Lack of self-averaging in neutral evolution of proteins

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(Dated: March 21, 2002)

We simulate neutral evolution of proteins imposing conservation of the thermodynamic stability of the native state in the framework of an effective model of folding thermodynamics. This procedure generates evolutionary trajectories in sequence space which share two universal features for all of the examined proteins. First, the number of neutral mutations fluctuates broadly from one sequence to another, leading to a non-Poissonian substitution process. Second, the number of neutral mutations displays strong correlations along the trajectory, thus causing the breakdown of self-averaging of the resulting evolutionary substitution process.

PACS numbers: 87.14.Ee, 87.15.Aa, 87.23.Kg, 87.15.Cc

Introduction. – The neutral theory of molecular evolution is the simplest and most elegant theory of protein evolution. It was formulated in the late 1960’s [1] to explain the high substitution rate of amino acids observed in proteins of many vertebrates and the large intra-specific genetic variations between most species. The theory assumes that most of the amino acid substitutions occurring in an evolving population do not bring any selective advantage but are selectively neutral, maintaining the biological activity of the protein at the original level. Such neutral mutations are assumed to occur at random at a rate $\mu x$, where $\mu$ represents the genomic mutation rate and $x$ is the fraction of mutations which happen to be neutral. The theory predicts that (i) the rate of substitutions (mutations which become fixed in the population) equals the neutral mutation rate $\mu x$ and depends only on the protein considered, independent of the size of the population and its ecology, and that (ii) the number of amino acid substitutions taking place in a time $t$ follows a Poisson distribution with mean value $\mu x t$, thus giving an explanation to the ‘molecular clock’ observed in the early 1960’s [2]. Later studies in the 1970’s and 1980’s have revealed, however, that the variance of the substitution process is larger than its mean value [3, 4], pointing to an underlying non-Poissonian process. Since then, different alternatives have been suggested to explain this feature. Some authors have extended the neutral theory by including into it slightly deleterious mutations [5], others have rejected the neutral theory completely and have suggested that most mutations are fixed by positive selection [6]. An interesting proposal within the realm of neutral theory is its modification in terms of a fluctuating neutral space model [7], which can account for the non-Poissonian statistics.

Progress in the understanding of the folding and thermodynamics of biomolecules has opened the way to assess the thermodynamical stability of biomolecules involved in evolution through computational methods, thus providing powerful tools to complement the traditional population genetic approach. This structural approach has been introduced in the study of neutral networks (i.e., the set of sequences connected by structure conserving mutations) of RNA secondary structures [8], and has found fruitful applications in the study of protein evolution [9]. Based on this structural approach, we have studied a model of neutral evolution denoted structurally constrained neutral model (SCN model), in which conservation of the thermodynamic stability of the native structure is imposed [10]. The SCN model yields evolutionary trajectories consisting of sequences connected through neutral mutations. In this Letter, we show that the fraction of neutral mutations obtained along the trajectories fluctuates strongly, and consequently the SCN model produces a non-Poissonian substitution process, consistent with a fluctuating neutral network scenario [11] and with the statistics of protein evolution [12]. Moreover, we find that the number of neutral neighbors displays strong auto-correlations along the trajectories, which deeply influence the statistics of protein evolution as we discuss below.

We have studied seven protein folds: myoglobin (Protein Database (PDB) code 1a6g), cytochrome c (PDB code 451c), lysozyme (PDB code 31zt), ribonuclease (PDB code 7rsa), rubredoxin (both from a mesophilic and a thermophilic species, PDB codes 1iro and 1brf), ubiquitin (PDB code 1u9a), and the TIM barrel (PDB code 7tim). Although these proteins cover a broad spectrum of different biological activities, according to the SCN model their neutral evolution occurs in a rather similar way, suggesting that our model captures ‘universal’ features of protein evolution. In what follows, we concentrate on myoglobin to illustrate this common scenario.

The SCN model [10, 11]. – The simulated trajectories are started from a given “wild-type” sequence $A^*$ of $N$ amino acids (residues) that folds onto the native structure $C^*$, where both $A^*$ and $C^*$ are taken from the PDB. The target structure $C^*$ is kept fixed throughout the simulation. Following the neutral theory, a given mutated sequence $A'$ is considered to be either neutral, if it folds onto $C^*$ preserving thermodynamic stability, or lethal otherwise. Neither advantageous nor slightly deleterious mutations

\[ \mu x t \]

arXiv:cond-mat/0209453v1 19 Sep 2002
are considered. At each iteration, we generate all possible sequences \( \{A'\} \) obtained through point mutations of the current sequence \( A \) \([12]\), and determine the fraction belonging to the neutral network. One of these neutral sequences is chosen at random and becomes the new current sequence. The whole process is iterated, resulting in an evolutionary trajectory \( \{A_0 = A^*, A_1, A_2, \ldots\} \).

