

Fitting Protein-Folding Free Energy Landscape for a Certain Conformation to an NK Fitness Landscape

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Abstract

The NK fitness landscape is a mathematical landscape model with a parameter k that governs the degree of ruggedness of the landscape. We presented a procedure to fit a given landscape to the NK fitness landscape by introducing the "apparent k -value" k_{app} . In this paper, we defined the protein free energy (ΔG) landscape in amino acid sequence space, where ΔG is the folding free energy from a random coil to a "certain conformation". Applying this landscape to our fitting procedure, we examined the statistical properties of the landscape. For calculation of a conformation energy, amino acid residues are represented by points, and interaction energies among amino acid residues are given as $(1 + K)$ -body interactions, that is, an unit of interacting $(1 + K)$ residues cooperatively contribute a single energy value to the conformational energy. Our results suggest that the apparent k -value of the free energy landscape is $k_{\text{app}} \approx K$, and that the number of possible interactions among residues is unrelated to the k_{app} value. This leads to the inference that k_{app} takes values about $1 \sim 3$ in real landscapes, if nature adopts two-body \sim four-body interaction energies.

Keywords

Fitness landscape; Sequence space; Canonical ensemble; NK model; *In vitro* evolution; *In silico* evolution; Thermostability

1 Introduction

Molecular evolution is comprehended as a hill-climbing process on a fitness landscape in the dark (Wright,1931;Maynard-Smith, 1970; Eigen, 1985; Kauffman, 1993; Voigt *et al.*, 2000). Therefore, the climbing ability depends on a statistical property of the fitness landscape. Therefore, fitness landscapes are regarded as the "evolutionary attribute" of biopolymers, and investigation of fitness landscapes is an important issue in the field of life science and evolutionary molecular engineering. In particular, it is important to estimate

the degree of ruggedness of these landscapes, as ruggedness governs the evolvability of biopolymers. We are interested in whether there is a common degree of ruggedness over all kinds of proteins.

In this paper, we focused on the protein-folding free energy from a random coil to a "certain conformation" and the free energy landscape in sequence space for the following reasons (note that the domain in the landscape is not conformation space but sequence space). Suppose that a certain sequence is given, and let a_l and f_l be the functional activity of a conformation l per molecule and the mole fraction of the conformation l at equilibrium, respectively. The overall fitness of the given sequence is observed as the mean functional activity, $\langle a \rangle = \sum_l a_l f_l$ with $\sum_l f_l = 1$ (Ancel & Fontana,2000). This means that the fitness landscape can be decomposed into two elements: one is the landscape of molecular activity (the term a_l) and the other is the landscape of the mole fraction (the term f_l) or the thermodynamic stability, in which the mole fraction f_l at equilibrium is related with the folding free energy ΔG_l of the conformation l by $f_l = 1/(1 + \exp(\Delta G_l/T))$. In this paper, we focused on the protein free energy (ΔG) landscape in amino acid sequence space, where ΔG is the folding free energy from a random coil to a "certain conformation", and we examined the degree of ruggedness of the landscape.

The NK model is a familiar mathematical model that describes a complex system in which an arbitrary element is affected by other k elements (Kauffman, 1993). For a protein, each amino acid site corresponds to an element in the NK model. We refer to the fitness landscape described by the NK model as the "NK fitness landscape" in this paper. The parameter k governs the degree of ruggedness of the NK fitness landscape. If $k = 0$, mutational additivity holds and the landscape then has a single peak. As the k -value increases, the surface of the landscape becomes more rugged and many local optima appear. We have studied theoretically the statistical properties of NK fitness landscapes (Aita *et al.*, 2007). Our aim is to estimate the apparent k -value of the free energy landscape by fitting it to an NK fitness landscape.

Fitting the protein folding problem to spin glass models has previously been studied (e.g. Bryngelson & Wolynes,1987;Shakhnovich & Gutin,1990). In a study related to ours,

Shakhnovich & Gutin (1990) presented the idea that a significant proportion of all possible protein sequences could have a thermodynamically dominant fold. The main difference between the spin glass model and NK model is that the former considers only one and two-body interactions, while the latter considers $(1 + K)$ -body interactions.

In the first part of this paper, we summarized a model of the NK fitness landscape and the statistical property of local fitness distributions around reference sequences. Next, we defined the "apparent k -value", k_{app} , for a given landscape from the viewpoint of local fitness distributions. In the second part, concerning a fixed conformation of a protein, we considered the energy landscape in amino acid sequence space. In the third part, the folding free energy landscapes in sequence space were composed from the elemental energy landscapes for all possible conformations. We made a prediction that if interaction energies among amino acid residues were given as $(1 + K)$ -body interactions, the apparent k -value of the free energy landscape would be $k_{\text{app}} \approx K$, and that the number of possible interactions among residues was unrelated to the k_{app} value. In the last part, this prediction was confirmed numerically by computer experiments using a diamond lattice model.

2 NK landscape and X landscape

2.1 Model of the NK fitness landscape

We consider all conceivable amino acid sequences with chain length ν , where λ ($= 20$) naturally occurring amino acids are available letters at every site. Each sequence is mapped into the corresponding point in λ -valued ν -dimensional sequence space. The fitness W for a given sequence "A₁A₂⋯A_ν" is defined by

$$W = \sum_{j=1}^{\nu} w_j(A_j | A_{j_1}, A_{j_2}, \dots, A_{j_k}), \quad (1)$$

where $w_j(A_j | A_{j_1}, A_{j_2}, \dots, A_{j_k})$ is the "site-fitness," *i.e.*, a fitness contribution from a particular letter A_j at the j th site when the k sites $\{j_1, j_2, \dots, j_k\}$ are occupied by the particular letters $\{A_{j_1}, A_{j_2}, \dots, A_{j_k}\}$. The k sites $\{j_1, j_2, \dots, j_k\}$ are randomly chosen from among all of the $\nu - 1$ sites except the j -th site. The assignment of site-fitness values is conducted as follows: With a given set of letters $\{A_{j_1}, A_{j_2}, \dots, A_{j_k}\}$, the site-fitness of an arbitrary letter a , $w_j(a | A_{j_1}, A_{j_2}, \dots, A_{j_k})$, is assigned randomly from the discrete uniform distribution with mean ε and variance σ^2 . In the assignment, degeneracy is not allowed, that is, $w_j(a | \dots) \neq w_j(a' | \dots)$ for $a \neq a'$.

The fitness landscape resulting from this model is called the "NK landscape." We note that our definition is slightly different from that of the original NK landscape (Kauffman, 1993). In the case of $k = 0$, the fitness landscape has a single peak. As the k -value increases, the surface of the fitness landscape becomes more rugged and many local optima appear.

Let $\mathcal{E}[W]$ and $\mathcal{V}[W]$ be the mean and variance, respectively, of the fitness W over all possible sequences in the whole sequence space. These are given by

$$\mathcal{E}[W] = \varepsilon \nu \quad \text{and} \quad \mathcal{V}[W] = \sigma^2 \nu, \quad (2)$$

because it is expected that there is no correlation between the site-fitness values of different sites. The derivation of eqn.(2) is shown in Appendix A. The probability density of fitness W over the whole sequence space is approximately described by a normal distribution with mean $\mathcal{E}[W]$ and variance $\mathcal{V}[W]$. We define the region $W = \mathcal{E}[W]$ as the foot of the

landscape. Let $\mathcal{O}[W]$ be the fitness of the global optimum (peak) on the landscape. We refer to $(\mathcal{O}[W] - \mathcal{E}[W])$ as the "height" of the landscape.

2.2 Fitness distribution over the d -boundary on the NK landscape

We focus on the set of all sequences at distance d from a parent sequence in the sequence space. That is, the set consists of all conceivable d -fold point mutants generated from the parent sequence. According to Fontana and Schuster (1998), we call the set the " d -boundary" of the parent sequence (Fig.1). The size of the d -boundary is $\binom{\nu}{d}(\lambda - 1)^d$. Let \mathcal{W} be the fitness of the parent, and let ΔW be the change in fitness from the parent to its arbitrary mutant on the d -boundary ($\Delta W = W - \mathcal{W}$). Let $\psi_d(\Delta W|\mathcal{W})$ be the probability density of ΔW over the d -boundary, when \mathcal{W} is fixed. $\psi_d(\Delta W|\mathcal{W})$ is approximately given by the normal distribution:

$$\psi_d(\Delta W|\mathcal{W}) \approx \frac{1}{\sqrt{2\pi\mathbf{V}[\Delta W|\mathcal{W}]}} \exp\left(-\frac{(\Delta W - \mathbf{E}[\Delta W|\mathcal{W}])^2}{2\mathbf{V}[\Delta W|\mathcal{W}]}\right), \quad (3)$$

where $\mathbf{E}[\Delta W|\mathcal{W}]$ and $\mathbf{V}[\Delta W|\mathcal{W}]$ are the conditional expectation and variance of ΔW over the d -boundary (Aita & Husimi,2003;Aita *et al.*,2007). These are respectively given as follows:

$$\mathbf{E}[\Delta W|\mathcal{W}] = -(\mathcal{W} - \mathcal{E}[W])\mathcal{D}/\nu \quad (4)$$

$$\mathbf{V}[\Delta W|\mathcal{W}] \approx a\mathcal{V}[W]\mathcal{D}/\nu, \quad (5)$$

where $\mathcal{E}[W]$ and $\mathcal{V}[W]$ are the mean and variance, respectively, of fitness W over all possible sequences in the whole sequence space (eqn.(2)). \mathcal{D} is defined as the mean number of sites that change their site-fitnesses as a result of random d -fold point mutations.

$$\mathcal{D} = \nu - (\nu - d) \left(1 - \frac{k}{\nu - 1}\right)^d \quad (6)$$

$$\approx d(1 + k) \quad \text{for } dk \ll \nu. \quad (7)$$

In Fig.1 in Aita *et al.*, (2007)¹, \mathcal{D} is demonstrated as a function of d . The a in eqn.(5) takes a value between 1 and 2. We note that the distribution shown in eqn.(3) has a truncation

¹In this paper, we wrote " d_{eff} " as \mathcal{D} .

because of the finitude of the number of mutants, but the truncation is actually small and negligible (Aita *et al.*,2007). Fig.1 is a schematic representation of eqn.(3).

