Selection maintaining protein stability at equilibrium

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Abstract

The common understanding of protein evolution has been that neutral mutations are fixed by random drift, and a proportion of neutral mutations depending on the strength of structural and functional constraints primarily determines evolutionary rate. Recently it was indicated that fitness costs due to misfolded proteins are a determinant of evolutionary rate and selection originating in protein stability is a driving force of protein evolution. Here we examine protein evolution under the selection maintaining protein stability.

Protein fitness is a generic form of fitness costs due to misfolded proteins; \( s = \kappa \exp(\Delta G/kT)(1 - \exp(\Delta\Delta G/kT)) \), where \( s \) and \( \Delta\Delta G \) are selective advantage and stability change of a mutant protein, \( \Delta G \) is the folding free energy of the wild-type protein, and \( \kappa \) is a parameter representing protein abundance and indispensability. The distribution of \( \Delta\Delta G \) is approximated to be a bi-Gaussian distribution, which represents structurally slightly- or highly-constrained sites. Also, the mean of the distribution is negatively proportional to \( \Delta G \).

The evolution of this gene has an equilibrium point \( \Delta G_e \) of protein stability, the range of which is consistent with observed values in the ProTherm database. The probability distribution of \( K_a/K_s \), the ratio of nonsynonymous to synonymous substitution rate per site, over fixed mutants in the vicinity of the equilibrium shows that nearly neutral selection is predominant only in low-abundant, non-essential proteins of \( \Delta G_e > -2.5 \) kcal/mol. In the other proteins, positive selection on stabilizing mutations is significant to maintain protein stability at equilibrium as well as random drift on slightly negative mutations, although the average \( \langle K_a/K_s \rangle \) is less than 1. Slow evolutionary rates can be caused by both high protein abundance/indispensability and large effective population size, which produces positive shifts of \( \Delta\Delta G \) through decreasing \( \Delta G_e \), and strong structural constraints, which directly make \( \Delta\Delta G \) more positive. Protein abun-

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dance/indispensability more affect evolutionary rate for less constrained proteins, and structural constraint for less abundant, less essential proteins. The effect of protein indispensability on evolutionary rate may be hidden by the variation of protein abundance and detected only in low-abundant proteins. Also, protein stability \((-\Delta G_e/kT)\) and \(\langle K_a/K_s \rangle\) are predicted to decrease as growth temperature increases.

**Highlights**

- Protein stability is kept at equilibrium by random drift and positive selection.
- Neutral selection is predominant only for low-abundant, non-essential proteins.
- Protein abundance more decreases evolutionary rate for less-constrained proteins.
- Structural constraint more decreases evolutionary rate for less-abundant, less-essential proteins.
- Protein stability \((-\Delta G_e/kT)\) and \(\langle K_a/K_s \rangle\) are predicted to decrease as growth temperature increases.

**Keywords:** neutral theory, positive selection, evolutionary rate, structural constraints, protein abundance

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1. **Introduction**

   The common understanding of protein evolution has been that amino acid substitutions observed in homologous proteins are neutral (Kimura, 1968; 1969; Kimura and Ohta, 1971; 1974) or slightly deleterious (Ohta, 1973; 1992), and random drift is a primary force to fix amino acid substitutions in population. The rate of protein evolution has been understood to be determined primarily by the proportion of neutral mutations, which may be measured by the ratio of nonsynonymous to synonymous substitution rate per site \((K_a/K_s)\) (Miyata and Yasunaga, 1980) and determined by functional density (Zuckerkandl, 1976) weighted by the relative variability at specific-function sites of a protein (Go and Miyazawa, 1980). Since then, these theories have been widely accepted, however, recently a question
has been raised on whether the diversity of protein evolutionary rate among genes can be explained only by the proportion and the variability of specific-function sites, and molecular and population-genetic constraints on protein evolutionary rate have been explored.

Recent works have revealed that protein evolutionary rate is correlated with gene expression level; highly expressed genes evolve slowly, accounting for as much as 34% of rate variation in yeast (Pál et al., 2001). Of course, there are many reports that support a principle of lower evolution rate for stronger functional density. Broadly expressed proteins in many tissues tend to evolve slower than tissue-specific ones (Kuma et al., 1995; Duret and Mouchirou, 2000). The connectivity of well-conserved proteins in a network is shown (Fraser et al., 2002) to be negatively correlated with their rate of evolution, because a greater proportion of the protein sequence is directly involved in its function. A fitness cost due to protein–protein misinteraction affects the evolutionary rate of surface residues (Yang et al., 2012). Protein dispensability in yeast is correlated with the rate of evolution (Hirsh and Fraser, 2001, 2003), although there is a report insisting on no correlation between them (Pál et al., 2003). Other reports indicate that the correlation between gene dispensability and evolutionary rate, although low, is significant (Zhang and He, 2005; Wall et al., 2005; Jordan et al., 2002).

It was proposed (Drummond et al., 2005; Drummond and Wilke, 2008; Geiler-Samerotte et al., 2011) that low substitution rates of highly expressed genes could be explained by fitness costs due to functional loss and toxicity (Stoebel et al., 2008; Geiler-Samerotte et al., 2011) of misfolded proteins. Misfolding reduces the concentration of functional proteins, and wastes cellular time and energy on production of useless proteins. Also misfolded proteins form insoluble aggregates (Geiler-Samerotte et al., 2011). Fitness cost due to misfolded proteins is larger for highly expressed genes than for less expressed ones.

Fitness cost due to misfolded proteins was formulated (Drummond and Wilke, 2008; Geiler-Samerotte et al., 2011) to be related to the proportion of misfolded proteins. Knowledge of protein folding indicates that protein folding primarily occurs in two-state transition (Miyazawa and Jernigan, 1982a,b), which means that the ensemble of protein conformations are a mixture of completely folded and unfolded conformations. Free energy ($\Delta G$) of protein stability, which is equal to the free energy of the denatured state subtracted from that of the native state, and stability change ($\Delta \Delta G$) due to amino acid substitutions are collected in the ProTherm database (Kumar et al., 2006), although the data are not sufficient. Prediction methods, however, for $\Delta \Delta G$ are improved enough to reproduce real distributions of $\Delta G$ (Schymkowitz et al., 2005; Yin et al., 2007). Therefore, on the biophys-
cal basis, the distribution of fitness can be estimated and protein evolution can be studied. Shakhnovich group studied protein evolution on the basis of knowledge of protein folding (Serohijos and Shakhnovich, 2014; Dasmeh et al., 2014) and showed (Serohijos et al., 2012) that the negative correlation between protein abundance and $K_a/K_s$ was caused by the distribution of $\Delta G$ that negatively correlates with the $\Delta G$ of a wild type. Also, it was shown (Serohijos et al., 2013) that highly abundant proteins had to be more stable than low abundant ones. Relationship between evolutionary rate and protein stability is studied from various points of view (Echave et al., 2015; Faure and Koonin, 2015).

Here we study relationship between evolutionary rate and selection on protein stability in a monoclonal approximation. A fitness assumed here for a protein is a generic form to which all formulations (Drummond and Wilke, 2008; Geiler-Samerotte et al., 2011; Serohijos et al., 2012, 2013; Serohijos and Shakhnovich, 2014; Dasmeh et al., 2014) previously employed for protein fitness are reduced in the condition of $\exp(\beta \Delta G) \ll 1$, which is satisfied in the typical range of folding free energies shown in Fig. 1; $\beta = 1/(kT)$, $k$ is the Boltzmann constant and $T$ is absolute temperature. The generic form of Malthusian fitness of a protein-coding gene is $m \equiv -\kappa \exp(\beta \Delta G)$, where $\kappa$ is a parameter, which may be a function of protein abundance and dispensability; see Methods for details. The distribution of stability change $\Delta \Delta G$ due to single amino acid substitutions is approximated as a weighted sum of two Gaussian functions that was shown (Tokuriki et al., 2007) to well reproduce actual distributions of $\Delta \Delta G$. One of the two Gaussian functions describes substitutions at structurally less-constrained surface sites, and the other at more-constrained core sites of proteins. The proportion of less-constrained surface sites is a parameter ($\theta$).

The fixation probability of a mutant with $\Delta \Delta G$ can be calculated for a diploid population with effective population size $N_e$ (Crow and Kimura, 1970). In the population of genes with such a fitness protein stability is evolutionarily maintained at equilibrium, and equilibrium stability ($\Delta G_e$) negatively correlates with protein abundance/dispensability ($\kappa$). The range of $\Delta G_e$ is consistent with the observed range of folding free energies shown in Fig. 1.

The probability density functions (PDF) of $K_a/K_s$, the ratio of nonsynonymous to synonymous substitution rate per site (Miyata and Yasunaga, 1980), at equilibrium and also in the vicinity of equilibrium are numerically examined over a whole domain of the parameters, $0 \leq \log 4N_e\kappa \leq 20$ and $0 \leq \theta \leq 1$. The dependences of evolutionary rate on protein abundance/dispensability and on structural constraint are quantitatively described, and it is shown that both factors cannot be ignored on protein evolutionary rate, although protein abundance/indispensability more
affect evolutionary rate for less constrained proteins, and structural constraint for less abundant, less essential proteins. Like protein abundance, protein indispensability must correlate with evolutionary rate, but a correlation between them may be hidden by the variation of protein abundance as well as effective population size, and detected only in low-abundant proteins. It has also become clear that nearly neutral selection is predominant only in low-abundant, non-essential proteins with log \( 4N_e\kappa < 2 \) or \( \Delta G_e > -2.5 \) kcal/mol, and in the other proteins positive selection is significant to more stabilize a less-stable wild type. Also, a significant amount of slightly negative mutants are fixed in population by random drift. This view of protein evolution is contrary to the previous understanding. The present model based on a biophysical knowledge of protein stability also indicates that protein stability \( (-\beta\Delta G_e) \) and the average of \( K_a/K_s \) decrease as growth temperature increases.
Figure 1: **Distribution of folding free energies of monomeric protein families.** Stability data of monomeric proteins for which the item of $dG_{H2O}$ or $dG$ was obtained in the experimental condition of $6.7 \leq \text{pH} \leq 7.3$ and $20^\circ C \leq T \leq 30^\circ C$ and their folding-unfolding transition is two state and reversible are extracted from the ProTherm [Kumar et al. 2006]; in the case of $dG$ only thermal transition data are used. Thermophilic proteins, and proteins observed with salts or additives are also removed. An equal sampling weight is assigned to each species of homologous protein, and the total sampling weight of each protein family is normalized to one. In the case in which multiple data exist for the same species of protein, its sampling weight is divided to each of the data. However, proteins whose stabilities are known may be samples biased from the protein universe. The value, $\Delta G_e = -5.24$ kcal/mol, of equilibrium stability at the representative parameter values, $\log 4N_\kappa = 7.55$ and $\theta = 0.53$, agrees with the most probable value of $\Delta G$ in the distribution above. Also, the range of $\Delta G$ shown above is consistent with that range, $-2$ to $-12.5$ kcal/mol, expected from the present model. The kcal/mol unit is used for $\Delta G$. A similar distribution was also compiled [Zeldovich et al. 2007].
2. Methods

