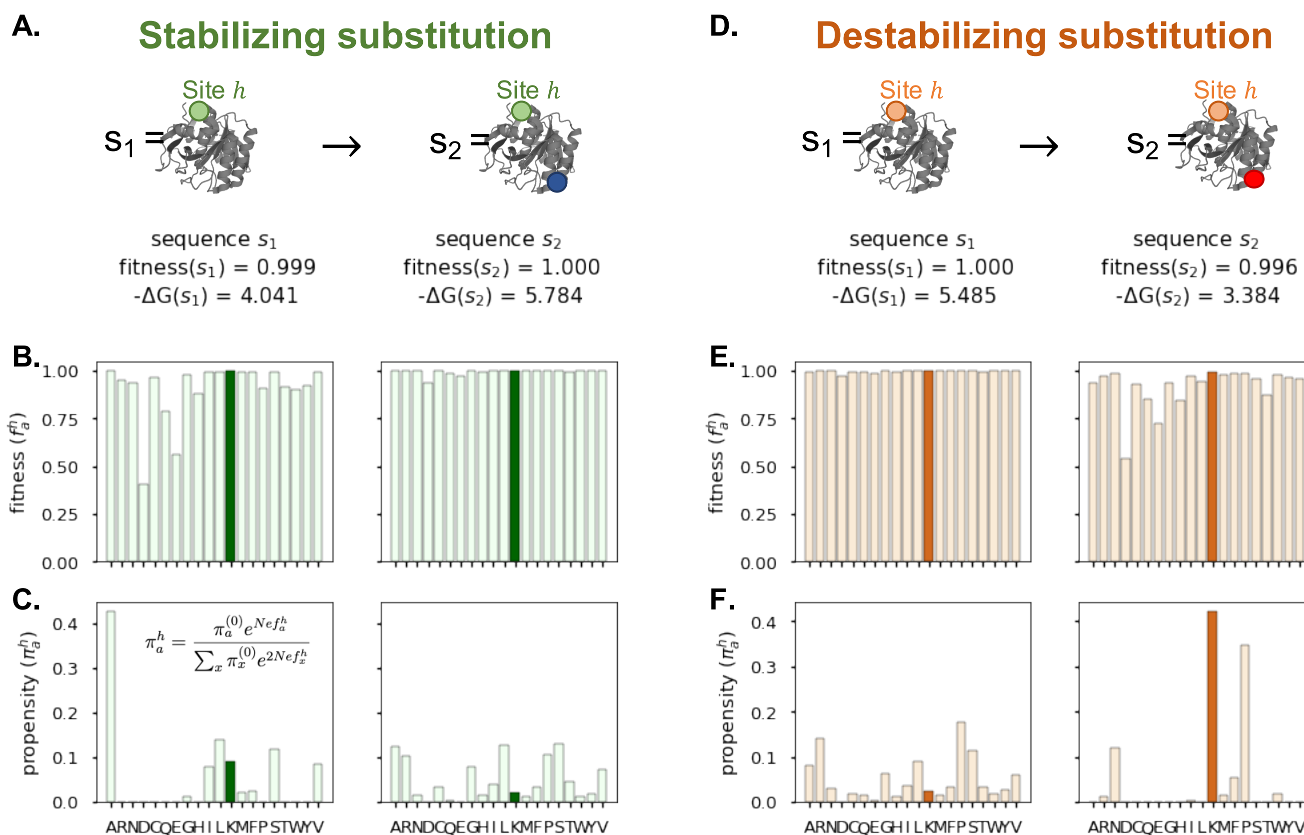

Figure 5: Epistatic dynamics following the fixations of stabilizing (A,B,C) and destabilizing (D,E,F) substitutions. (A) Let s_1 be the initial protein sequence, and s_2 be the sequence following the acceptance of a stabilizing substitution (blue dot). Given the "stability-buffered" background sequence s_2, deleterious mutations which would not have been fixed in the context of background sequence s_1 are now more likely to be fixed (e.g. R, N,P). (B) The fitness landscape at a non-substituted site h becomes more uniform because of the increase in overall protein stability. (C) Similarly, the propensity landscape becomes more uniform. The fitness and propensity of the resident amino acid is shown in dark green. (D, E, F) are the respective plots following the fixation of a destabilizing substitution (red dot). The fitness and propensity landscapes at the non-substituted site become less uniform.