Eqns (4) and (5) are easily derived as follows. Let \mathcal{W} be the fitness of a parent sequence, and let w_{out} and w_{in} be the "outgoing" and "incoming" changes, respectively, in site-fitness at a single mutated site. First, for the incoming site-fitness w_{in} , since the value of w_{in} is randomly chosen from the discrete uniform distribution with mean ε and variance σ^2 , the mean and variance of w_{in} are given by

$$\mathbf{E}[w_{\text{in}}] = \varepsilon = \mathcal{E}[W]/\nu, \quad \mathbf{V}[w_{\text{in}}] = \sigma^2 = \mathcal{V}[W]/\nu, \quad (8)$$

(using eqn. (2)). Second, for the outgoing site-fitness w_{out} , since the value of w_{out} is randomly chosen from among the ν site-fitnesses $\{w_1, w_2, \dots, w_\nu\}$ for the parent sequence, the mean and variance of w_{out} are given by

$$\mathbf{E}[w_{\text{out}}] = \mathcal{W}/\nu, \quad \mathbf{V}[w_{\text{out}}] = a'\sigma^2 = a'\mathcal{V}[W]/\nu. \quad (9)$$

The a' takes a value between 0 and 1. In the NK model mentioned above, random d -fold point mutations cause changes in site-fitness at \mathcal{D} sites on average. Therefore, eqns (4) and (5) are derived from

$$\mathbf{E}[\Delta W|\mathcal{W}] = (\mathbf{E}[w_{\text{in}}] - \mathbf{E}[w_{\text{out}}]) \times \mathcal{D} \quad (10)$$

$$\mathbf{V}[\Delta W|\mathcal{W}] = (\mathbf{V}[w_{\text{in}}] + \mathbf{V}[w_{\text{out}}]) \times \mathcal{D}. \quad (11)$$

2.3 Fitting a given landscape to the NK landscape

In this subsection, we refer to a given landscape, which can be explored by *in vitro* or *in silico* evolution experiments. If several statistical properties observed for the landscape are compatible with the theoretical framework of the NK landscape, we can estimate the apparent k -value for the given landscape from the observations. The apparent k -value is denoted by k_{app} in this paper.

For a given landscape, suppose the following:

- (1) the frequency distribution of the fitness W over the d -boundary of an arbitrary sequence with fitness \mathcal{W} is approximately a normal distribution.

(2) the mean and variance of the fitness change $\Delta W (= W - \mathcal{W})$ for the normal distribution are respectively described by

$$\mathbf{E}[\Delta W | \mathcal{W}] = -(\mathcal{W} - \mathcal{E}[W])d/\nu \times \gamma \quad \text{for } d \ll \nu \quad (12)$$

$$\mathbf{V}[\Delta W | \mathcal{W}] = a\mathcal{V}[W]d/\nu \times \gamma \quad \text{for } d \ll \nu, \quad (13)$$

where γ is a constant.

If these properties are observed everywhere on the given landscape, we call the landscape an "X landscape" and define the apparent k -value of the given landscape as

$$k_{\text{app}} = \gamma - 1. \quad (14)$$

This definition originates from eqns (4) and (5) with eqn.(7) in the previous subsection.

It should be noted that adaptive walks on a fitness landscape are significantly governed by the local fitness distributions rather than the details of the landscape structure (Aita *et al.*, 2007). Thus, we can expect that the statistical property of adaptive walks on the X landscapes with $k_{\text{app}} = k$ is comparable to that on NK landscapes with the same k -values.

Another procedure for estimating the apparent k -value of a given landscape is to calculate an autocorrelation function (Fontana *et al.*, 1993). Autocorrelation function of the NK landscape is given by

$$\rho(d) = 1 - \frac{\mathcal{D}}{\nu}. \quad (15)$$

The derivation is shown in Appendix B. According to eqn.(44) in Appendix B, $\rho(d)$ is derived from $\mathbf{E}[\Delta W | \mathcal{W}]$, and then includes the d -dependence of $\mathbf{E}[\Delta W | \mathcal{W}]$. Therefore, it is expected that the apparent k -value estimated by calculating the autocorrelation function is the same as that we proposed here.

3 Energy landscape in amino acid sequence space for a fixed conformation

In this section, we refer to an energy landscape in amino acid sequence space under the condition that a certain conformation is fixed, while the amino acid sequence is variable.

For simplicity, amino acid residues are represented by points. We adopt a $(1 + K)$ -body interaction energy function system, that is, an unit of interacting $(1 + K)$ residues cooperatively contribute a single energy value to a protein's conformational energy. For example, many researchers use a pairwise energy function ($K = 1$) (e.g. Miyazawa & Jernigan,1985) and a four-body energy function ($K = 3$) based on Delaunay tessellation of protein structures (e.g. Singh *et al.*1996). The energy E for an amino acid sequence $A_1A_2 \cdots A_\nu$ mounted on the fixed conformation is given by

$$E = \sum_{c=1}^C e(A_{j_0^c}, A_{j_1^c}, \cdots, A_{j_K^c}) \quad (16)$$

$$= \frac{1}{1 + K} \sum_{j=1}^{\nu} \sum_{b=1}^{B_j} e(A_j, A_{j_1^b}, \cdots, A_{j_K^b}). \quad (17)$$

The $e(A_{j_0^c}, A_{j_1^c}, \cdots, A_{j_K^c})$ in eqn.(16) (or $e(A_j, A_{j_1^b}, \cdots, A_{j_K^b})$ in eqn.(17)) represents the $(1 + K)$ -body energy for a set of $(1 + K)$ interacting residues $\{A_{j_0^c}, A_{j_1^c}, \cdots, A_{j_K^c}\}$ (eqn.(16)) (or $\{A_j, A_{j_1^b}, \cdots, A_{j_K^b}\}$ (eqn.(17))). We call the set an "interacting cluster" and call $e(\cdots)$ "cluster-energy" hereafter. C in eqn.(16) is the number of all of the interacting clusters and B_j in eqn.(17) is the number of interacting clusters to which the j -th site belongs. The mean of B_j over all sites is denoted by B : $B \equiv (\sum_{j=1}^{\nu} B_j)/\nu$. The j_K^b in $\{A_j, A_{j_1^b}, \cdots, A_{j_K^b}\}$ represents the K -th site in the b -th interacting cluster to which the j -th site belongs. We note that an interacting cluster appears when these residues are close to each other, but the detailed conditions for its appearance are not essential for our purpose. It should be noted that K is a "system" parameter given by nature, whereas C and B are "conformational" parameters determined by individual conformational states. K , C and B are related by

$$C(1 + K) = B\nu. \quad (18)$$

Consider the set of all conceivable interacting clusters, and let $\hat{\varepsilon}$ and $\hat{\sigma}^2$ be the mean and variance of the cluster-energy $e(\cdots)$ over the set. For example, a two-body energy function has $210(= 21 \times 20/2)$ elements in the set. We assume that the cluster-energies are assigned from a set of independent random values with mean $\hat{\varepsilon}$ and variance $\hat{\sigma}^2$ (Shakhnovich & Gutin,1990; Pande, *et al.*,1997). Then, the mean $\mathcal{E}[E]$ and variance

$\mathcal{V}[E]$ of the energy E over the whole sequence space are approximately given by

$$\mathcal{E}[E] = \hat{\varepsilon}C, \quad \mathcal{V}[E] = \hat{\sigma}^2 C. \quad (19)$$

Next, we consider the energy distribution over the d -boundary of an arbitrary sequence by a method similar to that mentioned in the previous section. Here, we derive the mean and variance for the energy distribution by following the derivation of eqns (8)-(11). Consider that a single point mutation occurs at a certain site. Let E be the energy of a parent sequence, and let e_{out} and e_{in} be the "outgoing" and "incoming" changes, respectively, in cluster-energy for a single interacting cluster to which the mutated site belongs. The mean and variance of e_{in} , and those of e_{out} are respectively given by

$$\mathbf{E}[e_{\text{in}}] = \hat{\varepsilon} = \mathcal{E}[E]/C, \quad \mathbf{V}[e_{\text{in}}] = \hat{\sigma}^2 = \mathcal{V}[E]/C \quad (20)$$

$$\mathbf{E}[e_{\text{out}}] = E/C, \quad \mathbf{V}[e_{\text{out}}] = a'\hat{\sigma}^2 = a'\mathcal{V}[E]/C. \quad (21)$$

The a' takes a value between 0 and 1. Random d -fold point mutations cause changes in cluster-energy for about $B \times d$ interacting clusters. Therefore, the mean and variance of change in energy, ΔE , are respectively given by

$$\mathbf{E}[\Delta E|E] = (\mathbf{E}[e_{\text{in}}] - \mathbf{E}[e_{\text{out}}]) \times Bd \quad (22)$$

$$= -(E - \mathcal{E}[E])(1 + K)d/\nu \quad (23)$$

$$\mathbf{V}[\Delta E|E] = (\mathbf{V}[e_{\text{in}}] + \mathbf{V}[e_{\text{out}}]) \times Bd \quad (24)$$

$$= a\mathcal{V}[E](1 + K)d/\nu. \quad (25)$$

The a in eqn.(25) takes a value between 1 and 2. When Bd is large, the energy distribution is approximately a normal distribution. According to Subsection 2.3, we can propose the following prediction:

Prediction 1: the energy landscape for a fixed conformation is an X landscape with

$$k_{\text{app}} = K. \quad (26)$$

We add that the flat landscape ($E \equiv 0$) for $C = 0$ is regarded as a special case of the X landscape. This result says that k_{app} of the landscape is affected by K and is not affected

by B (or C), whereas B (or C) affects the height of the landscape from the foot to the peak (see eqn.(19)). It may be surprising that the number of possible interactions among residues in a conformation is unrelated to the k_{app} value (Greene & Higman,2003).