2.1. Fitness costs due to misfolded proteins

Misfolding can impose costs in three distinct ways (Geiler-Samerotte et al., 2011): loss of function, diversion of protein synthesis resources away from essential proteins, and toxicity of the misfolded molecules. Fitness cost due to functional loss was formulated (Drummond and Wilke, 2008) by taking account of protein dispensability. Assuming that fitness cost of each gene is additive in the Malthusian fitness scale, the total Malthusian fitness of a genome was estimated as

\[ m_{\text{dispensability}} \equiv -\sum_i \gamma_i (1 - f_{i}^{\text{native}}) \]  

(1)

where \(-\gamma_i\) is defined as \(-\gamma_i \equiv \log(\text{deletion-strain growth rate} / \text{max growth rate})\), and \(f_{i}^{\text{native}}\) is the fraction of the native conformation for gene \(i\).

Protein folding primarily occurs in the two-state transition, which means that protein conformations are a mixture of completely folded and unfolded conformations (Miyazawa and Jernigan, 1982). Therefore, if the completely folded (native) state is more stable by a free energy difference \(\Delta G\) than the unfolded (denatured) state, then the native fraction in the conformational ensemble will be equal to

\[ f_{i}^{\text{native}} = \frac{e^{-\beta \Delta G_i}}{1 + e^{-\beta \Delta G_i}} \]  

(2)

where \(\beta = 1/kT\); \(k\) is the Boltzmann constant and \(T\) is absolute temperature.

Thus, Eq. (1) for the Malthusian fitness of a genome can be transformed as follows in terms of the folding free energy \(\Delta G\) of the native conformation:

\[ m_{\text{dispensability}} = -\sum_i \gamma_i \frac{e^{\beta \Delta G_i}}{1 + e^{\beta \Delta G_i}} \]  

(3)

Because of \(\exp(\beta \Delta G) \ll 1\) in the typical range of folding free energies shown in Fig. 1, the above definition of fitness is approximated by

\[ m_{\text{dispensability}} = -\sum_i \gamma_i [e^{\beta \Delta G_i} - O(e^{2\beta \Delta G_i})] \]  

(4)

Drummond and Wilke (Drummond and Wilke, 2008) took notice of toxicity of misfolded proteins as well as diversion of protein synthesis resources, and formulated the Malthusian fitness \(m_{\text{misfolds}}\) of a genome to be negatively proportional
to the total amount of misfolded proteins, which must be produced to obtain the necessary amount of folded proteins (Serohijos et al., 2012).

\[
m_{\text{misfolds}} = -c \sum_i A_i \frac{1 - f_{i}^{\text{native}}}{f_{i}^{\text{native}}} \quad (5)
\]

\[
= -c \sum_i A_i e^{\beta \Delta G_i} \quad (6)
\]

where \( c \) is a positive constant and assumed to be \( c = 0.0001 \), and \( A_i \) is the abundance of protein \( i \).

2.2. Fitness of a linear metabolic pathway

Serohijos and Shakhnovich (Serohijos and Shakhnovich, 2014) examined the evolution of a linear metabolic pathway whose Wrightian fitness was defined as

\[
w_{\text{linear pathway}} \equiv w_{\text{flux}} + w_{\text{misfolds}} \quad (7)
\]

\[
w_{\text{flux}} \equiv \frac{\sum_i \varepsilon_i A_i^{-1}}{\sum_i \varepsilon_i (A_i f_{i}^{\text{native}})^{-1}} \quad (8)
\]

\[
w_{\text{misfolds}} \equiv -c \sum_i A_i (1 - f_{i}^{\text{native}}) \quad (9)
\]

where \( \varepsilon_i \) was defined as enzyme efficiency and assumed to be \( \varepsilon_i = 1 \). The \( w_{\text{flux}} \) is a fitness originating from the enzymatic flux of a linear metabolic pathway, and \( w_{\text{misfolds}} \) represents the effect of toxicity of misfolded proteins, and is the same functional form as Eq. (1), although Eq. (1) is a definition for Malthusian fitness. Then, the Malthusian fitness corresponding to the Wrightian fitness above can be represented as

\[
m_{\text{linear pathway}} \equiv \log \left[ 1 + \sum_i \frac{\varepsilon_i A_i^{-1} e^{\beta \Delta G_i}}{\sum_i \varepsilon_i A_i^{-1}} \right] - c \sum_i A_i e^{\beta \Delta G_i} (1 + e^{\beta \Delta G_i} - 1)
\]

\[
= -\sum_i \left( \frac{\varepsilon_i A_i^{-1}}{\sum_i \varepsilon_i A_i^{-1}} + cA_i e^{\beta \Delta G_i} \right)
\]

\[
+ O(\left( \sum_i \frac{\varepsilon_i A_i^{-1}}{\sum_i \varepsilon_i A_i^{-1}} + cA_i e^{\beta \Delta G_i} \right)^2) \quad (11)
\]

Because \( cA_i \leq 0.459 \) (Serohijos and Shakhnovich, 2014), \( \Delta G < -3 \) and \( \sum_{i=1}^{10} cA_i \exp(\beta \Delta G_i) < 0.03 \), the higher order terms can be neglected in this case.
However, the fitness costs due to the flux and misfolded proteins may be formulated to be additive in the Malthusian scale rather than in the Wrightian scale, employing Eq. (6) for the fitness cost due to misfolded proteins;

\[
m_{\text{linear pathway}} \equiv - \sum_i \left\{ \frac{\varepsilon_i A_i^{-1}}{\sum_i \varepsilon_i A_i^{-1}} + cA_i \right\} e^{\beta \Delta G_i} \tag{12}\]

**2.3. Other formulations of protein fitness**

Also, the following simple definition for fitness to maintain protein stability was used [Dasmeh et al., 2014]:

\[
w \propto f_{\text{native}} \tag{13}\]

\[
m = -e^{\beta \Delta G} + O(e^{2\beta \Delta G}) + \text{constant} \tag{14}\]

In addition, Eq. (3) for functional loss was employed with \(\gamma_i \Rightarrow cA_i\) to represent toxicity of misfolded proteins in [Serohijos et al., 2012, 2013].

**2.4. A generic form of protein fitness**

Thus, all expressions above for Malthusian fitness of protein can be well approximated by the following expression, because of \(\exp(\beta \Delta G) \ll 1\) in the typical range of folding free energies shown in Fig. 1.

\[
m \equiv - \sum_i \kappa_i e^{\beta \Delta G_i} \quad \text{with } \kappa_i \geq 0 \tag{15}\]

where \(\kappa_i\) is a parameter. If the fitness costs of functional loss and toxicity due to misfolded proteins are taken into account, \(\kappa_i\) will be defined as

\[
\kappa_i = cA_i + \gamma_i \geq 0 \tag{16}\]

assuming their additivity in the Malthusian fitness scale.

The selective advantage of a mutant, in which each protein is destabilized by \(\Delta \Delta G_i\), to the wild type can be represented by

\[
s \equiv m_{\text{mutant}} - m_{\text{wildtype}} = \sum_i s_i \tag{17}\]

\[
s_i = \kappa_i e^{\beta \Delta G_i} (1 - e^{\beta \Delta \Delta G_i}) \quad \text{with } \kappa_i \geq 0 \tag{18}\]
3. Results

3.1. Protein stability and fitness

Here, we consider the evolution of a single protein-coding gene in which the selective advantage of mutant proteins in Malthusian parameters is assumed to be

\[ s = \kappa e^{\beta \Delta G} (1 - e^{\beta \Delta \Delta G}) \]  

with \( \kappa \geq 0 \) (19)

and therefore \( s \) is upper-bounded by

\[ s \leq \kappa e^{\beta \Delta G} \]  

where \( \Delta G \) is the stability of a wild-type protein, \( \Delta \Delta G \) is a stability change of a mutant protein, \( \beta = 1/kT \); unless specified, \( \beta = 1/0.593 \) kcal\(^{-1}\)mol corresponding to \( T = 298^\circ \)K. \( \kappa \) is a parameter whose meaning may depend on the situation; refer to Method for details. If the fitness costs of functional loss and toxicity due to misfolded proteins are both taken into account and assumed to be additive in the Malthusian fitness scale, \( \kappa \) will be defined as

\[ \kappa = cA + \gamma \]  

(21)

where \( c \) is fitness cost per misfolded protein (Drummond and Wilke, 2008; Geiler-Samerotte et al., 2011), \( A \) is the cellular abundance of the protein (Drummond and Wilke, 2008; Geiler-Samerotte et al., 2011), and \( \gamma \) is indispensability (Drummond and Wilke, 2008) and defined to be \( \gamma = -\log(\text{deletion-strain growth rate} / \text{max growth rate}) \). Equation (19) indicates that the selective advantage \( s \) is upper-bounded by \( \kappa e^{\beta \Delta G} \). The parameter \( \kappa \) is assumed in the present analysis to take values in the range of \( 0 \leq \log 4N_e \kappa \leq 20 \) with effective population size \( N_e \), taking account of the values of the parameters, \( c \sim 10^{-4} \) (Drummond and Wilke, 2008), \( 10 < A < 10^6 \) (Ghaemmaghami et al., 2003), \( \gamma = 10 \) for essential genes (Drummond and Wilke, 2008), and \( N_e \sim 10^4 \) to \( 10^5 \) for vertebrates, \( \sim 10^5 \) to \( 10^6 \) for invertebrates, \( \sim 10^7 \) to \( 10^8 \) for unicellular eukaryotes, and \( > 10^8 \) for prokaryotes (Lynch and Conery, 2003). The above ranges of the parameters indicate that the effect of protein indispensability (\( \gamma \)) may be hidden by the variation of protein abundance (\( cA \)) as well as effective population size (\( N_e \)), and may be detected only in low-abundant proteins.