To assess the conservation of the thermodynamic stability of a test sequence in the target structure, we rely on two well-established empirical parameters, the normalized energy gap \( \alpha \) \([11]\), measuring the minimal value of the energy gap between an alternative conformation and the target one divided by their structural distance, and the Z-score \( Z \) \([13]\), measuring the difference between the native energy and the average energy of alternative compact conformations in units of the standard deviation of the energy. A positive and large value of the \( \alpha \)-parameter ensures both that the target conformation has lowest energy and that the energy landscape is well correlated, the latter in the sense that conformations very different from the native have much higher energy. Moreover, the native energy allows a rough estimate of the folding free energy. Alternative structures are generated by aligning in all possible ways the test sequence with all non-redundant protein structures in the PDB. Conformational energies are computed through the effective energy function described in Ref. \([14]\). The resulting estimate of the thermodynamic stability provides a realistic genotype to phenotype mapping, although the energy function used is approximate and alternative structures are non-exhaustively sampled.

**Neutral connectivity.** In the SCN model, the quantity of interest is the fraction of neutral neighbors \( x_1 = X_1/X_{\text{max}} \in (0, 1] \) associated to each sequence \( A_i \), as this quantity determines the evolutionary substitution process (see below). Here, \( X_i \) is the number of neutral neighbors of sequence \( A_i \), and \( X_{\text{max}} \) is its maximal possible value \([12]\). Thus, an evolutionary trajectory is an (ideally infinite) series \( x = \{x_0, x_1, x_2, \ldots\} \). For each position \( k \), we define analogously evolutionary trajectories \( x^{(k)} \), where \( x_i^{(k)} \) counts the fraction of the 18 possible changes at position \( k \) which are neutral. The probability distribution \( P(x) \), where \( P(x) \, dx \) is the probability to find a fraction of neutral neighbors between \( x \) and \( x + dx \) in the trajectory \( x \), obtained for myoglobin is shown in Fig. 1(a). The fraction of neutral neighbors is broadly distributed, reminiscent of a fluctuating neutral network \([10]\).

To get a deeper insight into the mechanism of neutral evolution, it is helpful to investigate the auto-correlation of the trajectory \( x \). For this purpose, we study the ‘profile’ \( p(n) \) of walks of \( n \) steps, defined as \( p(n) = \sum_{i=1}^{n} [x_i - \bar{x}] \), where \( \bar{x} = \int_0^1 x \, P(x) \, dx \) is the average over all \( x_i \) in the trajectory (cf. inset in Fig. 1(b)). The ‘roughness’ of the profile tells us about the presence or absence of correlations along the trajectory \( x \). They can be determined quantitatively by calculating the fluctuations of \( p(n) \) within a window of width \( \ell \) as

\[
F(\ell) = \langle [p(n) - p(n + \ell)]^2 \rangle^{1/2}
\]

(see Ref. \([12]\)). If \( x \) is a series of independent variables, \( F(\ell) \) scales as \( \ell^{1/2} \). Instead, if the series \( x \) is correlated (anti-correlated) over a length \( \ell_0 \), the scaling is modified into \( F(\ell) \sim \ell^\nu \) with \( \nu > 1/2 \) (\( \nu < 1/2 \)) on the same length scale. The information provided by \( F(\ell) \) is equivalent to the one given by the auto-correlation function, but the former has the advantage that different regimes and intermediate crossovers are more easily detected.

The plot of the fluctuations of an evolutionary trajectory \( x \) obtained for myoglobin is shown in Fig. 1(b), together with the analogous analysis of the fraction of neutral neighbors for residues 25 and 115, respectively the residues with the largest and the smallest mean fraction of neutral neighbors. The fraction of neutral neighbors for the whole protein is strongly auto-correlated, with an apparent exponent \( \nu \approx 0.82 \) for about 20 evolutionary steps, after which the random walk exponent \( \nu = 1/2 \) is attained. Single residues have a somewhat larger apparent exponent between \( \nu \approx 0.85 \) (residue 25) and \( \nu \approx 0.9 \) (residue 115) for about \( 10^2 \) to \( 10^3 \) evolutionary steps, which is roughly the product of number of steps it takes the correlations to vanish for the whole protein times the protein length. These auto-correlations can be