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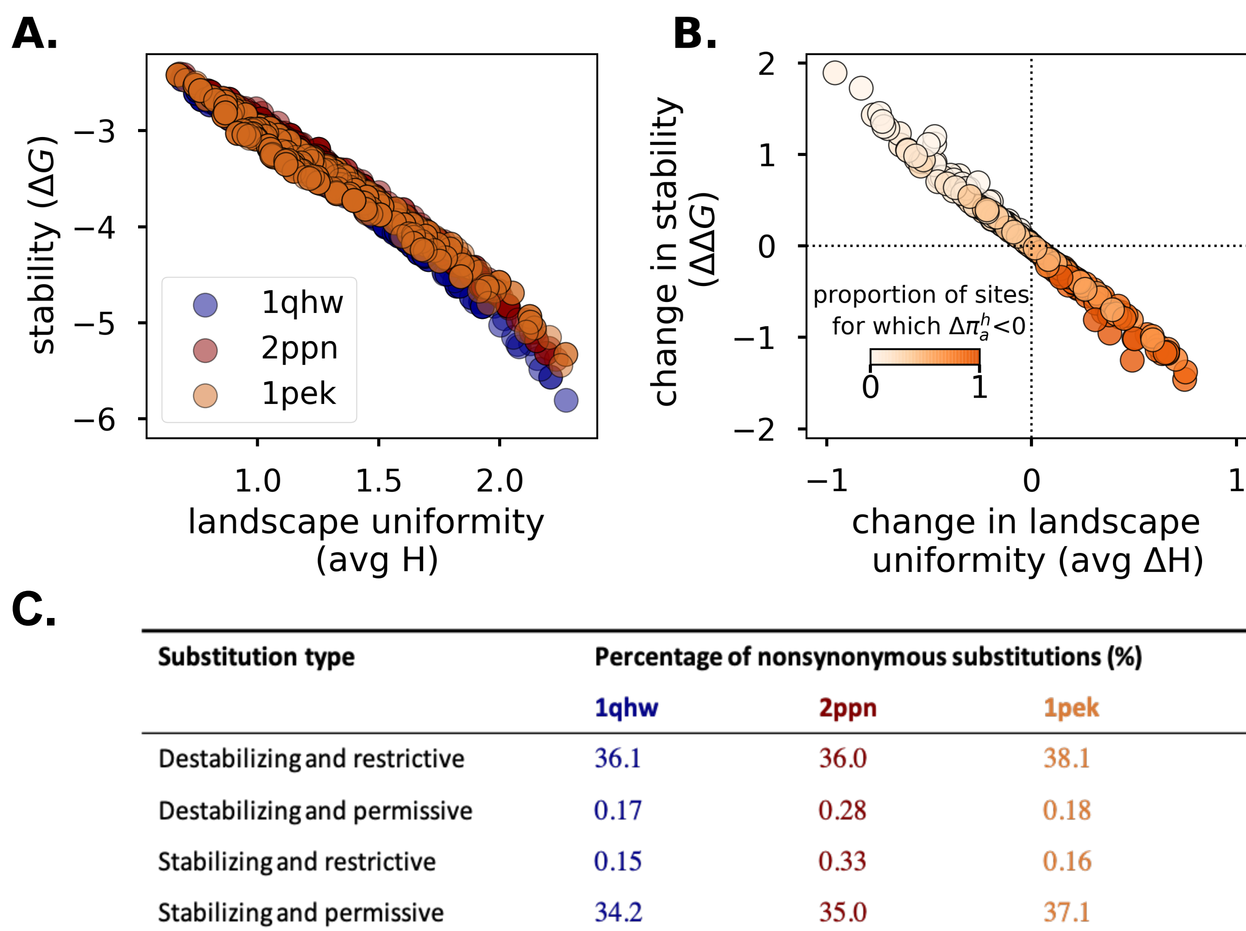

Substitution type	Percentage of nonsynonymous substitutions (%)		
	1qhw	2ppn	1pek
Destabilizing and restrictive	36.1	36.0	38.1
Destabilizing and permissive	0.17	0.28	0.18
Stabilizing and restrictive	0.15	0.33	0.16
Stabilizing and permissive	34.2	35.0	37.1

Figure 6: Most stabilizing substitutions are permissive and most destabilizing substitutions are restrictive. (A) The relationship between protein stability (ΔG) and landscape uniformity, measured as the entropy of the propensity landscape averaged over all sites in the protein (avg H). (B) The relationship between the stability effect of a substitutions ($\Delta\Delta G$) and the resulting average change in landscape uniformity (avg ΔH). Color bar represents the proportion of sites for which the propensity for the resident amino acid decreased ($\Delta\pi_a^h < 0$). Positive avg ΔH values imply that, on average, the landscapes became more uniform. Therefore, the substitution is deemed permissive. Negative avg ΔH are indicative of restrictive substitutions. Plotted results are based on a single simulation of the 1pek protein. (C) The percentages of different types of substitutions for each of three proteins (1qhw, 2ppn, and 1pek). Percentages are calculated from 500 protein-specific trials

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