Based on measurements of stability changes due to single amino acid substitutions in proteins, which are collected in the ProTherm database (Kumar et al., 2006), Serohijos et al. (Serohijos et al., 2012) reported that the distribution of
$\Delta \Delta G$ is approximately a Gaussian distribution with mean $= 1$ kcal/mol and standard deviation $= 1.7$ kcal/mol. In addition, it was shown (Serohijos et al., 2012) that the mean of $\Delta \Delta G$ is negatively proportional to $\Delta G$, and that this dependence of the mean of $\Delta \Delta G$ on $\Delta G$ is not large but still important to cause the observed negative correlation between protein abundance and evolutionary rate. On the other hand, Tokuriki et al. (Tokuriki et al., 2007) computationally predicted $\Delta \Delta G$ for all possible single amino acid substitutions in 21 different globular, single domain proteins, and showed that the predicted distributions of $\Delta \Delta G$ were strikingly similar despite a range of protein sizes and folds and largely follow a bi-Gaussian distribution: one of the two Gaussian distributions results from substitutions on protein surfaces and is a narrow distribution with a mildly destabilizing mean $\Delta \Delta G$, whereas the other due to substitutions in protein cores is a wider distribution with a stronger destabilizing mean (Tokuriki et al., 2007).

![Figure 2](image-url)  

Figure 2: Dependence of stability changes, $\Delta \Delta G$, due to single amino acid substitutions on the protein stability, $\Delta G$, of the wild type. A solid line shows the regression line, $\Delta \Delta G = -0.14 \Delta G + 0.49$; the correlation coefficient and p-value are equal to $-0.20$ and $< 10^{-7}$, respectively. Broken lines show two means of bi-Gaussian distributions, $\mu_s$ in blue and $\mu_c$ in red. Blue dotted lines show $\mu_s \pm 2 \sigma_s$ and red dotted lines $\mu_c \pm 2 \sigma_c$. See Eqs. (22), (23) and (24) for the bi-Gaussian distribution. Stability data of single amino acid mutants for which the items $dG_{H2O}$ and $ddG_{H2O}$ or $dG$ and $ddG$ were obtained in the experimental condition of $6.7 \leq pH \leq 7.3$ and $20^\circ C \leq T \leq 30^\circ C$ and their folding-unfolding transitions are two state and reversible are extracted from the ProTherm (Kumar et al., 2006). In the case of $dG$ only thermal transition data are used. In the case in which multiple data exist for the same protein, only one of them is used. The kcal/mol unit is used for $\Delta \Delta G$ and $\Delta G$. A similar distribution was also compiled (Serohijos et al., 2012).
Here, according to (Tokuriki et al., 2007), the distribution of $\Delta \Delta G$ due to single amino acid substitutions is approximated as a bi-Gaussian function with the dependence of mean $\Delta \Delta G$ on $\Delta G$, in order to examine the effects of structural constraint on evolutionary rate. The probability density function (PDF) of $\Delta \Delta G$, $p(\Delta \Delta G)$, for nonsynonymous substitutions is assumed to be

$$p(\Delta \Delta G) = \theta N(\mu_s, \sigma_s) + (1 - \theta) N(\mu_c, \sigma_c)$$  \hspace{1cm} (22)

where $0 \leq \theta \leq 1$, and $N(\mu, \sigma)$ is a normal distribution with mean $\mu$ and standard deviation $\sigma$. Since the majority of substitutions appear to be single nucleotide substitutions, the values of the standard deviations ($\sigma_s$ and $\sigma_c$) estimated in (Tokuriki et al., 2007) for single nucleotide substitutions are employed here; in kcal/mol units,

$$\begin{align*}
\mu_s &= -0.14 \Delta G - 0.17 , & \sigma_s &= 0.90 \hspace{1cm} (23) \\
\mu_c &= -0.14 \Delta G + 1.23 , & \sigma_c &= 1.93 \hspace{1cm} (24)
\end{align*}$$

To analyze the dependences of the means, $\mu_s$ and $\mu_c$, on $\Delta G$, we plotted the observed values of $\Delta \Delta G$ of single amino acid mutants against $\Delta G$ of the wild type, which are collected in the ProTherm database (Kumar et al., 2006); the same analysis was done by (Serohijos et al., 2012). Fig. 2 shows a significant dependence of $\Delta \Delta G$ on $\Delta G$; the regression line is $\mu = -0.14 \Delta G + 0.49$. The linear slopes of $\mu_s$ and $\mu_c$ are taken to be equal to the slope ($-0.14$) of the regression line. The intercepts have been estimated to satisfy the following two conditions.

1. Equations (23) and (24) satisfy $\mu_s(\Delta G_0) = 0.56$ and $\mu_c(\Delta G_0) = 1.96$, which were estimated for single nucleotide substitutions in (Tokuriki et al., 2007), at a certain value ($\Delta G_0$) of $\Delta G$.

2. The total mean of the two Gaussian functions agrees with the regression line, $\mu = -0.14 \Delta G + 0.49$. The value of $\theta$ is taken to be 0.53, which is equal to the average of $\theta$ over proteins used in (Tokuriki et al., 2007).

A representative value, 7.550, of $\log 4N_e \kappa$ is determined in such way that the equilibrium value of $\Delta G$ is equal to $\Delta G_0 = -5.24$ introduced above; $\Delta G_e$ is explicitly defined later. It is interesting that this value $\Delta G_e = -5.24$ kcal/mol agrees with the most probable value of $\Delta G$ in the observed distribution of protein stabilities shown in Fig. 1. The fraction $\theta$ of less-constrained residues such as most residues on protein surface is correlated with protein length for globular, monomeric proteins; $\theta = 1.27 - 0.33 \cdot \log_{10}(\text{protein length})$ for $50 \leq \text{length} \leq 330$ (Tokuriki et al., 2007).
et al. (2007). However, residues taking part in protein–protein interactions may be regarded as core residues rather than surface residues.

The dependence of the PDF, $p(\Delta \Delta G)$, of $\Delta \Delta G$ on $\theta$ is shown in Fig. 3. Also, the PDF of selective advantage, $p(4N_e s) = -p(\Delta \Delta G)d\Delta \Delta G/d4N_e s$, is shown in Fig. S.4 to have a peak at a small, positive value of selective advantage, which moves toward more positive values as $\theta$ and/or $4N_e \kappa$ increase.
Figure 3: PDFs of stability changes, $\Delta \Delta G$, due to single amino acid substitutions in all mutants and in fixed mutants at equilibrium of protein stability, $\Delta G = \Delta G_e$. The PDF of $\Delta \Delta G$ due to single amino acid substitutions in all arising mutants is assumed to be bi-Gaussian; see Eq. (22). Unless specified, $\log 4N_e \kappa = 7.55$ and $\theta = 0.53$ are employed. The kcal/mol unit is used for $\Delta \Delta G$ and $\Delta G_e$. 
3.2. Equilibrium state of protein stability in protein evolution

The fixation probability \( u \) for a mutant gene with selective advantage \( s \) and gene frequency \( q \) in a duploid system of effective population size \( N_e \) was given as a function of \( 4N_es \) and \( q \) by (Crow and Kimura, 1970)

\[
u(4N_es) = \frac{1 - e^{-4N_es}}{1 - e^{-4N_es}}
\]

(25)

where \( q = 1/(2N) \) for a single mutant gene in a population of size \( N \). Population size is taken to be \( N = 10^6 \). The ratio of the substitution rate per nonsynonymous site (\( K_a \)) for nonsynonymous substitutions with selective advantage \( s \) to the substitution rate per synonymous site (\( K_s \)) for nonsynonymous substitutions with \( s = 0 \) is

\[
\frac{K_a}{K_s} = \frac{u(4N_es)}{u(0)} = \frac{u(4N_es)}{q} \quad \text{with} \quad q = \frac{1}{2N}
\]

(26)

\[
\approx \frac{4N_es}{1 - e^{-4N_es}} \quad \text{for} \quad \frac{|4N_esq|}{2} \ll 1
\]

(27)

assuming that synonymous substitutions are completely neutral and mutation rates at both types of sites are the same. Eqs. (19) and (25) indicate that \( 4N_e\kappa \) can be regarded as a single parameter for \( K_a/K_s \). Furthermore, if the dependence of the mean \( \Delta\Delta G \) on \( \Delta G \) could be neglected, \( 4N_e\kappa \exp(\beta\Delta G) \) could be regarded as a single parameter. In the range of \( |4N_esq|/2 \ll 1 \), both \( K_a/K_s \) and the PDF of \( K_a/K_s \) do not depend on \( q = 1/(2N) \); see Eqs. (27) and (S.15).