4 Folding free energy landscape in amino acid sequence space from a random coil to a certain conformation

In this section, we refer to the protein free energy (ΔG) landscape in amino acid sequence space, where ΔG is the folding free energy from a random coil to a "certain conformation."

Consider the set of all conceivable conformations with chain length ν . The energy E_l of a conformation l in the set is given by eqn. (16). We focus on a particular reference conformation, which is one of the most compact ones with large C . This reference conformation is denoted by $l = 0$ and its energy is then denoted by E_0 . Let S be the set defined by subtracting the element $l = 0$ from the set of all conceivable conformations, and let T be a temperature parameter corresponding to the room temperature.

The folding free energy ΔG from a random coil to the reference conformation $l = 0$ is given by

$$\Delta G = E_0 + T \ln Z', \quad \text{where} \quad (27)$$

$$Z' \equiv \sum_{l \in S} e^{-E_l/T}. \quad (28)$$

It should be noted that E_l ($l \in S$) is the X landscape with $k_{\text{app}} = K$. Let $|S|$ be the number of conformations in the set S . We assume that the density function of energy E in the set S is given by the truncated normal distribution with mean $m \equiv (\sum_{l \in S} E_l)/|S|$ and variance $s^2 \equiv (\sum_{l \in S} (E_l - m)^2)/|S|$, as well as E_{min} as the minimal energy of all conformations in the set S :

$$\frac{1}{|S|} \sum_{l \in S} \delta(E - E_l) \approx \frac{e^{-(E-m)^2/2s^2}}{\int_{E_{\text{min}}}^{\infty} e^{-(E-m)^2/2s^2} dE}, \quad (29)$$

where $\delta(w)$ is Dirac's delta function. Although there is room for argument on this assumption, we leave the details to other studies (e.g. Shakhnovich & Gutin,1990; Pande,

et al.,1997; Wolynes & Luthey-Schulten,1997). Z' in eqn.(28) is calculated as follows:

$$Z' = \frac{\int_{E_{\min}}^{\infty} e^{-E/T} \times |S| e^{-(E-m)^2/2s^2} dE}{\int_{E_{\min}}^{\infty} e^{-(E-m)^2/2s^2} dE}, \quad (30)$$

$$\approx \begin{cases} |S| e^{-m/T+s^2/2T^2} & \text{for } T \gg \frac{s^2}{m-E_{\min}}, \\ e^{-E_{\min}/T} & \text{for } T \ll \frac{s^2}{m-E_{\min}}. \end{cases} \quad (31)$$

Substituting the upper one in eqn. (31) into eqn. (27), the folding free energy is approximately given by

$$\Delta G \approx E_0 - m + s^2/2T + T \ln |S|. \quad (32)$$

Here, we consider the protein free energy (ΔG) landscape in amino acid sequence space, regarding ΔG as a function of amino acid sequences. Assuming that $E_0 - m$ in eqn.(32) is more sensitive to sequences than $s^2/2T$ for large T , we focus on

$$E_0 - m = E_0 - \frac{1}{|S|} \sum_{l \in S} E_l. \quad (33)$$

This term gives an energy landscape resulting from a linear composition of the elemental energy landscapes, which are the X landscapes with $k_{\text{app}} = K$.

Here, we introduce the following theorem:

Theorem 1: when several independent X landscapes with $k_{\text{app}} = k$ are linearly composed, the resultant landscape becomes an X landscape with $k_{\text{app}} = k$.

The proof is shown in Appendix C. Following these findings and assuming that these elemental energy landscapes are independent, we can infer that

Prediction 2: the folding free energy landscape from a random coil to a reference conformation (eqn.(32)) is also an X landscape with

$$k_{\text{app}} \approx K. \quad (34)$$

This prediction was numerically confirmed by computer experiments using a diamond lattice model, shown in the next section. Correlation among energy landscapes for different conformations is an interesting issue and will be examined elsewhere.

Here, we return to our motivation mentioned in the Introduction. The fraction f of the structure $l = 0$ at equilibrium is given by

$$f = \frac{1}{1 + e^{\Delta G/T}}. \quad (35)$$

Then, the natural logarithm of f is

$$\ln f \approx \begin{cases} -\Delta G/T & \text{for } \Delta G/T \gg 0, \text{ that is } f \ll 1/2, \\ -e^{\Delta G/T} & \text{for } \Delta G/T \ll 0, \text{ that is } f \gg 1/2. \end{cases} \quad (36)$$

We consider the "ln f landscape", which is defined by assigning the $\ln f$ value of a sequence to the corresponding point in sequence space. We can see that there are two different phases on the $\ln f$ landscape: one is the region of $f \ll 1/2$, in which the statistical properties of the landscape are the same as those of the free energy landscape, and the other is the region of $f \gg 1/2$. Details are provided in the next section.

5 Confirmation of the theoretical prediction by computer experiments

To confirm Prediction 1 (in Sections 3) and Prediction 2 (in Sections 4), we performed computer experiments using a 3-D diamond lattice model (Blackburne & Hirst,2003), in which amino acid residues are located at lattice points and there are four neighboring points around each point. The spatial distance between neighboring lattice points was set to be the unity (=1). The number of sites was set to be $\nu = 26$, and twenty naturally occurring amino acids were available at each site ($\lambda = 20$).

Energy landscape of a fixed conformation

First, we performed computer experiments to confirm Prediction 1 (in Sections 3). We used the structure shown in Fig.2 as a fixed conformation. This structure is one of the most compact of all of the possible conformations. Concerning the definition of energy function, we examined the following three systems:

System #1: We set $K = 1$ (this corresponds to a pairwise energy), and defined the energy of a given sequence "A₁A₂ ··· A_ν" by

$$E = \sum_{i=1}^{24} \sum_{j=i+2}^{26} \delta(A_i, A_j) e(A_i, A_j), \quad \text{with} \quad (37)$$

$$\delta(A_i, A_j) = \begin{cases} 1 & \text{if } D(A_i, A_j) = 1, \\ 0 & \text{otherwise,} \end{cases} \quad (38)$$

where $D(A_i, A_j)$ is the spatial distance between the i th residue A_i and the j th residue A_j . In this system, the maximal number of C among all the conformations is $C_{\max} = 12$. The structure in Fig.2 gives $C = 12 (= C_{\max})$ and then $B = 0.92$. We used the Miyazawa-Jernigan energy function (Table 5 in Miyazawa & Jernigan, 1985) as values of $e(A_i, A_j)$.

System #2: Almost all conditions were the same as in System #1, except that eqn.(38) was replaced by

$$\delta(A_i, A_j) = \begin{cases} 1 & \text{if } D(A_i, A_j) \leq 1.63, \\ 0 & \text{otherwise.} \end{cases} \quad (39)$$

The structure in Fig.2 gives $C = 60$ and $B = 4.62$.

System #3: We set $K = 3$ (this corresponds to a four-body energy function), and defined the energy by

$$E = \sum_{j=1}^{26} \sum_{j_1 < j_2 < j_3} \delta(A_j, A_{j_1}) \delta(A_j, A_{j_2}) \delta(A_j, A_{j_3}) e(A_j, A_{j_1}, A_{j_2}, A_{j_3}) \quad (40)$$

with eqn.(38). The structure in Fig.2 gives $C = 20$ and $B = 3.08$. A cluster-energy value $e(A_j, A_{j_1}, A_{j_2}, A_{j_3})$ is assigned randomly from a set of Miyazawa-Jernigan energy values.

By performing down-hill walks on each energy landscape of the three systems, we chose several reference points (parent sequences) along the walks from random points to the optimum. Then, we obtained the energy distribution over the d -boundary of each reference point, where $d = 1, 2, 3$ and 4 . We found that the energy distributions were approximately a normal distribution. For the three systems, we show in Fig.3 the relationship between $\mathbf{E}[\Delta E|E]$ and $-(E - \mathcal{E}[E])d/\nu$ and the relationship between $\mathbf{V}[\Delta E|E]$ and $2\mathcal{V}[E]d/\nu$ (see

equations (23) and (25)). By conducting regression analysis on the plots, we calculated the k_{app} values from the slope γ of the regression line (see eqn.(14)). As a result, k_{app} equaled 0.2 for System #1 ($K = 1, C = 12, B = 0.92$) and #2 ($K = 1, C = 60, B = 4.62$), and k_{app} equaled 2.5 for System #3 ($K = 3, C = 20, B = 3.08$). These k_{app} values were found to reflect their respective K values. The k_{app} -values ($=0.2$) for Systems #1 and #2 are much less than 1.0 due to the correlation between amino acid pairs and their energy values in the Miyazawa-Jernigan potential (Li *et al.*,1997). Shuffling the assignment of cluster-energy values to amino acid pairs randomly and performing the same experiment, we obtained $k_{\text{app}} = 0.8$ for the two systems. These results support Prediction 1 (in Sections 3).