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**FIG. 1:** (a) Probability distribution \( P(x) \) of the fraction \( x \) of neutral neighbors for myoglobin. (b) Analysis of the fluctuations of \( x \) along an evolutionary trajectory of myoglobin, displayed as \( F(\ell) \) vs \( \ell \). We present the analysis of the whole protein (triangles) and the analysis for residues 25 (circles) and 115 (squares). The full lines have the slopes 0.82 (for the triangles, up to \( \ell \approx 20 \)), 0.85 (for the circles, up to \( \ell \approx 10^2 \)) and 0.9 (for the squares, up to \( \ell \approx 10^3 \)) indicating strong correlations, whereas the dashed lines have the slope 1/2, indicating the absence of correlations. The inset in (b) shows part of the profile \( p(n) \) vs \( n \) for the total fraction of neutral neighbors (see text).
qualitatively explained by the fact that more stable sequences have a larger number of neutral neighbors (they are more tolerant to mutations), and stability itself is auto-correlated along an evolutionary trajectory as long as the number of mutations is small with respect to sequence length. These correlations, although being short range, have an important influence on the evolutionary substitution process, as shown below.

Modeling the substitution process. – In order to construct the substitution process, we have to obtain the number of mutations accepted within a time interval \( t \). The substitution process consists of three steps: (i) generation of an evolutionary trajectory using the SCN model; (ii) determination of the number \( k \) of mutation events taking place within the time \( t \), which is assumed to be a Poissonian variable of average \( \mu t \), so that the probability of \( k \) mutations within time \( t \) is \( P_k(k) = \exp(-\mu t)(\mu t)^k/k! \); (iii) determination of the number \( n \) of accepted mutation events out of \( k \), where the corresponding conditional probability \( P_{\text{acc}}(n \mid k) = \prod_{i=1}^{n} x_i / \prod_{j=1}^{n+1} (1 - x_j)^{m_j} \). Here, the \( \{m_j\} \) are all integer numbers between zero and \( k - n \) satisfying \( \sum_{j=1}^{n+1} m_j = k - n \). In other words, the probability that a mutation is accepted is \( x_0 = x(A_0) \) as long as the protein sequence is \( A_0 \), \( x_1 = x(A_1) \) as long as the sequence is \( A_1 \), and so on. If all \( x_i = x \) are equal, \( P_{\text{acc}}(n \mid k) \) reduces to a binomial distribution \( P_{\text{acc}}(n \mid k) = \binom{k}{n} x^n (1 - x)^{k-n} \). The probability \( P_t(n) \) that \( n \) mutations are accepted within time \( t \) is the weighted sum over \( k \) of the acceptance probability, \( P_t(n) = \sum_{k \geq n} P_t(k) P_{\text{acc}}(n \mid k) \), which in the case of equal \( x_i \) reduces to a simple Poisson distribution \( P_t(n) = \exp(-\mu x t)(\mu t x)^n/n! \) with average value \( \mu x t \) and substitution rate \( \mu x \), as in the original neutral model.

Statistics of the substitution process. – In our analysis, two kinds of averages must be distinguished. We indicate by angular brackets \( \langle \cdot \rangle \) the average over the mutation and acceptance process for a given realization of the evolutionary trajectory, and by an overline \( \overline{\cdot} \) the average over evolutionary trajectories. We determine the mean number of substitutions \( \langle S_t \rangle \) within a time \( t \), and show this quantity in Fig. 2(a) together with the normalized mutation variance \( R_{\mu} = \langle (S_t)^2 \rangle - \langle S_t \rangle^2 \rangle / \langle S_t \rangle \), the normalized trajectory variance \( R_{x} = \langle (S_t)^2 \rangle - \langle S_t \rangle^2 \rangle / \langle S_t \rangle \), and the normalized total variance \( R = R_{\mu} + R_{x} \). The latter quantity is also known as dispersion index. Notice that if all \( x_i = x \) are the same, one gets \( R_{\mu} = 1 \) as for all Poissonian processes, and the normalized trajectory variance \( R_{x} = 0 \), since \( x \) is constant in all sequence space.

The results of the substitution process based on the evolutionary trajectories are shown in Fig. 2(a). We also show in Fig. 2(b) results based on annealed trajectories, obtained by extracting at random the values of \( x_i \) at each substitution event according to the observed distribution \( P(x) \). In this case, the different \( x_i \) along the trajectories are independent variables. Note that the annealed trajectories ‘interpolate’ between the Poissonian case (all \( x_i \) are equal) and the correlated trajectories obtained through the SCN model: (i) The comparison between the annealed trajectories and the simple Poissonian case allows us to identify the effect of the broad distribution of the fraction of neutral neighbors, whereas (ii) the comparison between the actual and the annealed trajectories allows us to identify the effect of correlations.