The PDF of \( \Delta\Delta G \) of fixed mutant genes, \( p(\Delta\Delta G_{\text{fixed}}) \), is

\[
p(\Delta\Delta G_{\text{fixed}}) \equiv p(\Delta\Delta G)\frac{u(4N_es)}{\langle u \rangle}
\]

(28)

\[
\langle u \rangle \equiv \int_{-\infty}^{\infty} u(4N_es)p(\Delta\Delta G)d\Delta\Delta G
\]

(29)

where \( \langle u \rangle \) is the average fixation rate. Fig. 3 shows the PDF of \( \Delta\Delta G \) of fixed mutant genes. The PDF of \( 4N_es \) in fixed mutants is also shown in Fig. 5A: \( p(4N_es_{\text{fixed}}) = -p(\Delta\Delta G_{\text{fixed}})d\Delta\Delta G/d4N_es \). Then, the average of \( \Delta\Delta G \) in fixed mutant genes can be calculated; \( \langle \Delta\Delta G \rangle_{\text{fixed}} \equiv \int_{-\infty}^{\infty} \Delta\Delta G \ p(\Delta\Delta G_{\text{fixed}}) \ d\Delta\Delta G \).
Figure 4: The average, \(\langle \Delta \Delta G \rangle_{\text{fixed}}\), of stability changes over fixed mutants versus protein stability, \(\Delta G\), of the wild type. \(\Delta G_e\), where \(\langle \Delta \Delta G \rangle = 0\), is the stable equilibrium value of folding free energy, \(\Delta G\), in protein evolution. The averages of \(\Delta \Delta G\), \(4N_e\), and \(K_a/K_s\) over fixed mutants are plotted against protein stability, \(\Delta G\), of the wild type by solid, broken, and dash-dot lines, respectively. Thick dotted lines show the values of \(\langle \Delta \Delta G \rangle_{\text{fixed}} \pm \Delta \Delta G_{\text{sd}}\), where \(\Delta \Delta G_{\text{sd}}\) is the standard deviation of \(\Delta \Delta G\) over fixed mutants. \(\log 4N_e \kappa = 7.55\) and \(\theta = 0.53\) are employed. The kcal/mol unit is used for \(\Delta \Delta G\) and \(\Delta G\).
Fig. 4 shows the average of the ΔΔG over fixed mutant genes, ⟨ΔΔG⟩_{fixed}, to monotonically decrease with ΔG, indicating that the temporal process of ΔG is stable at ⟨ΔΔG⟩_{fixed}(ΔG_e) = 0 due to the balance between random drift on destabilizing mutations and positive selection on stabilizing mutations; ΔG_e is the folding free energy at the equilibrium state. If a wild-type protein becomes less stable than the equilibrium, ΔG > ΔG_e, more stabilizing mutants will fix due to primarily positive selection and secondarily random drift, because stabilizing mutants will increase due to negative shifts of ΔΔG and also the effect of stability change on selective advantage will be more amplified; see Eqs. (23) and (24) for the dependence of ΔΔG on ΔG, and Eq. (19) for the fitness of stability change. As shown in Fig. S.6, the probability of \( \frac{K_a}{K_s} > 1.0 \), that is, positive selection, significantly increases as ΔG becomes more positive than the equilibrium stability ΔG_e. On the other hand, if a wild-type protein becomes more stable than the equilibrium, ΔG < ΔG_e, more destabilizing mutants will fix due to random drift, because destabilizing mutants will increase due to positive shifts of ΔΔG and also more destabilizing mutants become nearly neutral due to the less-amplified effect of stability change on selective advantage. As shown later, the PDF of \( \frac{K_a}{K_s} \) in the vicinity of equilibrium confirms this mechanism for maintaining protein stability at equilibrium.

It was claimed (Serohijos et al., 2012, 2013) that the equilibrium point would correspond to the minimum of the average fixation probability. However, in Fig. 4 for log 4N_eκ = 7.550 and θ = 0.53, the average ⟨s⟩_{fixed} of selective advantage in fixed mutants has a minimum at ΔG = −5.50 kcal/mol and changes its sign at ΔG = −4.58 kcal/mol, where the average ⟨K_a/K_s⟩ = ⟨u⟩/q has a minimum and which is more positive than the equilibrium stability ΔG_e = −5.24 kcal/mol. In other words, Figs. 4 and S.16 show that the values of ΔG at ⟨ΔΔG⟩_{fixed} = 0 and at the minimum of ⟨K_a/K_s⟩ may be close but differ from each other, and indicate that the value of ΔG corresponding to the minimum of ⟨K_a/K_s⟩ is not a good approximation for the equilibrium stability, because ⟨K_a/K_s⟩ gently changes in the vicinity of the equilibrium stability as shown in Fig. S.16.
Figure 5: Dependence of equilibrium stability, $\Delta G_e$, on parameters, $4N_e\kappa$ and $\theta$. $\Delta G_e$ is the equilibrium value of folding free energy, $\Delta G$, in protein evolution. The value of $\beta \Delta G_e + \log 4N_e\kappa$ is the upper bound of $\log 4N_e\kappa$, and would be constant if the mean of $\Delta\Delta G$ in all arising mutants did not depend on $\Delta G$; see Eq. (19). The kcal/mol unit is used for $\Delta G_e$. 
3.3. Equilibrium stability, $\Delta G_e$

The equilibrium value, $\Delta G_e$, of $\Delta G$ that satisfies $\langle \Delta \Delta G_{\text{fixed}} \rangle = 0$ in fixed mutants depends directly on $\theta$ and indirectly on $4N_e\kappa$ through fixation probability; see Eqs. (19) and (25). As shown in Fig. 5, $\Delta G_e$ depends weakly on $\theta$. On the other hand, $\Delta G_e$ depends more strongly on and is almost negatively proportional to $\log 4N_e\kappa$, as also shown in real proteins (Serohijos et al., 2013). If the dependence of the means, $\mu_s$ and $\mu_c$, in Eqs. (23) and (24), of $\Delta \Delta G$ in all mutants on $\Delta G$ could be neglected, $4N_e\kappa \exp(\beta \Delta G_e)$ could be regarded as a single parameter, and so $4N_e\kappa \exp(\beta \Delta G_e)$ would be constant, irrespective of $4N_e\kappa$. Thus, the dependence of $\log 4N_e\kappa \exp(\beta \Delta G_e)$ on $\log 4N_e\kappa$ shown in Fig. 5 is caused solely by the linear dependence of the means $\mu_s$ and $\mu_c$ of $\Delta \Delta G$ on $\Delta G$ (Serohijos et al., 2012). It is interesting to know that as $\log 4N_e\kappa$ varies from 0 to 20, $\Delta G_e$ changes from $-1.5$ to $-12.5$ kcal/mol, the range of which is consistent with experimental values of protein folding free energies shown in Fig. 1.

3.4. $K_a/K_s$ at equilibrium, $\Delta G = \Delta G_e$

Equations (23) and (24) indicate that the distribution of $\Delta \Delta G$ shifts toward the positive direction as $\Delta G$ becomes more negative. Hence, increasing $4N_e\kappa$ that makes $\Delta G_e$ more negative results in positive shifts of the distribution of $\Delta \Delta G$, which increase destabilizing mutations. In addition, as indicated by Eq. (19), the upper bound of $4N_e\kappa \exp(\beta \Delta G_e)$, scales the effect of $\Delta \Delta G$ on protein fitness. The larger $4N_e\kappa \exp(\beta \Delta G_e)$ is, the larger the effect of $\Delta \Delta G$ on selective advantage becomes. Thus, the increase of $4N_e\kappa \exp(\beta \Delta G_e)$ caused by the increase of $\kappa$ and/or $N_e$ increases both destabilizing mutations and their fitness costs, and results in slow evolutionary rates for proteins with large $\kappa$ and/or $N_e$. In other words, highly expressed and indispensable genes, and genes with a large effective population size must evolve slowly. On the other hand, the decrease of $\theta$, that is, the increase of highly constrained residues directly shifts the average of $\Delta \Delta G$ in all arising mutants toward the positive direction, and causes slow evolutionary rates.
Figure 6: The average of $K_a/K_s$ over all mutants or over fixed mutants only at equilibrium of protein stability, $\Delta G = \Delta G_e$. 
The average of $K_a/K_s$ over all mutants, which can be observed as the ratio of average nonsynonymous substitution rate per nonsynonymous site to average synonymous substitution rate per synonymous site, and also that over fixed mutants only are shown in Fig. 6. At any value of $\theta$, $\langle K_a/K_s \rangle$ decreases as $\log 4N_e \kappa$ increases, explaining the observed relationship that highly expressed and indispensable genes evolve slowly (Drummond et al., 2005; Drummond and Wilke, 2008; Serohijos et al., 2012). Likewise, at any value of $\log 4N_e \kappa$, $\langle K_a/K_s \rangle$ increases as $\theta$ increases. In other words, the more structurally constrained a protein is, the more slowly it evolves. The effect of protein abundance/indispensability on evolutionary rate is more remarkable for less constrained proteins and the effect of structural constraint is more remarkable for less abundant, less essential proteins.

The average of $K_a/K_s$ over all mutants, $\langle K_a/K_s \rangle$, is less than 1.0 on the whole domain shown in the figure, indicating that the average of $K_a/K_s$ over a long time interval and over many sites should not show any positive selection. Even the average of $K_a/K_s$ over fixed mutants is less than 1.0, and falls into a narrow range of 0.97–0.85, which is much narrower than a range of 0.96–0.15 for that over all mutants; the average of $K_a/K_s$ over fixed mutants is equal to $\langle (K_a/K_s)^2 \rangle/\langle K_a/K_s \rangle$, and as a matter of course must be equal to or larger than the averages of $K_a/K_s$ over all mutants. However, the average of $K_a/K_s$ over a short time interval and over a small number of sites may exhibit values larger than one. In Fig. 7 the PDFs of $K_a/K_s$ for all mutants and also for fixed mutants only are shown; $p(K_a/K_s) = p(4N_e s) d(4N_e s)/d(K_a/K_s)$. A significant fraction of fixed mutants fix with $K_a/K_s > 1$. 

21
Figure 7: PDFs of $K_a/K_s$ in all mutants and in fixed mutants only at equilibrium of protein stability, $\Delta G = \Delta G_e$. Unless specified, $\log 4N_{a,K} = 7.55$ and $\theta = 0.53$ are employed.
Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories whose $K_a/K_s$ are within the range of $0.5 \leq K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively. Then, the probabilities of each selection category in all mutants and in fixed mutants are calculated and shown in Figs. S.10 and S.8, respectively. At the largest abundance ($\log 4N_e\kappa = 20$) most arising mutations are negative mutations whose $K_a/K_s$ are less than 0.5. This is reasonable, because at this condition the wild-type protein is very stable with low equilibrium values $\Delta G_e$ as shown in Fig. 5 and therefore most mutations destabilize the wild type and tend to be removed from population. Most fixed mutants are positive mutants or slightly negative mutants fixed by random drift. Nearly neutral mutants are less than 3% of all mutants, and less than 15% of fixed mutants.
Figure 8: Probability of each selection category in fixed mutants at equilibrium of protein stability, $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively.
On the other hand, at the other extreme of log $4N_e \kappa < 2$, there are no mutations of the positive selection category, this is because the upper bound of $K_a/K_s$, which corresponds to the upper bound $(4N_e \kappa \exp(\beta \Delta G))$ of $4N_e \kappa$, at the equilibrium stability $\Delta G_e$ becomes less than 1.05 that is the lower bound for the positive selection category; see Eq. (19). The significant amount of mutations become nearly neutral. As $\theta$ changes from 1 to 0, that is, structural constraints increase, the proportion of nearly neutral mutations changes from 0.75(0.56) to 0.31(0.22), and instead negative mutations increase and most of them are removed from population. Thus, the selection mechanism for fixing stabilizing mutants in little expressed, non-essential genes (log $4N_e \kappa < 2$) is not positive selection but nearly neutral selection, that is, random drift.