Folding free energy landscape of a reference conformation

Second, we performed the same computer experiments to confirm Prediction 2 (in Sections 4). Definition of the energy function followed the conditions shown in System #1 ($K = 1$ and $C = 0 \sim 12$). We used the structure shown in Fig.2 as a reference conformation ($l = 0$). Let S_C be the set of all conceivable conformations (except $l = 0$) with a given C , that is $S = \sum_{C=0}^{12} S_C$. For the size of each subset, $|S_{12}| = 246, |S_{11}| = 872, |S_{10}| = 6.7 \times 10^4, |S_9| = 1.1 \times 10^6, \dots$. Roughly, $|S_C| \approx 10^{13} \exp(-2.0C)$. The partition function Z' defined in eqn.(28) was calculated in the following way. For $S_7 \sim S_{12}$, all possible conformations and their energies were enumerated, while for $S_0 \sim S_6$, an approximation similar to eqn.(31) was applied to S_C :

$$Z' = \sum_{C=7}^{12} \sum_{l \in S_C} e^{-E_l/T} + \sum_{C=0}^6 |S_C| e^{-m_C/T + s_C^2/2T^2} \quad (41)$$

with $m_C \equiv (\sum_{l \in S_C} E_l)/|S_C|$ and $s_C^2 \equiv (\sum_{l \in S_C} (E_l - m_C)^2)/|S_C|$. The temperature parameter T was set to be unity: $T = 1$, which corresponds to the room temperature. Then, the folding free energy ΔG was calculated using eqn.(27).

The free energy landscape was explored by the same manner as for the energy landscape. Let ΔG be the free energy of a reference sequence, and let $\Delta\Delta G$ be the change in free energy from the reference sequence to an arbitrary mutant sequence located on its

d -boundary ($d = 1, 2, 3, 4$). We observed that, for each d , the frequency distributions of $\Delta\Delta G$ were approximately the normal distribution. Fig.4 is a plot similar to Fig.3. Fig.4 shows the relationship between $\mathbf{E}[\Delta\Delta G|\Delta G]$ and $-(\Delta G - \mathcal{E}[\Delta G])d/\nu$ and the relationship between $\mathbf{V}[\Delta\Delta G|\Delta G]$ and $2\mathcal{V}[\Delta G]d/\nu$. By conducting regression analysis of these plots, we obtained $\gamma = 1.3$ and then $k_{\text{app}} = 0.3$. This value is close to $k_{\text{app}} = 0.2$ for the energy landscape of System #1. These observations are almost consistent with Prediction 2 (in Sections 4).

$\ln f$ landscape of a reference conformation

Finally, we mention a statistical property of the $\ln f$ landscape. Fig.5(a) shows the frequency distribution of $\ln f$ on the d -boundary ($d = 1, 2, 3, 4$) of each of four reference points on the $\ln f$ landscape. We can see that the frequency distribution in the region of $f \ll 1/2$ is approximately a normal distribution, while this is not the case in the region of $f \gg 1/2$. Fig.5(b,c) shows observed values of $\mathbf{E}[\Delta \ln f | \ln f]$ and $\mathbf{V}[\Delta \ln f | \ln f]$ as a function of the $\ln f$ of reference sequences. In this figure, we can clearly confirm that there are two different phases: the region of $f \ll 1/2$ and that of $f \gg 1/2$. The critical point $f_c = 1/2$ corresponds to $\Delta G = 0$. In the former region, $\mathbf{E}[\Delta \ln f | \ln f]$ decreases linearly, obeying $-\gamma(\ln f - \mathcal{E}[\ln f])d/\nu$, while $\mathbf{V}[\Delta \ln f | \ln f]$ obeys $2\gamma\mathcal{V}[\ln f]d/\nu$, with $\gamma = 1.3$.

6 Discussions

In this paper, we focused on the protein-folding free energy from a random coil to a "certain conformation" and fitted the free energy landscape to an NK fitness landscape (note that the domain in the landscape is not conformation space but sequence space). For calculation of a conformation energy, amino acid residues are represented by points, and interaction energies among amino acid residues are given as $(1+K)$ -body interactions. Our results suggest that the apparent k -value of the free energy landscape is $k_{\text{app}} \approx K$, and that the number of possible interactions among residues is unrelated to the k_{app} value. Additionally, this suggests that k_{app} is independent of protein's model, for example, cubic

lattice, diamond lattice, off-lattice, 2D, 3D, and so on, if these protein's model give a normal distribution of conformational energies (eqn. (29)).

In nature, the number of K is inherently determined as a physicochemical property of polypeptides. Almost all of the energy functions used in physics adopt two-body \sim four-body energies (Ponder & Case, 2003; Munson & Singh, 1997), which corresponds to $K = 1 \sim 3$. According to our results, this fact leads to a conclusion that the folding free energy landscape takes $k_{\text{app}} \approx 1 \sim 3$. Moreover, for a real energy function system such as the Miyazawa-Jernigan energy function, the assignment of a cluster-energy value to a cluster of interacting amino acids is somewhat orderly. Therefore we can expect $k_{\text{app}} < 1 \sim 3$ in real landscapes. Actually, we roughly estimated $k_{\text{app}} < 0.3$ for several real proteins listed in Table 3 of Bloom *et al.*(2005) (details are in Appendix D). Bloom *et al.*(2005) succeeded in predicting the probability that a protein retains its wild-type structure after one or more random amino acid substitutions, based on the assumption of mutational additivity. Mutational additivity means $k_{\text{app}} = K = 0$. Although our estimation ($k_{\text{app}} < 0.3$) was very rough, it was compatible with Bloom's scheme.

On the contrary, many researchers consider real fitness landscapes to be rugged ones. For example, Kauffman and Weinberger applied the NK model to affinity maturation of the V region in immunoglobulin and estimated that k was about 40 (Kauffman & Weinberger, 1989; Kauffman, 1993). In our previous paper, we estimated the k -value of the infectivity landscape for fd phages to be 18-27 (Hayashi *et al.*, 2006; Aita *et al.*, 2007). There is a large discrepancy between these estimations and the estimation in this paper ($k_{\text{app}} < 3$). There are three possibilities to explain the large ruggedness level observed. The first possibility is that, at the atomic level, nature uses multi-body energy with large K such as $K = 18 - 27$, but this seems unlikely to occur in nature. The second possibility lies in that kinetic traps make landscapes more rugged (Li *et al.*, 2004). The third possibility lies on the following mesoscopic level. Suppose that a certain sequence is given, and let a_l and f_l be the functional activity of a conformation l per molecule and the mole fraction of the conformation l at equilibrium, respectively. The overall fitness of the given sequence is observed as the mean functional activity, $\langle a \rangle = \sum_l a_l f_l$. Significant

ruggedness seems to manifest at this stage. Schuster's group mapped each of the RNA sequences into the most probable secondary structure minimizing free energy, and examined the free energy landscape, which is defined by assigning the minimum free energy of a base sequence to the corresponding point in the base sequence space (Schuster,1995). They estimated $k = 7 - 8$ for the free energy landscape in terms of autocorrelation on the landscape (Fontana *et al.*,1993). They obtained a statistical relationship between Hamming distance and structure distance. This relationship is called the "structure density surface" (Fig.4 in Schuster,1995). We tried to interpret the structure density surface shown in Fig.4 in Schuster (1995) from our original view. Under several assumptions, we roughly estimated the k_{app} -value of the relevant landscape to be about 9-12, which is similar to Fontana's estimation (details are in Appendix E). This suggests that the significant ruggedness originated from the spatial distribution, in sequence space, of the most probable structure minimizing free energy.

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References

- [1] Aita, T. & Husimi, Y., 2003. Thermodynamical Interpretation of an adaptive walk on a Mt. Fuji-type fitness landscape: Einstein relation-like formula holds in a stochastic evolution. *J.theor.Biol.* **225**, 215-228.
- [2] Aita, T., Hayashi, Y., Toyota, H., Husimi, Y., Urabe, I., Yomo, T, 2007. Extracting characteristic properties of fitness landscape from in vitro molecular evolution: A

- case study on infectivity of fd phage to E.coli. *J.theor.Biol.* **246**, 538-550.
- [3] Ancel, L.W. & Fontana, W., 2000. Plasticity, evolvability, and modularity in RNA. *J. Exp. Zool.* **288**, 242-283.
- [4] Bloom, J.D., Wilke, C.O., Arnold, F.H., Adamiy, C., 2004. Stability and the Evolvability of Function in a Model Protein. *Biophys. J.* **86**, 2758-2764.
- [5] Bloom, J.D., Silberg, J.J., Wilke, C.O., Drummond, D.A., Adami, C. & Arnold, F.H., 2005. Thermodynamic prediction of protein neutrality. *Proc. Natl. Acad. Sci. U S A.* **102**, 606-611.
- [6] Blackburne, B.P. & Hirst, J.D., 2003. Three-dimensional functional model proteins: Structure function and evolution. *J. Chem. Phys.* **119**, 3453-3460.
- [7] Bryngelson, J.D. & Wolynes, P.G., 1987. Spin glasses and the statistical mechanics of protein folding. *Proc. Natl. Acad. Sci. U S A.* **84**, 7524-7528.
- [8] Eigen, M., 1985. Macromolecular evolution: dynamical ordering in sequence space. *Ber. Bunsenges. Phys. Chem.* **89**, 658-667.
- [9] Fontana, W. & Schuster, P., 1998. Shaping space: the possible and the attainable in RNA genotype-phenotype mapping. *J.theor.Biol.* **194**, 491-515.
- [10] Fontana, W., Stadler, P.F., Bornberg-Bauer, E.G., Griesmacher, T., Hofacker, I.L., Tacker, M., Tarazona, P., Weinberger, E.D. & Schuster, P., 1993. RNA folding and combinatorial landscapes. *Phys. Rev. E* **47**, 2083-2099.
- [11] Greene, L.H. & Higman, V.A., 2003. Uncovering network systems within protein structures. *J. Mol. Biol.* **334**, 781-791.
- [12] Hayashi, Y., Aita, T., Toyota, H., Husimi, Y., Urabe, I., Yomo, T, 2006. Experimental Rugged Fitness Landscape in Protein Sequence Space. *PLoS ONE* **1**, e96.