In the annealed case, the time spans \( \tau \) between subsequent substitutions are independent variables distributed with the density \( D(\tau) = \int_0^1 P(x)(\mu x)^{-1} \exp(-\mu x \tau) \, dx \), whose average value is \( \tau = \int_0^1 P(x)(\mu x)^{-1} \, dx \). Thus, the average substitution rate \( \langle S_t \rangle / \tau \) is not constant in time as in the Poissonian case. Initially, \( S_t \) is a Poissonian variable with average rate \( \mu \tau \). At large time, however, the rate converges to the smaller value \( \langle S_t \rangle / \tau \approx 1/\tau \), since the process spends more and more time in sequences with small \( x \). Hence, the standard deviation of \( \tau \) is slightly larger than its average value, and the normalized mutation variance \( R_{\mu} \) is larger than the Poissonian value \( R_{\mu} = 1 \), although the actual difference is small (see Fig. 2(b)). The normalized trajectory variance \( R_{x} \) is very small, of the order of the ratio between variance and average value of the \( x_i \).

Using the actual evolutionary trajectories (see Fig. 2(a)), we note that the presence of correlations has only a weak effect both on the average number of substitutions \( \langle S_t \rangle \) and on the normalized mutation variance \( R_{\mu} \). However, the normalized trajectory variance increases considerably in response to the correlations, as \( R_{x} \approx 1 \) for \( \mu t = 200 \). It even grows with time, al-

FIG. 2: Statistics of the substitution process for myoglobin, shown are the average number of substitutions \( \langle S_t \rangle \) divided by \( \mu t \) (circles), the normalized mutation variance \( R_{\mu} \) (squares), the normalized trajectory variance \( R_{x} \) (triangles), and the normalized total variance \( R = R_{\mu} + R_{x} \) (diamonds). We present in (a) the statistics for the trajectories obtained through the SCN model (full symbols), and in (b) the same for the corresponding annealed trajectories (open symbols).
though more and more sequences are used to compute the mutational averages and one could naively expect that such averages approach typical values. Hence, the large fluctuations between different trajectories caused by the strong auto-correlations result in the breakdown of self-averaging in the substitution process, in the sense that even averaging over an arbitrary long trajectory does not give values representative of typical trajectories. (We note, however, that the variable $\langle S_t \rangle/t$ is still self-averaging as its variance vanishes in the long $t$ limit, but the normalized variable $\langle S_t \rangle/\sqrt{t}$ has a variance which increases with time.) The normalized total variance becomes larger than 2 already for $\mu t \approx 100$, in agreement with empirical estimates, varying in the range $1 < R \lesssim 5$ for most of the proteins studied. Hence, these large dispersion indices may be to a large extent due to the correlations present in the evolutionary process.

Conclusions. – We have shown that the evolutionary trajectories in sequence space generated by the SCN model are characterized both by a broad distribution of neutral connectivities and by strong correlations, which deeply influence the statistics of neutral evolution and the substitution process. For example, fluctuations of the evolutionary rate from one branch of the evolutionary tree to the other can obscure lineage effects, i.e. variations of the substitution rate among different taxonomic groups. One such effect is the generation time effect: Since the natural time unit for measuring substitution events is the generation time (at which reproduction takes place), the longer the latter the slower the substitution rate is expected to be. This has been verified by comparing for instance substitution rates in rodents and primates. However, the effect is significantly larger for synonymous substitutions (DNA changes which still code for the same amino acid) than for non-synonymous ones. Non-synonymous substitutions are superimposed with the large and correlated fluctuations in the substitution rate that we just described, while synonymous ones are not. Thus the statistics of neutral substitutions could explain this quantitative difference. Additionally, a better understanding of the mechanisms of neutral evolution will help to single out the more interesting cases of positive selection as, for instance, changes in the protein function and responses to variations of the environment. The best current bioinformatics method to identify such cases of positive selection, recently used to study the evolution of Drosophila genes, assumes a neutral substitution process with constant fraction of neutral neighbors $x$. The broad distribution and the correlations that we observe can mimic the presence of positive selection, and they should be taken into account to improve the performance of such methods.

[1] M. Kimura, Nature 217, 624 (1968); J.-L. King and T.H. Jukes, Science 164, 788 (1969).