The probability of each selection category in fixed mutants depends strongly on $4N_e \kappa$, but much less on $\theta$. Current common understanding is that amino acid substitutions in protein evolution are either neutral (Kimura 1968) or lethal, at most slightly deleterious (Ohta 1973) or lethal, unless functional selection operates and functional changes occur. On the contrary, nearly neutral fixations are predominant only in proteins with log $4N_e \kappa < 2$ or $\Delta G_e > -2.5$ kcal/mol, and positive selection is significant in the other proteins. On the other hand, slightly negative selection is always significant. An interesting result is that the effects of structural constraint on $K_a/K_s$ are the most remarkable in proteins with log $4N_e \kappa < 2$ or instead $\Delta G_e > -2.5$ kcal/mol in which nearly neutral fixations are predominant.

3.5. $K_a/K_s$ in the vicinity of equilibrium

In Fig. 4 the $\langle \Delta \Delta G \rangle_{\text{fixed}} \pm$ standard deviation of $\Delta \Delta G$ of fixed mutants are also drawn. The standard deviation of $\Delta \Delta G$ of fixed mutants is equal to 0.84 kcal/mol at the equilibrium, $\Delta G_e$, indicating that protein stability $\Delta G$ fluctuates more or less within $\Delta G_e \pm 0.84$ kcal/mol instantaneously. Such a deviation from the equilibrium must be canceled by compensatory substitutions that consecutively occur, otherwise the protein stability would far depart from its equilibrium point.

In Figs. 9 and 10 and Figs. S.12 and S.14, the probabilities of each selection category in fixed mutants and in all arising mutants are shown as a function of $\Delta G$ and $4N_e \kappa$ or $\theta$, respectively. The range of $\Delta G$ around $\Delta G_e$ shown by a blue line on the surface grid is within two times of the standard deviation of $\Delta \Delta G$ over fixed mutants at $\Delta G_e$. 

25
Figure 9: **Dependence of the probability of each selection category in fixed mutants on $4N_e\kappa$ and $\Delta G$.** A blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta \Delta G_{\text{sd}}^{\text{fixed}}$, where $\Delta \Delta G_{\text{sd}}^{\text{fixed}}$ is the standard deviation of $\Delta \Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively. $\theta = 0.53$ is employed. The kcal/mol unit is used for $\Delta G$. 
Figure 10: Dependence of the probability of each selection category in fixed mutants on $\theta$ and $\Delta G$. A blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta G_{fd}^{\text{fixed}}$, where $\Delta G_{fd}^{\text{fixed}}$ is the standard deviation of $\Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively. $\log 4N_e \kappa = 7.55$ is employed. The kcal/mol unit is used for $\Delta G$. 

27
As indicated by Eqs. (23) and (24), it is shown in Figs. S.12 and S.14 that stabilizing mutations increase due to negative shifts of $\Delta\Delta G$ as the wild type becomes less stable than the equilibrium, $\Delta G > \Delta G_e$, and that destabilizing mutations increase due to positive shifts of $\Delta\Delta G$ as $\Delta G < \Delta G_e$. In addition, as indicated by Eq. (19), it is shown in Figs. 9 and 10 that positive selection on stabilizing mutants is more amplified as $\Delta G > \Delta G_e$, and that more destabilizing mutations become nearly neutral due to the less-amplified effect of stability change on selective advantage as $\Delta G < \Delta G_e$. This is a mechanism of maintaining protein stability at equilibrium.
Figure 11: Dependence of the average of $K_a/K_s$ over all mutants or over fixed mutants only on protein stability, $\Delta G$, of the wild type. A blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta \Delta G_{sd}$, where $\Delta \Delta G_{sd}$ is the standard deviation of $\Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Unless specified, $\log 4N_e \kappa = 7.55$ and $\theta = 0.53$ are employed. The kcal/mol unit is used for $\Delta G$. 

29
3.6. Lower bound of $K_a/K_s$ for adaptive substitutions on protein function

The observed value of $K_a/K_s > 1$ is often used to indicate functional selection. The averages of $K_a/K_s$ over all mutants and even over fixed mutants are less than 1 as shown in Fig. 6. Therefore, the average of $K_a/K_s$ over long time or many sites does not indicate positive selection. However, the probability of $K_a/K_s > 1$ is not negligible as shown in Figs. 7 and 8. Then, a question is how large $K_a/K_s$ due to selection on protein stability can be.

The distribution of $K_a/K_s$ significantly changes with $\Delta G$, as shown in Figs. S.6 and 11. It may be appropriate to see the average of $K_a/K_s$, $\langle K_a/K_s \rangle_{\text{fixed}}$, in mutants fixed at $\Delta G > \Delta G_e$, because the upper bound of $K_a/K_s$ becomes larger for $\Delta G > \Delta G_e$ than at the equilibrium, and also positive mutants must fix to improve the protein stability of the wild type. Fig. 11 shows that $\langle K_a/K_s \rangle_{\text{fixed}}$ can be very large for proteins with low equilibrium stabilities (large $4N_e\kappa$ and small $\theta$), although $\langle K_a/K_s \rangle_{\text{fixed}} \sim 1$ in $\Delta G < \Delta G_e$ in which nearly neutral and slightly negative selections are predominant; 1.7(1.2) at $\Delta G_e + \Delta \Delta G_{\text{fixed}}$ and 6.1(5.6) at $\Delta G_e + 2 \cdot \Delta \Delta G_{\text{fixed}}$ for log $4N_e\kappa = 20$ ($\theta = 0.0$), where $\Delta \Delta G_{\text{fixed}}$ means the standard deviation of $\Delta \Delta G$ in fixed mutants at $\Delta G_e$. The 85% of fixed mutants have $\Delta \Delta G$ within the standard deviation. Therefore, a lower bound for adaptive substitutions may be taken to be about 1.7, which is almost equal to the upper bound of $K_a/K_s$ at the equilibrium for log $4N_e\kappa = 20$ and $\theta = 1$; see Fig. S.17. However, as shown in Fig. S.17, the more genes are expressed and/or the stronger structural constraints are, the larger the upper bound of $K_a/K_s$ at the equilibrium is. Judging of adaptive changes may need not only $K_a/K_s > 1$ but also other supporting evidences; such that substitutions are localized at specific sites.
Figure 12: Dependence of equilibrium stability, $\Delta G_e$, on parameters, $4N_e \kappa$ and $T$. $\Delta G_e$ is the equilibrium value of folding free energy, $\Delta G$, in protein evolution. $T$ is absolute temperature; $\beta = 1/kT$, where $k$ is the Boltzmann constant. Equations (22), (23) and (24) are assumed for the distribution of $\Delta \Delta G$ and its dependency on $\Delta G$; they are assumed to be independent of $T$. $\theta = 0.53$ is employed. The value of $\beta \Delta G_e + \log 4N_e \kappa$ is the upper bound of $\log 4N_e s$, and would not depend on $\log 4N_e \kappa$ if the mean of $\Delta \Delta G$ in all arising mutants did not depend on $\Delta G$; see Eq. (19). The kcal/mol unit is used for $\Delta G_e$. 
3.7. Dependences of protein stability (\(\beta \Delta G_e\)) and evolutionary rate (\(\langle K_a/K_s \rangle\)) on growth temperature

It is natural that the folding free energies, \(\Delta G_e\), of proteins in organisms growing at higher temperatures must be lower than those at the normal temperature, in order to attain the same stabilities and fitnesses as in the normal temperature. Equations (2) and (15) indicate that the same stability and fitness will be attained if \(\beta \Delta G_e\) is constant. It means that it is sufficient for \(\Delta G_e\) at the 100°C to decrease 373/298 = 1.25 times of that at the normal temperature (25°C). Is this a figure expected for folding free energies of thermophilic proteins at high growth temperature? It is not enough data of \(\Delta G\) at high temperature in the ProTherm (Kumar et al., 2006) to answer this question; \(\Delta G(T = 75°C) = 10.76 \text{ kcal/mol for oxidized and 4.3 for reduced CuA domain of cytochrome oxidase from Thermus thermophilus (Wittung-Stafshede et al., 1998)}\) and \(\Delta G(T = 60°C) = 13.01 \text{ kcal/mol for pyrrolidone carboxyl peptidase from Pyrococcus (Ogasahara et al., 1998)}\). The present model indicates that \(\beta \Delta G_e\) slightly increases as growth temperature increases.

In Figs. 12 and S.18 \(\beta \Delta G_e + \log 4N_e\kappa\) is shown as a function of absolute temperature \(T\) and \(\log 4N_e\kappa\) or \(\theta\), assuming that the distribution of \(\Delta \Delta G\) and its dependency on \(\Delta G\) do not depend on \(T\), that is, Eqs. (22), (23) and (24). At fixed values of \(\log 4N_e\kappa\) and \(\theta\), \(\beta \Delta G_e + \log 4N_e\kappa\) increases as \(T\) increases, meaning that protein stability, \(-\beta \Delta G_e\), decreases as growth temperature increases. This tendency is slightly larger at smaller values of \(\log 4N_e\kappa\), that is, for less abundant proteins.

The effects of growth temperature on \(K_a/K_s\) are shown in Fig. S.19. The present model predicts that \(\langle K_a/K_s \rangle\) decreases as growth temperature increases unless any other parameter does not change.

4. Discussion

Recently, fitness costs due to misfolded proteins have been widely noticed, particularly neurological disorder linked to misfolded protein toxicity (Bucciantini et al., 2002). Fitness costs that originate in functional loss (Geiler-Samerotte et al., 2011) and in diversion of protein synthesis and aggregation of proteins have been evaluated (Drummond and Wilke, 2008) to be related to the proportion of misfolded proteins. Also, previous studies indicate that factors that relate protein stability to protein fitness are protein abundance, protein indispensability, and structural constraints of protein. Current knowledge of protein folding can
provide an exact formulation for the proportion of misfolded proteins as a function of folding free energy, and reasonable predictions (Schymkowitz et al., 2005; Yin et al., 2007; Tokuriki et al., 2007) of stability changes due to single amino acid substitutions in protein native structures. Thus, on the basis of knowledge of protein biophysics it became possible to study the effects of amino acid substitutions on protein stability and then the evolution of protein (Drummond and Wilke, 2008; Serohijos et al., 2012; Serohijos and Shakhnovich, 2014; Echave et al., 2015; Faure and Koonin, 2015).