- [13] Kauffman, S.A. & Weinberger, E.D., 1989. The NK model of rugged fitness landscapes and its application to maturation of the immune response. *J.theor.Biol.* **141**, 211-245.
- [14] Kauffman, S.A., 1993. *The Origin of Order*. Oxford: Oxford University Press.
- [15] Li, H., Tang, C. & Wingreen, N.S., 1997. Nature of driving force for protein folding: a result from analyzing the statistical potential. *Phys. Rev. Lett.* **79**, 765-768.
- [16] Li, J., Wang, J., Zhang, J. & Wang, W., 2004. Thermodynamic stability and kinetic foldability of a lattice protein model. *J. Chem. Phys.* **120**, 6274-6287.
- [17] Maynard-Smith, J., 1970. Natural selection and the concept of a protein space. *Nature* **225**, 563-564.
- [18] Miyazawa, S. & Jernigan, R.L., 1985. Estimation of effective interresidue contact energies from protein crystal structure: quasi-chemical approximation. *Macromolecules* **18**, 534-552.
- [19] Munson, P.J. & Singh, R.K., 1997. Statistical significance of hierarchical multi-body potentials based on Delaunay tessellation and their application in sequence-structure alignment. *Protein Sci.* **6**, 1467-1481.
- [20] Pande, V.S., Grosberg, A.Y. & Tanaka, T., 1997. Statistical mechanics of simple models of protein folding and design. *Biophys. J.* **73**, 3192-3210.
- [21] Ponder, J.W. & Case, D.A., 2003. Force fields for protein simulations. *Adv. Protein Chem.* **66**, 27-85.
- [22] Yomo, T., Saito, S. & Sasai, M., 1999. Gradual development of protein-like global structures through functional selection. *Nat. Struct. Biol.* **6**, 743-746.
- [23] Schuster, P., 1995. How to search for RNA structures: theoretical concepts in evolutionary biotechnology. *J.Biotech.* **41**, 239-257.

- [24] Shakhnovich, E.I. & Gutin, A.M., 1990. Implications of thermodynamics of protein folding for evolution of primary sequences. *Nature* **346**, 773-775.
- [25] Singh, R.K., Tropsha, A. & Vaisman, I.I., 1996. Delaunay tessellation of proteins: four body nearest-neighbor propensities of amino acid residues. *J. Comput. Biol.* **3**, 213-221.
- [26] Voigt, C.A., Kauffman, S. & Wang, Z.G., 2000. Rational evolutionary design: the theory of *in vitro* protein evolution. *Adv Protein Chem.* **55**, 79-160.
- [27] Wilke, C.O., Bloom, J.D., Drummond, D.A. & Raval, A., 2005. Predicting the tolerance of proteins to random amino acid substitution. *Biophys. J.* **89**, 3714-3720.
- [28] Wright, S., 1931. Evolution in Mendelian populations. *Genetics* **16**, 97-159.
- [29] Wolynes, P.G. & Luthey-Schulten, Z., 1997. The energy landscape theory of protein folding, in: Flyvbjerg, H., Hertz, J., Jensen, M.H., Mouritsen, O.G. and Sneppen, K. (Eds.), *Physics of biological systems: from molecules to species*, Springer, pp. 61-79.

Appendix A: Derivation of eqn.(2)

Consider the following sum of fitness over all the sequences of λ^ν :

$$S_1 \equiv \sum_{\text{all sequences}} W = \sum_{\text{all sequences}} \sum_{j=1}^{\nu} w_j(A_j | A_{j_1}, A_{j_2}, \dots, A_{j_k}).$$

In the set of all the sequences, there are $\lambda^{\nu-(1+k)}$ sequences which include a set of letters, $\{A_j, A_{j_1}, \dots, A_{j_k}\}$. Then,

$$\begin{aligned} S_1 &= \sum_{j=1}^{\nu} \lambda^{\nu-(1+k)} \sum_{\{A_j, A_{j_1}, \dots, A_{j_k}\}} w_j(A_j | A_{j_1}, \dots, A_{j_k}) \\ &= \sum_{j=1}^{\nu} \lambda^{\nu-(1+k)} \sum_{\{A_{j_1}, \dots, A_{j_k}\}} \sum_a w_j(a | A_{j_1}, \dots, A_{j_k}). \end{aligned}$$

Here, let us focus on

$$\sum_{\{A_{j_1}, \dots, A_{j_k}\}} \sum_a w_j(a | A_{j_1}, \dots, A_{j_k}).$$

With a set $\{A_{j_1}, \dots, A_{j_k}\}$ fixed, we can see

$$\sum_a w_j(a | A_{j_1}, \dots, A_{j_k}) = \varepsilon \lambda.$$

This equation holds for all possible λ^k states of $\{A_{j_1}, \dots, A_{j_k}\}$. Then

$$S_1 = \nu \lambda^{\nu-(1+k)} \varepsilon \lambda \lambda^k = \varepsilon \nu \lambda^\nu.$$

Therefore, the mean of fitness over all the sequences is given by

$$\mathcal{E} = \frac{S_1}{\lambda^\nu} = \varepsilon \nu.$$

Next, consider the following sum of the square of fitness over all the sequences of λ^ν :

$$\begin{aligned} \sum_{\text{all sequences}} W^2 &= S_2 + S_3 \\ S_2 &\equiv \sum_{\text{all sequences}} \sum_{j=1}^{\nu} w_j(A_j | A_{j_1}, \dots, A_{j_k})^2 \\ S_3 &\equiv \sum_{\text{all sequences}} 2 \sum_{i < j} w_i(A_i | A_{i_1}, \dots, A_{i_k}) w_j(A_j | A_{j_1}, \dots, A_{j_k}). \end{aligned}$$

We can easily calculate S_2 through the procedure similar to the calculation of S_1 :

$$S_2 = \nu \lambda^{\nu-(1+k)} (\varepsilon^2 + \sigma^2) \lambda \lambda^k = (\varepsilon^2 + \sigma^2) \nu \lambda^\nu.$$

The calculation of S_3 is done in the following way. Let q_{ij} be the number of sites included in $\{i_1, \dots, i_k, j_1, \dots, j_k\}$ except sites i and j . For example, if there are no overlapped sites between $\{i_1, \dots, i_k\}$ and $\{j_1, \dots, j_k\}$, and sites i and j are not included in them, we get $q_{ij} = 2k$. The number of sites that affect the site-fitness of the i th and j th sites (w_i and w_j) is then $2 + q_{ij}$. In the set of all the sequences, there are $\lambda^{\nu-(2+q_{ij})}$ sequences which include a set of $2 + q_{ij}$ letters, $\{A_i, A_{i_1}, \dots, A_{i_k}, A_j, A_{j_1}, \dots, A_{j_k}\}$. Then,

$$\begin{aligned} S_3 &= 2 \sum_{i < j} \lambda^{\nu-(2+q_{ij})} \sum_{\{A_i, A_{i_1}, \dots, A_{i_k}, A_j, A_{j_1}, \dots, A_{j_k}\}} w_i(A_i | A_{i_1}, \dots, A_{i_k}) w_j(A_j | A_{j_1}, \dots, A_{j_k}) \\ &= 2 \sum_{i < j} \lambda^{\nu-(2+q_{ij})} \sum_{\{A_{i_1}, \dots, A_{i_k}, A_{j_1}, \dots, A_{j_k}\}} \sum_a \sum_b w_i(a | A_{i_1}, \dots, A_{i_k}) w_j(b | A_{j_1}, \dots, A_{j_k}). \end{aligned}$$

Here, let us focus on

$$\sum_a \sum_b w_i(a | A_{i_1}, \dots, A_{i_k}) w_j(b | A_{j_1}, \dots, A_{j_k}).$$

Since the site-fitness values are assigned randomly from the discrete uniform distribution with mean ε and variance σ^2 without degeneracy (see Section 2), then the covariance of site-fitnesses is zero for large λ :

$$\frac{1}{\lambda^2} \sum_a \sum_b w_i(a | A_{i_1}, \dots, A_{i_k}) w_j(b | A_{j_1}, \dots, A_{j_k}) - \varepsilon^2 = 0.$$

Therefore,

$$\sum_a \sum_b w_i(a | A_{i_1}, \dots, A_{i_k}) w_j(b | A_{j_1}, \dots, A_{j_k}) = (\varepsilon \lambda)^2.$$

This equation holds for all possible $\lambda^{q_{ij}}$ states of $\{A_{i_1}, \dots, A_{i_k}, A_{j_1}, \dots, A_{j_k}\}$. Then

$$\begin{aligned} S_3 &= 2 \sum_{i < j} \lambda^{\nu-(2+q_{ij})} (\varepsilon \lambda)^2 \lambda^{q_{ij}} \\ &= 2 \frac{\nu(\nu-1)}{2} \varepsilon^2 \lambda^\nu = \varepsilon^2 \nu(\nu-1) \lambda^\nu. \end{aligned}$$

Therefore, the variance of fitness over all the sequences is given by

$$\begin{aligned} \mathcal{V} &= \frac{S_2 + S_3}{\lambda^\nu} - \mathcal{E}^2 = (\varepsilon^2 + \sigma^2) \nu + \varepsilon^2 \nu(\nu-1) - (\varepsilon \nu)^2 \\ &= \sigma^2 \nu. \end{aligned}$$

Appendix B: Autocorrelation function of the NK landscape

Autocorrelation function of a fitness landscape is defined by

$$\rho(d) \equiv \frac{\langle W(\mathbf{A})W(\mathbf{B}) \rangle_{d(\mathbf{A},\mathbf{B})=d} - \mathcal{E}^2}{\mathcal{V}}, \quad (42)$$

where $W(\mathbf{A})$ is the fitness of an arbitrary sequence \mathbf{A} (Fontana *et al.*,1993). \mathbf{A} and \mathbf{B} are variable under the restriction that the Hamming distance between them is d ($d(\mathbf{A}, \mathbf{B}) = d$). The average $\langle \dots \rangle$ is conducted over the whole sequence space. Applying the definition to the NK landscape, we obtain

$$\rho(d) = \frac{\langle \mathcal{W} \int_{-\infty}^{\infty} (\Delta W + \mathcal{W}) \psi_d(\Delta W | \mathcal{W}) d\Delta W \rangle - \mathcal{E}^2}{\mathcal{V}}, \quad (43)$$

$$= \frac{\langle \mathcal{W} \mathbf{E}[\Delta W | \mathcal{W}] + \mathcal{W}^2 \rangle - \mathcal{E}^2}{\mathcal{V}}, \quad (44)$$

$$= 1 - \frac{\mathcal{D}}{\nu} = \left(1 - \frac{d}{\nu}\right) \left(1 - \frac{k}{\nu - 1}\right)^d, \quad (45)$$

where $\psi_d(\Delta W | \mathcal{W})$ is the probability density of fitness change ΔW from a reference point to its d -boundary, when the reference point takes a fitness-value of \mathcal{W} , and $\mathbf{E}[\Delta W | \mathcal{W}]$ is its mean (see Section 2.2). The derivation of eqn.(45) from eqn.(44) can be easily carried out by using eqns (4) and (6). We note that the righthand side of eqn.(45) is the same form shown in Table I of Fontana *et al.* (1993).