Here, the effects of protein abundance and indispensability ($\kappa$) and of structural constraint ($\theta$) on protein evolutionary rate ($K_a/K_s$) have been examined in detail. Both the effects are represented with different functional forms. Structural constraints affect the distribution of stability change $\Delta\Delta G$ due to mutations. On the other hand, protein abundance/indispensability affects the effectiveness of stability change on protein fitness as well as the distribution of $\Delta\Delta G$.

The common understanding of protein evolution has been that amino acid substitutions found in homologous proteins are selectively neutral (Kimura, 1968, 1969; Kimura and Ohta, 1971, 1974) or slightly deleterious (Ohta, 1973, 1992), and random drift is a primary force to fix amino acid substitutions in population. However, there is a selection maintaining protein stability at equilibrium (Drummond and Wilke, 2008; Serohijos et al., 2012; Serohijos and Shakhnovich, 2014). From the present analysis of the PDF of $K_a/K_s$, it has become clear how the equilibrium of stability is maintained; see Figs. 9 and 10. In less-stable proteins of $\Delta G > \Delta G_e$, more stabilizing mutations fix due to positive selection, because negative shifts of $\Delta\Delta G$ increase stabilizing mutants and also more amplify the effect of stability change on selective advantage; see Eqs. (23), (24) and (19). In more-stable proteins of $\Delta G < \Delta G_e$, more destabilizing mutations are fixed by random drift, because positive shifts of $\Delta\Delta G$ increase destabilizing mutants and also make more destabilizing mutants nearly neutral with the less-amplified effect of stability change on selective advantage. It has been revealed that contrary to the neutral theory nearly neutral selection is predominant only in low-abundant, non-essential proteins with log $4N_e\kappa < 2$ or with low equilibrium stability ($\Delta G_e > -2.5$ kcal/mol); see Fig. 8.

The average $\langle K_a/K_s \rangle$ and even $\langle K_a/K_s \rangle_{\text{fixed}}$ at equilibrium stability $\Delta G = \Delta G_e$ are less than one over the whole parameter range; see Fig. 6. Hence, as far as selection is on protein stability, the average of $K_a/K_s$ over a long time interval and over many sites will be expected to be less than one, if all synonymous mutations are neutral (Spielman and Wilke, 2015). However, because the probability of $K_a/K_s > 1$ is significant, branches with $K_a/K_s > 1$ in phylogenetic trees
may be observed, as observed in a population dynamics simulation (Serohijos and
Shakhnovich, 2014), even though synonymous mutations are neutral and no adap-
tive selection operates on protein function. According to the present estimate, a
lower bound of $K_a/K_s$ to indicate adaptive substitutions must be at least as large
as 1.7.

Protein equilibrium stability ($\Delta G_e$) has been clearly described here as a func-
tion of $4N_e \kappa$ and $\theta$. The more expressed a gene is (the larger $4N_e \kappa$ is), the stabler
the wild-type protein at equilibrium is (the more negative $\Delta G_e$ becomes); see Fig.
5. The decrease of $\Delta G_e$ shifts the distribution of $\Delta \Delta G$ toward the positive di-
rection, generating more highly destabilizing mutants; see Eqs. (23) and (24). In
addition, as $4N_e \kappa$ increases, the net effect, $4N_e \kappa \exp(\beta \Delta G_e)$, increases and more
amplifies the effects of stability changes ($\Delta \Delta G$) on selective advantage ($s$); see
Fig. 5 and Eq. (19). As a result, highly expressed and indispensable genes, and
genes with a large effective population size evolve slowly; see Fig. 6. However, if
the distribution of $\Delta \Delta G$ did not depend on $\Delta G$, $4N_e \kappa \exp(\beta \Delta G_e)$ would be constant,
and $K_a/K_s$ would not depend on $4N_e \kappa$, that is, protein abundance/indispensability
and effective population size.

On the other hand, structural constraints on protein affect protein evolution-
ary rate by changing the distribution of $\Delta \Delta G$ due to amino acid substitutions.
As shown in Fig. 6 at any value of log $4N_e \kappa$, $\langle K_a/K_s \rangle$ decreases as $\theta$ decreases.
In other word, the more a protein is structurally constrained, the more slowly it
evolves, as claimed by Zuckerkandl (Zuckerkandl 1976). Fig. 6 shows that the
effect of protein abundance/indispensability on evolutionary rate is more remark-
able for less constrained proteins, and the effect of structural constraint is more
remarkable for less abundant, less essential proteins.

In the result, the average of $K_a/K_s$ over all arising mutants decreases roughly
by $0.4 - 0.8$ as log $4N_e \kappa$ increases from 0 to 20; see Fig. 6. On the other hand,
it decreases by $0.1 - 0.4$ as the proportion of the residues of the surface type,
$\theta$, decreases from 1 to 0. For monomeric, globular proteins, the proportion of
protein surface may range from 0.7 to 0.45. Thus, in typical globular proteins,
protein abundance/indispensability may cause larger differences of evolutionary
rate between proteins than structural constraint. However, proteins that interact
with other molecules on protein surface effectively reduce residues of the protein-
surface type (Franzosa and Xia, 2009). Both protein abundance/indispensability
and structural constraint must be taken into account for protein evolutionary rate.

Protein abundance and indispensability both affect evolutionary rate similarly
through protein fitness. It was shown in real proteins that protein abundance corre-
lates with evolutionary rate (Pál et al., 2001). The present model of protein fitness
(Eq. (19)) also indicates that protein indispensability must correlate with evolutionary rate \cite{Hirsh2001,Hirsh2003}, but a correlation between them may be hidden by the variation of protein abundance and detected only in low-abundant proteins \cite{Pal2003}; see Eq. (21). In addition, effective population size must affect $\Delta G_e$ and $\langle K_a/K_s \rangle$ together with $\kappa$ as $4N_e \kappa$.

In the present model, protein equilibrium stability ($\Delta G_e$) and evolutionary rate ($\langle K_a/K_s \rangle$) are predictable from $\theta$ and $4N_e \kappa$. The proportion of the surface type of residues may be estimated as those whose surface accessibility values (ASA) are less than 0.25 \cite{Tokuriki2007}, but experimental measurements of protein abundance, indispensability, and effective population size to determine $4N_e \kappa$ may be relatively hard. Instead the experimental value of protein stability may be employed as equilibrium stability to predict evolutionary rate and others, although it is not an independent variable. Fig. \ref{fig:13} shows evolutionary rate as a function of $\Delta G_e$ and $\theta$. Needless to say, mutational effects on $\Delta G$, such as $\theta$ and the distribution of $\Delta \Delta G$, must be well estimated for various categories of proteins \cite{Faure2015} to obtain successful predictions. Also, accurate estimations of $\Delta G$ for various proteins are needed to examine the present predictions. It is interesting to examine if protein stability ($-\beta \Delta G$) and $K_a/K_s$ decrease as growth temperature increases.

![Figure 13: The average of $K_a/K_s$ over all mutants as a function of $\Delta G_e$ and $\theta$.](image)

Figure 13: The average of $K_a/K_s$ over all mutants as a function of $\Delta G_e$ and $\theta$. 

35
5. Conclusions

- The range, $-2$ to $-12.5$ kcal/mol, of equilibrium values, $\Delta G_e$, of protein stability calculated with the present fitness model is consistent with the distribution of experimental values shown in Fig. 1.

- Contrary to the neutral theory, nearly neutral selection is predominant only in low-abundant, non-essential proteins of $\log 4N_e\kappa < 2$ or $\Delta G_e > -2.5$ kcal/mol. In the other proteins, positive selection on stabilizing mutations is significant to maintain protein stability at equilibrium as well as random drift on slightly negative mutations. However, $\langle K_a/K_s \rangle$ and even $\langle K_a/K_s \rangle_{\text{fixed}}$ at $\Delta G = \Delta G_e$ are less than 1.

- Protein abundance/indispensability ($\kappa$) and effective population size ($N_e$) more affect evolutionary rate for less constrained proteins, and structural constraint ($1 - \theta$) for less abundant, less essential proteins.

- Protein indispensability must negatively correlate with evolutionary rate like protein abundance, but the correlation between them may be hidden by the variation of protein abundance and detected only in low-abundant proteins.

- Evolutionary rates of proteins may be predicted from equilibrium stability ($\Delta G_e$) and structural constraints (PDF of $\Delta \Delta G$) of the protein.

- The present model indicates that protein stability ($-\beta \Delta G_e$) and $\langle K_a/K_s \rangle$ decrease as growth temperature increases.