Appendix C: Proof of Theorem 1: fitness landscape resulting from a linear composition of independent NK fitness landscapes

Consider that there are several NK fitness landscapes ($l = 1, 2, 3, \dots$) that are independent and uncorrelated with each other, although they are formed in the same sequence space. These landscapes have the same k -value, k . Let $W^{(l)}$ be the fitness function for the l -th landscape. And let $\mathcal{E}^{(l)}$ and $\mathcal{V}^{(l)}$ be the mean and variance, respectively, of fitness $W^{(l)}$ over the whole sequence space. Therefore, the statistical properties of the l -th landscape are characterized by $\mathcal{E}^{(l)}$, $\mathcal{V}^{(l)}$ and k . Next, consider that these independent landscapes

are composed linearly as follows:

$$W \equiv \sum_l A^{(l)} W^{(l)}, \quad (46)$$

where $A^{(l)}$ is the constant weight for the l -th and W represents the fitness function resulting from the composition. Hereafter, we call the landscape(s) before and after the composition the "elemental landscapes" and the "resultant landscape," respectively. Since the elemental landscapes are not correlated with one another, mean \mathcal{E} and variance \mathcal{V} of the resultant fitness W over the whole sequence space are given by

$$\mathcal{E} = \sum_l A^{(l)} \mathcal{E}^{(l)}, \quad \mathcal{V} = \sum_l A^{(l)2} \mathcal{V}^{(l)}.$$

For the l -th elemental landscape, focus on the d -boundary of an arbitrary sequence with fitness value $\mathcal{W}^{(l)}$ on the landscape. We have theoretically deduced that the frequency distribution of fitness $W^{(l)}$ over the d -boundary is approximately a normal distribution. For the normal distribution, the mean and variance of the fitness change $\Delta W^{(l)}$ ($= W^{(l)} - \mathcal{W}^{(l)}$, where $\mathcal{W}^{(l)}$ is fixed) are given by

$$\mathbf{E}[\Delta W^{(l)} | \mathcal{W}^{(l)}] = -(\mathcal{W}^{(l)} - \mathcal{E}^{(l)}) \mathcal{D} \quad (47)$$

$$\mathbf{V}[\Delta W^{(l)} | \mathcal{W}^{(l)}] \approx a \mathcal{V}^{(l)} \mathcal{D}, \quad (48)$$

respectively (see equations (4) and (5)). The a in eqn.(48) takes a value between 1 and 2.

For the resultant landscape (eqn.(46)), focus on the d -boundary of an arbitrary sequence with fitness value \mathcal{W} ($= \sum_l A^{(l)} \mathcal{W}^{(l)}$). We can easily observe that the frequency distribution of fitness W over the d -boundary is also a normal distribution, which is deduced by the convolution of the elemental normal distributions with mean shown in eqn.(47) and variance shown in eqn.(48). For the resultant normal distribution, the mean and variance of the fitness change ΔW ($= \sum_l A^{(l)} \Delta W^{(l)}$) are given by

$$\begin{aligned} \mathbf{E}[\Delta W | \mathcal{W}] &= \sum_l A^{(l)} \mathbf{E}[\Delta W^{(l)} | \mathcal{W}^{(l)}] = -(\mathcal{W} - \mathcal{E}) \mathcal{D} \\ \mathbf{V}[\Delta W | \mathcal{W}] &= \sum_l A^{(l)2} \mathbf{V}[\Delta W^{(l)} | \mathcal{W}^{(l)}] \approx a \mathcal{V} \mathcal{D}, \end{aligned}$$

respectively. $\mathcal{D} \approx d(1+k)$. According to Subsection 2.3, the resultant landscape becomes an X landscape with $k_{\text{app}} = k$.

In conclusion, we found that when several independent (elemental) NK landscapes with the same parameter k are linearly composed, the resultant landscape becomes an X landscape with $k_{\text{app}} = k$. This conclusion also holds when the elemental landscapes are X landscapes with $k_{\text{app}} = k$.

Appendix D: Rough estimation of k_{app} of the folding free energy landscape for real proteins listed in Table 3 in Bloom *et al.* (2005)

Bloom *et al.* defined a protein's "d-neutrality" as the fraction of sequences with d substitutions that still fold to the native structure (Bloom *et al.*,(2005)), and they found the d-neutrality $P_f(d)$ for large d obeys

$$P_f(d) \propto \langle v_{\text{aa}} \rangle^d, \quad (49)$$

where $\langle v_{\text{aa}} \rangle$ ² is a constant (< 1) inherent in proteins. The estimated $\langle v_{\text{aa}} \rangle$ values for several real proteins are listed in Table 3 in Bloom *et al.* (2005).

Here, assuming that the folding free energy landscape is an NK landscape or X landscape, we derive the form of $P_f(d)$ in our theoretical framework. Let ΔG be the free energy of a reference sequence, and $\Delta\Delta G$ be the change in free energy from the reference sequence to its arbitrary mutant on the d -boundary. The probability density of $\Delta\Delta G$ over the d -boundary is approximately given by a normal distribution with the following mean and variance:

$$\mathbf{E}[\Delta\Delta G] \approx -(\Delta G - \mathcal{E}[\Delta G])d(1+k)/\nu \quad \text{for } d \ll \nu \quad (50)$$

$$\mathbf{V}[\Delta\Delta G] \approx a\mathcal{V}[\Delta G]d(1+k)/\nu \quad \text{for } d \ll \nu, \quad (51)$$

where $\mathcal{E}[\Delta G]$ and $\mathcal{V}[\Delta G]$ are the mean and variance, respectively, of the free energy over all possible sequences in the whole sequence space. The a in eqn.(51) takes a value between 1 and 2. Then, by introducing $\Delta G^{\text{extra}}(\leq 0)$ as the minimal threshold for folding

²Bloom *et al.* (2005) used " ν_{aa} " as their original description.

(Bloom *et al.*,(2005)), $P_f(d)$ is given by

$$P_f(d) = \int_{-\infty}^{-\Delta G^{\text{extra}}} \frac{1}{\sqrt{2\pi\mathbf{V}[\Delta\Delta G]}} \exp\left(-\frac{(\Delta\Delta G - \mathbf{E}[\Delta\Delta G])^2}{2\mathbf{V}[\Delta\Delta G]}\right) d\Delta\Delta G \quad (52)$$

$$\approx \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{(-\Delta G^{\text{extra}} - \mathbf{E}[\Delta\Delta G])^2}{2\mathbf{V}[\Delta\Delta G]}\right) \quad \text{for large } d \quad (53)$$

$$\approx \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{\mathbf{E}[\Delta\Delta G]^2}{2\mathbf{V}[\Delta\Delta G]}\right) \quad \text{for large } d \quad (54)$$

$$\approx \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{(\Delta G - \mathcal{E}[\Delta G])^2 d(1+k)}{2a\mathcal{V}[\Delta G]\nu}\right) \quad \text{for large } d. \quad (55)$$

Here, we introduce the following two assumptions. One is that the ΔG of the reference sequence takes almost the global optimum (minimum): $\Delta G \approx \mathcal{O}[\Delta G]$. The other is that site free energy is distributed according to the uniform distribution with the mean ε and variance σ^2 (see Section 2). Let ω be the width of the uniform distribution. ω is related with σ by $\omega \approx 2\sqrt{3}\sigma$. In addition, $|\mathcal{O}[\Delta G] - \mathcal{E}[\Delta G]| = \omega\nu/2$ and $\mathcal{V}[\Delta G] = \sigma^2\nu$. Therefore, the following relationship holds:

$$\frac{(\Delta G - \mathcal{E}[\Delta G])^2}{\mathcal{V}[\Delta G]\nu} = \left(\frac{\omega\nu/2}{\sigma\nu}\right)^2 \approx 3, \quad (56)$$

Substituting eqn.(56) into eqn.(55), we obtain

$$P_f(d) \approx \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{3d(1+k)}{2a}\right). \quad (57)$$

By comparing eqn.(57) with eqn.(49), k_{app} is related with $\langle v_{\text{aa}} \rangle$ by

$$k_{\text{app}} \approx -\frac{2a \ln\langle v_{\text{aa}} \rangle}{3} - 1. \quad (58)$$

Using the values of $\langle v_{\text{aa}} \rangle$ (that is $\langle \nu_{\text{aa}} \rangle$ in their original description) listed in Table 3 in Bloom *et al.* (2005), we obtained $k_{\text{app}} < 0.3$ for the relevant proteins. Wilke *et al.* (2005) investigated asymptotic expressions of $P_f(d)$ using more accurate approximations. They discussed the deviation when they used Gaussian approximation.