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Malthusian fitness originating in protein stability in the present model

For typical proteins whose folding free energy $\Delta G$ satisfies $\exp(\beta \Delta G) \ll 1$, without loss of generality we can assume the Malthusian fitness of the single protein-coding genes to be equal to

$$m = -\kappa e^{\beta \Delta G} \quad \text{with } \kappa \geq 0 \quad \text{(S.1)}$$

where $\beta = 1/kT$, $k$ is the Boltzmann constant, $T$ is absolute temperature, and $\kappa$ is a parameter whose meaning may depend on the situation; refer to Method for details. If the fitness costs of functional loss and toxicity due to misfolded proteins are both taken into account and assumed to be additive in the Malthusian fitness scale, $\kappa$ will be defined as

$$\kappa = cA + \gamma \quad \text{(S.2)}$$

where $c$ is fitness cost per misfolded protein (Geiler-Samerotte et al., 2011), $A$ is the cellular abundance of a protein (Geiler-Samerotte et al., 2011), and $\gamma$ is indispensability (Drummond and Wilke, 2008) and defined as $\gamma \equiv -\log(\text{deletion-strain growth rate} / \text{max growth rate})$. The parameter $\kappa$ is assumed in the present analysis to take values in the range of $0 \leq \log 4N_e \kappa \leq 20$ with effective population size $N_e$, taking account of the values of the parameters, $c \sim 10^{-4}$ (Drummond and Wilke, 2008), $10 < A < 10^6$ (Ghaemmaghami et al., 2003), $\gamma = 10$ for essential genes (Drummond and Wilke, 2008), and $N_e \sim 10^4$ to $10^5$ for vertebrates, $\sim 10^5$ to $10^6$ for invertebrates, $\sim 10^4$ to $10^8$ for unicellular eukaryotes, and $> 10^8$ for prokaryotes (Lynch and Conery, 2003).
Then, the selective advantage of a mutant protein is given as follows in Malthusian parameters as a function of the folding free energy ($\Delta G$) of the wild-type protein, the stability change ($\Delta\Delta G$) of a mutant protein, and the parameter $\kappa$:

$$s \equiv m^{\text{mutant}} - m^{\text{wildtype}} = \kappa e^{\beta \Delta G} (1 - e^{\beta \Delta\Delta G}) \quad (S.3)$$

Equation (S.3) indicates that $s$ is upper-bounded.

$$s \leq \kappa e^{\beta \Delta G} \quad (S.4)$$

**Distribution of stability changes ($\Delta\Delta G$) due to single amino acid substitutions**

Here, according to (Tokuriki et al., 2007), the distribution of folding free energy changes, $\Delta\Delta G$, of mutant proteins is assumed to be a bi-Gaussian function with mean depending on $\Delta G$, in order to take into account the effects of structural constraint on evolutionary rate. The probability density function (PDF), $p(\Delta\Delta G)$, of $\Delta\Delta G$ for nonsynonymous substitutions is defined as

$$p(\Delta\Delta G) = \theta N(\mu_s, \sigma_s) + (1 - \theta) N(\mu_c, \sigma_c) \quad (S.5)$$

where $0 \leq \theta \leq 1$, and $N(\mu, \sigma)$ is a normal distribution with mean $\mu$ and standard deviation $\sigma$. One of the two Gaussian distributions above, $N(\mu_s, \sigma_s)$, results from substitutions on protein surfaces and is a narrow distribution with a mildly destabilizing mean $\Delta\Delta G$, whereas the other, $N(\mu_c, \sigma_c)$, due to substitutions in protein cores is a wider distribution with a stronger destabilizing mean (Tokuriki et al., 2007). Since the majority of substitutions appear to be single nucleotide substitutions, the values of standard deviations ($\sigma_s$ and $\sigma_c$) estimated in (Tokuriki et al., 2007) for single nucleotide substitutions are employed here; in kcal/mol units,

$$\mu_s = -0.139 \Delta G - 0.168 \quad , \quad \sigma_s = 0.90 \quad (S.6)$$
$$\mu_c = -0.139 \Delta G + 1.232 \quad , \quad \sigma_c = 1.93 \quad (S.7)$$

To analyze the dependences of the means, $\mu_s$ and $\mu_c$, on $\Delta G$, we plotted the observed values of $\Delta\Delta G$ of single amino acid mutants against $\Delta G$ of the wild type, which are collected in the ProTherm database (Kumar et al., 2006); the same analysis was done in (Serohijos et al., 2012). Fig. S.2 shows a significant dependence of $\Delta\Delta G$ on $\Delta G$; the regression line is $\mu = -0.139\Delta G + 0.490$. The linear slopes of $\mu_s$ and $\mu_c$ are taken to be equal to the slope ($-0.139$) of the regression line. The intercepts have been estimated to satisfy the following two conditions.
1. Equations (S.6) and (S.7) satisfy $\mu_x(\Delta G_0) = 0.56$ and $\mu_x(\Delta G_0) = 1.96$, which were estimated for single nucleotide substitutions in (Tokuriki et al., 2007), at a certain value ($\Delta G_0$) of $\Delta G$.

2. The total mean of the two Gaussian functions agrees with the regression line, $\mu = -0.139\Delta G + 0.490$. The value of $\theta$ is taken to be 0.53, which is equal to the average of $\theta$ over proteins used in (Tokuriki et al., 2007).

A representative value, 7.550, of $\log 4N_e\kappa$ is determined in such way that the equilibrium value of $\Delta G$ is equal to $\Delta G_0 = -5.24$ introduced above. It is interesting that this value $\Delta G_e = -5.24$ kcal/mol agrees with the most probable value of $\Delta G$ in the observed distribution of protein stabilities shown in Fig. 1. The fraction $\theta$ of less-constrained residues such as most residues on protein surface is correlated with protein length for globular, monomeric proteins (Tokuriki et al., 2007); $\theta = 1.27 - 0.33 \cdot \log_{10}$(protein length) for $50 \leq \text{length} \leq 330$. (Tokuriki et al., 2007). However, residues taking part in protein–protein interactions may be regarded as core residues rather than surface residues.

**Probability distributions of selective advantage, fixation rate and $K_a/K_s$**

Now, we can consider the probability distributions of characteristic quantities that describe the evolution of genes. First of all, the PDF of selective advantage $s$, $p(s)$, of mutant genes can be represented by

$$p(s) = -p(\Delta \Delta G) \frac{d\Delta \Delta G}{ds} = \frac{p(\Delta \Delta G)}{\beta(\kappa e^{\beta \Delta G} - s)}$$

where $\Delta \Delta G$ must be regarded as a function of $s$, that is, $\Delta \Delta G = \beta^{-1} \log(1 - s(\kappa e^{\beta \Delta G})^{-1})$. The PDF of $4N_e s$, $p(4N_e s)$, may be more useful than $p(s)$.

$$p(4N_e s) = p(s) \frac{1}{4N_e}$$

The fixation probability $u$ of a mutant gene with selective advantage $s$ and gene frequency $q$ in a duploid system is equal to (Crow and Kimura, 1970)

$$u(4N_e s) = \frac{1 - e^{-4N_e sq}}{1 - e^{-4N_e s}}$$

where $q = 1/(2N)$ for a single mutant gene in a population of size $N$. Population size is taken to be $N = 10^6$. Thus, the PDF of fixation probability $u$ can be
represented by

\[
p(u) = p(4N_e s) \frac{d4N_e s}{du} = p(4N_e s) \left( \frac{(e^{4N_e s} - 1)^2 e^{4N_e s(q-1)}}{q(e^{4N_e s} - 1) - (e^{4N_e s} - 1)} \right) \quad (S.11)
\]

where \( s \) must be regarded as a function of \( u \).

The ratio of the substitution rate per nonsynonymous site \( (K_a) \) for nonsynonymous substitutions with selective advantage \( s \) to the substitution rate per synonymous site \( (K_s) \) for nonsynonymous substitutions with \( s = 0 \) is

\[
\frac{K_a}{K_s} = \frac{u(4N_e s)}{u(0)} = \frac{u(4N_e s)}{q} \quad (S.12)
\]

\[
\approx \frac{4N_e s}{1 - e^{-4N_e s}} \quad \text{for } \frac{|4N_e s q|}{2} \ll 1 \quad (S.13)
\]

assuming that synonymous substitutions are completely neutral and mutation rates at both types of sites are the same. The PDF of \( K_a/K_s \) is

\[
p(K_a/K_s) = p(u) \frac{du}{d(K_a/K_s)} = p(u) q \quad (S.14)
\]

\[
\approx p(4N_e s) \left( \frac{(e^{4N_e s} - 1)^2}{e^{4N_e s}(e^{4N_e s} - 1 - 4N_e s)} \right) \quad \text{for } \frac{|4N_e s q|}{2} \ll 1 \quad (S.15)
\]

In the range of \( |4N_e s q|/2 \ll 1 \), both \( K_a/K_s \) and \( p(K_a/K_s) \) do not depend on \( q = 1/(2N) \).

**Probability distributions of \( \Delta \Delta G \), \( 4N_e s \), \( u \), and \( K_a/K_s \) in fixed mutant genes**

Now, let us think about fixed mutant genes. The PDF of the \( \Delta \Delta G \) of fixed mutant genes is

\[
p(\Delta \Delta G_{\text{fixed}}) = p(\Delta \Delta G) \frac{u(4N_e s(\Delta \Delta G))}{\langle u(4N_e s(\Delta \Delta G)) \rangle} \quad (S.16)
\]

\[
\langle u \rangle \equiv \int_{-\infty}^{\infty} u(4N_e s) p(\Delta \Delta G) d\Delta \Delta G \quad (S.17)
\]

\[
= \int_{-\infty}^{4N_e s \exp(\beta \Delta G)} u(4N_e s) p(4N_e s) d4N_e s \quad (S.18)
\]

Likewise, the PDF of the selective advantage of fixed mutant genes is

\[
p(4N_e s_{\text{fixed}}) = p(4N_e s) \frac{u(4N_e s)}{\langle u(4N_e s) \rangle} \quad (S.19)
\]
and those of the $u$ and $K_a/K_s$ of fixed mutant genes are

\[
p(u_{\text{fixed}}) = p(u) \frac{u}{\langle u \rangle} \quad \text{(S.20)}
\]

\[
p\left(\frac{K_a}{K_s}\right)_{\text{fixed}} = p\left(\frac{K_a}{K_s}\right) \frac{u}{\langle u \rangle} = p\left(\frac{K_a}{K_s}\right) \frac{\frac{K_a}{K_s}}{\langle \frac{K_a}{K_s} \rangle} \quad \text{(S.21)}
\]

Then, the probabilities of $a < K_a/K_s < b$ and the averages of $K_a/K_s$ over all mutants and also in fixed mutants can be calculated. The average of $K_a/K_s$ in fixed mutants is equal to the ratio of the second moment to the first moment of $K_a/K_s$ in all arising mutants.