Appendix E: Rough estimation of k_{app} from the structure density surface in Fig.4 in Schuster (1995)

We assume that the replication rate constant of an RNA sequence with a structure distance t from an optimal structure is given by $\exp(-ct)$ with a constant c , in which ct corresponds

to the activation energy. Here, we note that Schuster's group used a hyperbolic function of t as the replication rate constant. However, it was reported that their results also hold under exponential form (Ancel & Fontana,2000). Our definition of fitness W is a quantity at the energy level, and then we define $W = -ct$. Consider the d -boundary of a reference sequence. The Hamming distance and structure distance between the reference sequence and its d -fold point mutants located on the d -boundary are denoted by d and t , respectively. We assume $\mathbf{E}[\Delta W|\mathcal{W}]$ on the lefthand-side of eqn.(12) is given by

$$\mathbf{E}[\Delta W|\mathcal{W}] = -(\mathcal{W} - \mathcal{E}) \frac{\mathbf{E}[t|d]}{\mathbf{E}[t|\nu]}, \quad (59)$$

where $\mathbf{E}[t|d]$ is the conditional mean of t with a given d ($d = 1 \sim \nu$). Comparing eqn.(49) with eqn.(12), we obtain

$$\frac{\mathbf{E}[t|d]}{\mathbf{E}[t|\nu]} = \frac{\gamma d}{\nu}. \quad (60)$$

Considering the ridge on the structure density surface in Fig.4 in Schuster (1995) to be $\mathbf{E}[t|d]$ and analyzing only the linear phase at small d 's, we roughly estimated $k_{\text{app}} \approx 9-12$, with $\nu = 100$ and $\mathbf{E}[t|\nu] \approx 36$.

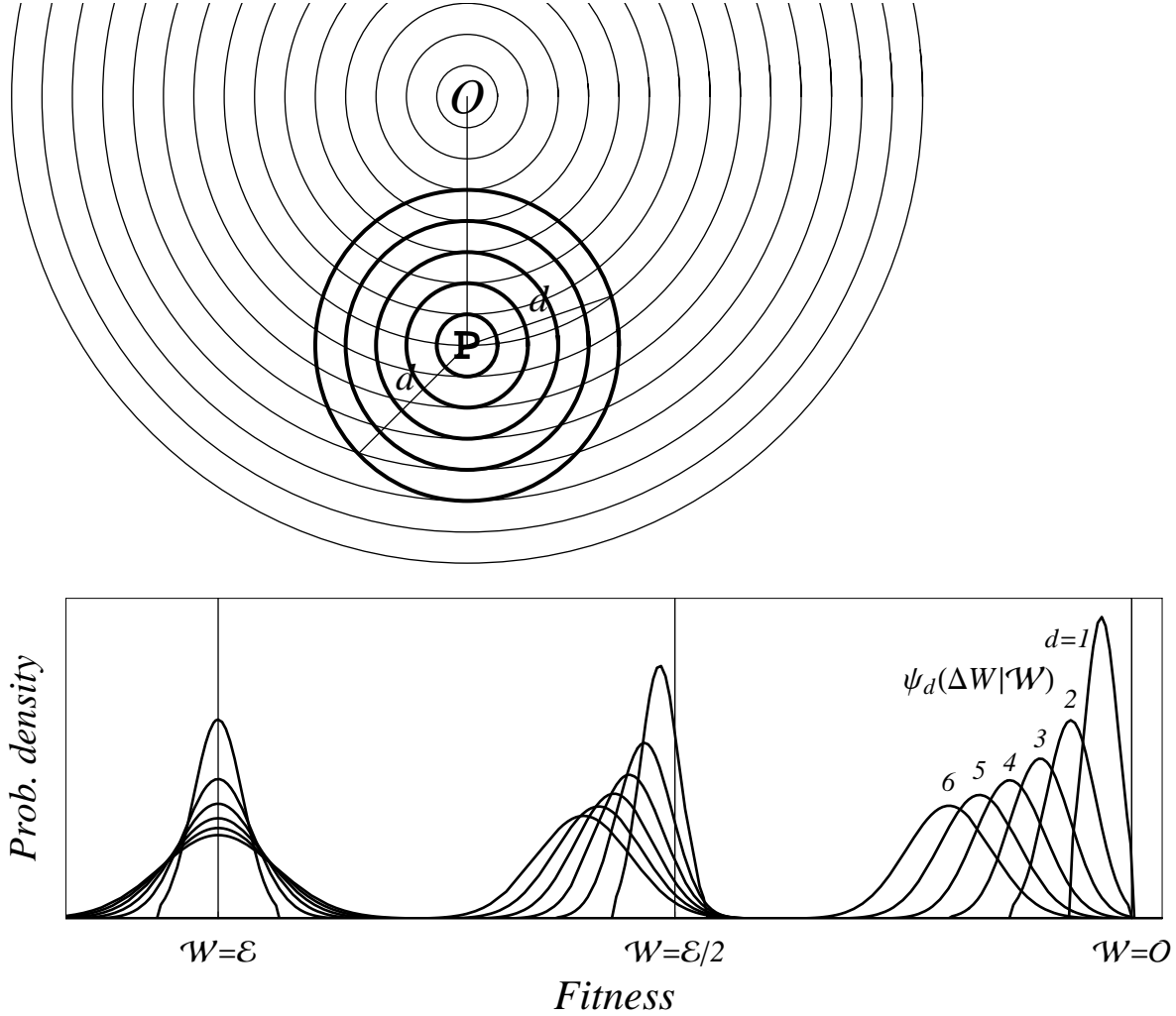


Figure 1: **The d -boundary and its fitness distribution.** (Top) Global sequence space is schematically represented by concentric circles centering at the global peak \mathcal{O} . The radius of each concentric circle represents the Hamming distance. The thick concentric circle with radius d centering at a parent sequence \mathbf{P} represents the d -boundary of the parent sequence. (Bottom) Each Gaussian-like distribution represents the probability density of the fitnesses on the d -boundary ($= \psi_d(\Delta W|\mathcal{W})$). Three cases for the location of parent sequences are shown: $\mathcal{W} = \mathcal{O}$ (peak), $\mathcal{W} = \mathcal{E}/2$ (middle) and $\mathcal{W} = \mathcal{E}$ (foot).

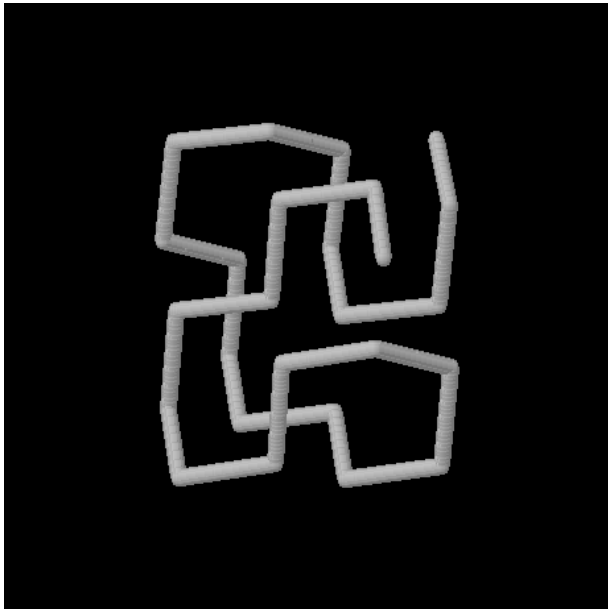


Figure 2: Three-dimensional diamond lattice as a simplified protein model (Blackburne & Hirst,2003). The number of residues is $\nu = 26$. This structure takes the 12 nearest neighbor contacts and is one of the most compact structures in the conformation space.