\[
\langle \frac{K_a}{K_s} \rangle_{\text{fixed}} = \frac{\langle (\frac{K_a}{K_s})^2 \rangle}{\langle \frac{K_a}{K_s} \rangle} \quad \text{(S.22)}
\]
Figure S.1: **Distribution of folding free energies of monomeric protein families.** Stability data of monomeric proteins for which the item of \( \text{dG}_{\text{H2O}} \) or \( \text{dG} \) was obtained in the experimental condition of \( 6.7 \leq \text{pH} \leq 7.3 \) and \( 20^\circ C \leq T \leq 30^\circ C \) and their folding-unfolding transition is two state and reversible are extracted from the ProTherm (Kumar et al., 2006); in the case of \( \text{dG} \) only thermal transition data are used. Thermophilic proteins, and proteins observed with salts or additives are also removed. An equal sampling weight is assigned to each species of homologous protein, and the total sampling weight of each protein family is normalized to one. In the case in which multiple data exist for the same species of protein, its sampling weight is divided to each of the data. However, proteins whose stabilities are known may be samples biased from the protein universe. The value, \( \Delta G_e = -5.24 \text{ kcal/mol} \), of equilibrium stability at the representative parameter values, \( \log 4N_e \kappa = 7.550 \) and \( \theta = 0.53 \), agrees with the most probable value of \( \Delta G \) in the distribution above. Also, the range of \( \Delta G \) shown above is consistent with that range, \(-2\) to \(-12.5\) kcal/mol, expected from the present model. The kcal/mol unit is used for \( \Delta G \). A similar distribution was also compiled (Zeldovich et al., 2007).
Figure S.2: Dependence of stability changes, ΔΔG, due to single amino acid substitutions on the protein stability, ΔG, of the wild type. A solid line shows the regression line, ΔΔG = −0.139ΔG + 0.490; the correlation coefficient and p-value are equal to −0.20 and < 10⁻⁷, respectively. Broken lines show two means of bi-Gaussian distributions, μs in blue and μc in red. Blue dotted lines show μs ± 2σs and red dotted lines μc ± 2σc. See Eqs. (S.5), (S.6) and (S.7) for the bi-Gaussian distribution. Stability data of single amino acid mutants for which the items dG_H2O and ddG_H2O or dG and ddG were obtained in the experimental condition of 6.7 ≤ pH ≤ 7.3 and 20°C ≤ T ≤ 30°C and their folding-unfolding transitions are two state and reversible are extracted from the ProTherm [Kumar et al., 2006]. In the case of dG only thermal transition data are used. In the case in which multiple data exist for the same protein, only one of them is used. The kcal/mol unit is used for ΔΔG and ΔG. A similar distribution was also compiled [Seroñíos et al., 2012].
Figure S.3: PDFs of stability changes, $\Delta \Delta G$, due to single amino acid substitutions in all mutants and in fixed mutants at equilibrium of protein stability, $\Delta G = \Delta G_e$. The PDF of $\Delta \Delta G$ due to single amino acid substitutions in all arising mutants is assumed to be bi-Gaussian; see Eq. (S.5). Unless specified, log $4N_\kappa = 7.550$ and $\theta = 0.53$ are employed. The kcal/mol unit is used for $\Delta \Delta G$ and $\Delta G_e$. 
PDFs of $4N_e s$ in all mutants and in fixed mutants at equilibrium of protein stability, $\Delta G = \Delta G_e$. Unless specified, log $4N_e \kappa = 7.550$ and $\theta = 0.53$ are employed.
Figure S.5: The average, \( \langle \Delta \Delta G \rangle_{\text{fixed}} \), of stability changes over fixed mutants versus protein stability, \( \Delta G \), of the wild type. \( \Delta G_e \), where \( \langle \Delta \Delta G \rangle = 0 \), is the stable equilibrium value of folding free energy, \( \Delta G \), in protein evolution. The averages of \( \Delta \Delta G \), \( 4N_e \), and \( K_a/K_s \) over fixed mutants are plotted against protein stability, \( \Delta G \), of the wild type by solid, broken, and dash-dot lines, respectively. Thick dotted lines show the values of \( \langle \Delta \Delta G \rangle_{\text{fixed}} \pm \Delta \Delta G_{\text{fixed}} \), where \( \Delta \Delta G_{\text{fixed}} \) is the standard deviation of \( \Delta \Delta G \) over fixed mutants. \( \log 4N_e \kappa = 7.550 \) and \( \theta = 0.53 \) are employed. The kcal/mol unit is used for \( \Delta \Delta G \).
Figure S.6: Dependence of the PDF of $K_a/K_s$ on protein stability, $\Delta G$, of the wild type in all mutants or in fixed mutants only. $\Delta\Delta G_{\text{sd}}^{\text{fixed}}$ is the standard deviation (0.84 kcal/mol) of $\Delta\Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. $\log 4N_c\kappa = 7.550$ and $\theta = 0.53$ are employed. The kcal/mol unit is used for $\Delta G_e$. 
Figure S.7: **Dependence of equilibrium stability, \( \Delta G_e \), on parameters, \( 4N_e \kappa \) and \( \theta \). \( \Delta G_e \) is the equilibrium value of folding free energy, \( \Delta G \), in protein evolution. The value of \( \beta \Delta G_e + \log 4N_e \kappa \) is the upper bound of \( \log 4N_e s \), and would be constant if the mean of \( \Delta \Delta G \) in all arising mutants did not depend on \( \Delta G \); see Eq. (S.3). The kcal/mol unit is used for \( \Delta G_e \).
Figure S.8:  The average of $K_a/K_r$ over all mutants or over fixed mutants only at equilibrium of protein stability, $\Delta G = \Delta G_{x}$. 
Figure S.9: PDFs of $K_a/K_s$ in all mutants and in fixed mutants only at equilibrium of protein stability, $\Delta G = \Delta G_e$. Unless specified, log $4N_e\kappa = 7.550$ and $\theta = 0.53$ are employed.
Figure S.10: Probability of each selection category in all mutants at equilibrium of protein stability, $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively.
Figure S.11: Probability of each selection category in fixed mutants at equilibrium of protein stability, $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively.
Figure S.12: Dependence of the probability of each selection category in all mutants on $4N_eK$ and $\Delta G$. A blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta \Delta G_{sd}^{\text{fixed}}$, where $\Delta \Delta G_{sd}^{\text{fixed}}$ is the standard deviation of $\Delta \Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively. $\theta = 0.53$ is employed. The kcal/mol unit is used for $\Delta G$. 

S.17
**Figure S.13**: Dependence of the probability of each selection category in fixed mutants on $4N_e \kappa$ and $\Delta G$. A blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta \Delta G_{sd}^{fixed}$, where $\Delta \Delta G_{sd}^{fixed}$ is the standard deviation of $\Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively. $\theta = 0.53$ is employed. The kcal/mol unit is used for $\Delta G$. 

S.18
Figure S.14: Dependence of the probability of each selection category in all mutants on $\theta$ and $\Delta G$. A blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta \Delta G_{\text{fixed}}$, where $\Delta \Delta G_{\text{fixed}}$ is the standard deviation of $\Delta \Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes: negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively. $\log 4N_e\kappa = 7.550$ is employed. The kcal/mol unit is used for $\Delta G$. 

S.19
Figure S.15: **Dependence of the probability of each selection category in fixed mutants on $\theta$ and $\Delta G$.** The blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta \Delta G_{sd}^{\text{fixed}}$, where $\Delta \Delta G_{sd}^{\text{fixed}}$ is the standard deviation of $\Delta \Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Arbitrarily, the value of $K_a/K_s$ is categorized into four classes; negative, slightly negative, nearly neutral, and positive selection categories in which $K_a/K_s$ is within the ranges of $K_a/K_s \leq 0.5$, $0.5 < K_a/K_s \leq 0.95$, $0.95 < K_a/K_s \leq 1.05$, and $1.05 < K_a/K_s$, respectively. $\log 4N_e k = 7.550$ is employed. The kcal/mol unit is used for $\Delta G$. 

$\text{S.20}$
Figure S.16: Dependence of the average of $K_a/K_s$ over all mutants or over fixed mutants only on protein stability, $\Delta G$, of the wild type. A blue line on the surface grid shows $\Delta G = \Delta G_e$, which is the equilibrium value of $\Delta G$ in protein evolution. The range of $\Delta G$ shown in the figures is $|\Delta G - \Delta G_e| < 2 \cdot \Delta \Delta G_{sd}$, where $\Delta \Delta G_{sd}$ is the standard deviation of $\Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. Unless specified, $\log 4N_e \kappa = 7.550$ and $\theta = 0.53$ are employed. The kcal/mol unit is used for $\Delta G$. 

S.21
Figure S.17: Dependence of $\max K_a/K_s$ on $4N_e \kappa$ and $\theta$. The maximum values of $K_a/K_s$, which correspond to the upper bound of selective advantage $s$ (Eq. (S.4)), at $\Delta G = \Delta G_e$ and at $\Delta G = \Delta G_e + \Delta \Delta G_{\text{sd}}^{\text{fixed}}$ are plotted as a function of $\log 4N_e \kappa$ and $\theta$; $\Delta \Delta G_{\text{sd}}^{\text{fixed}}$ is the standard deviation of $\Delta \Delta G$ over fixed mutants at $\Delta G = \Delta G_e$. 
Figure S.18: Dependence of equilibrium stability, $\Delta G_e$, on parameters, $4N_e\kappa$, $\theta$ and $T$. $\Delta G_e$ is the equilibrium value of folding free energy, $\Delta G$, in protein evolution. $T$ is absolute temperature; $\beta = 1/kT$, where $k$ is the Boltzmann constant. Equations (22), (23) and (24) are assumed for the distribution of $\Delta \Delta G$ and its dependency on $\Delta G$; they are assumed to be independent of $T$. Unless specified, $\log 4N_e\kappa = 7.550$ and $\theta = 0.53$ are employed. The value of $\beta \Delta G_e + \log 4N_e\kappa$ is the upper bound of $\log 4N_e\kappa$, and would not depend on $\log 4N_e\kappa$ if the mean of $\Delta \Delta G$ in all arising mutants did not depend on $\Delta G$; see Eq. (S.3). The kcal/mol unit is used for $\Delta G_e$. 
Figure S.19: The average of $K_a/K_s$ over all mutants or over fixed mutants only at equilibrium of protein stability, $\Delta G = \Delta G_e$: Dependence on temperature. $T$ is absolute temperature. Equations (22), (23) and (24) are assumed for the distribution of $\Delta \Delta G$ and its dependency on $\Delta G$; they are assumed to be independent of $T$. Unless specified, $\log 4N_e \kappa = 7.550$ and $\theta = 0.53$ are employed.
Figure S.20: The average of $K_a/K_x$ over all mutants as a function of $\Delta G_e$ and $\theta$. 