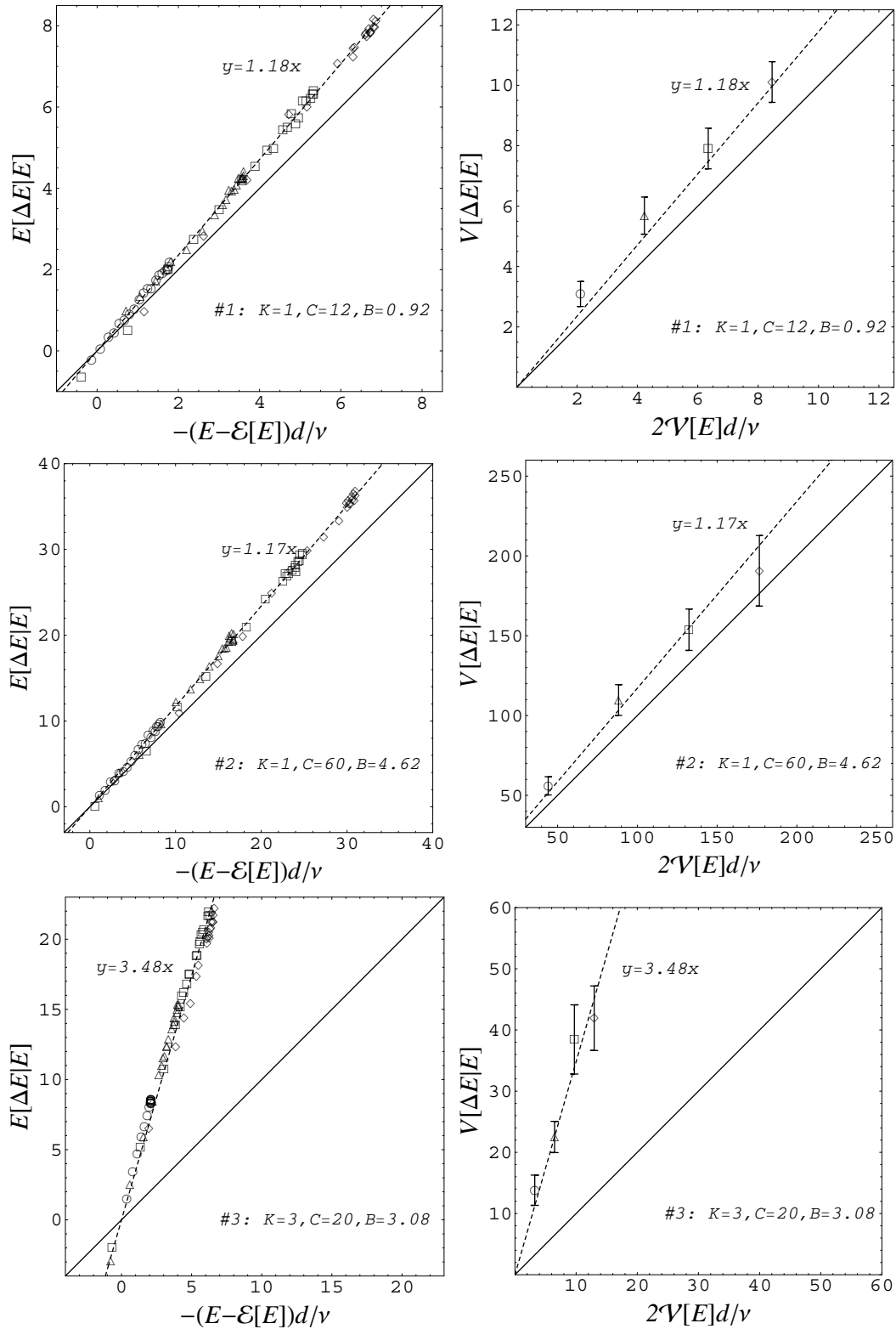


Figure 3: Plots to determine the apparent k -value, k_{app} , for the energy landscape. The energy is for the structure shown in Fig.2. The data were obtained for several reference points on the landscape. The #1,#2 and #3 represent Systems #1,#2 and #3, respectively. The symbols \bigcirc , \triangle , \square and \diamond indicate $d = 1, 2, 3$ and 4 , respectively. For right figures, the mean and standard deviation are indicated. The dashed line is the regression line: $y = \gamma x$. Details are shown in the text.

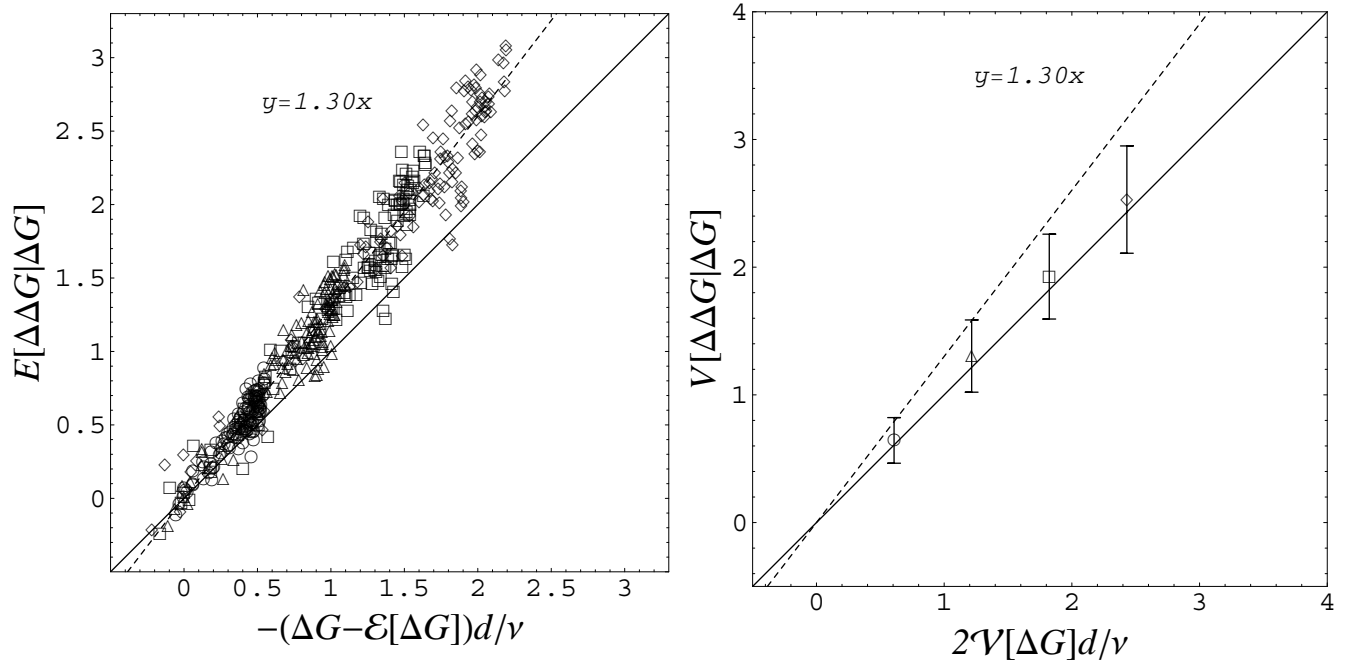


Figure 4: Plots to determine the apparent k -value, k_{app} , for the folding free energy landscape. The free energy is for the reference structure shown in Fig.2. The data were obtained for several reference points on the landscape. These figures were depicted in the same manner as those in Fig.3 by replacing E and ΔE with ΔG and $\Delta\Delta G$, respectively. The symbols \circ , \triangle , \square and \diamond indicate $d = 1, 2, 3$ and 4 , respectively. Details are shown in the text.

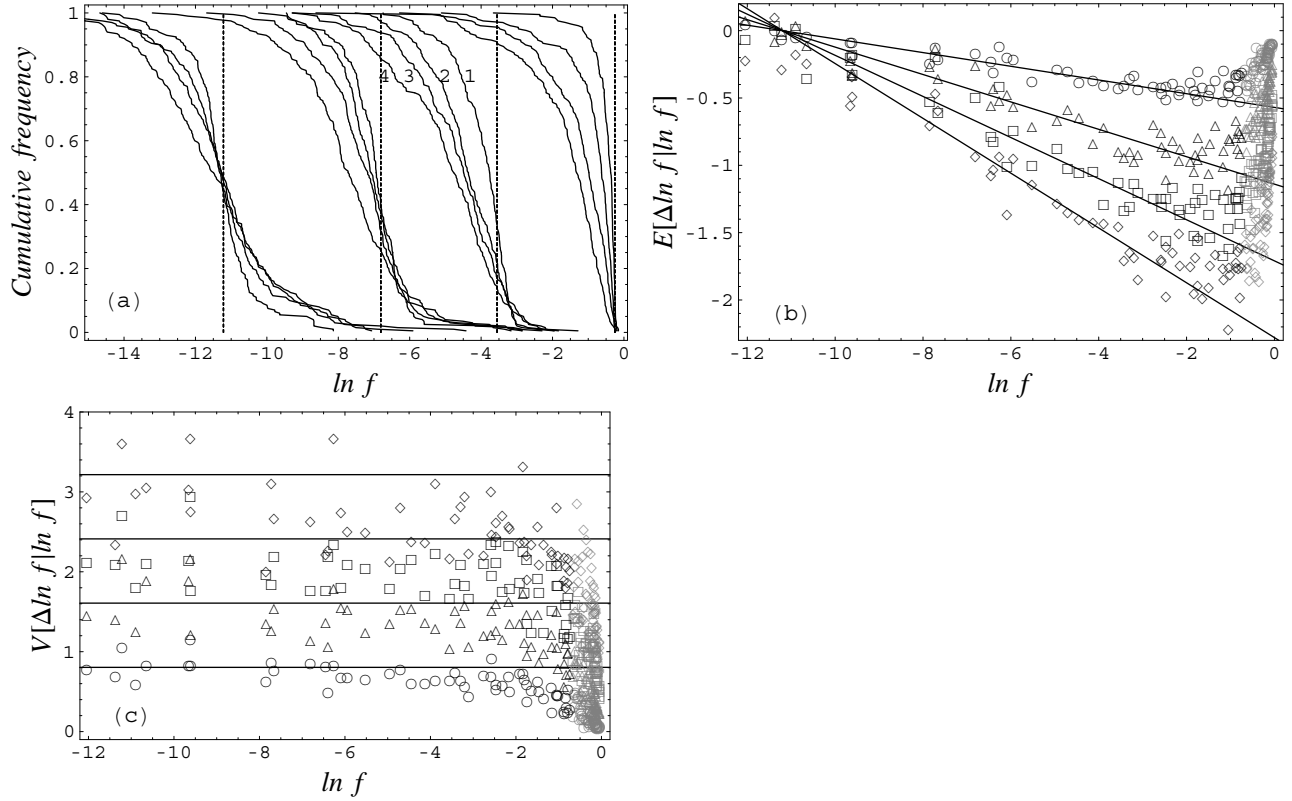


Figure 5: (a) The distribution of $\ln f$ over the d -boundary ($d = 1, 2, 3, 4$) of each of four reference points on the $\ln f$ landscape. The reference structure is shown in Fig.2. Sample size is 200 for each one. The vertical dotted line indicates the $\ln f$ value of each reference sequence. For each reference sequence, the distribution of $\ln f$ becomes broader as d increases, as shown in the figure. (b,c) $\mathbf{E}[\Delta \ln f | \ln f]$ and $\mathbf{V}[\Delta \ln f | \ln f]$ as functions of the $\ln f$ of reference sequences. The solid line represents the function $-\gamma(\ln f - \mathcal{E}[\ln f])d/\nu$ (for b) and $2\gamma\mathcal{V}[\ln f]d/\nu$ (for c) with $\gamma = 1.3$. The quantities for $f > 1/2$ are plotted in gray.