Connectivity of neutral networks, overdispersion and structural conservation in protein evolution

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Abstract

Protein structures are much more conserved than sequences during evolution. Based on this observation, we investigate the consequences of structural conservation on protein evolution. We study seven of the most studied protein folds, finding out that an extended neutral network in sequence space is associated to each of them. Within our model, neutral evolution leads to a non-Poissonian substitution process, due to the broad distribution of connectivities in neutral networks. The observation that the substitution process has non-Poissonian statistics has been used against the original Kimura’s neutral theory, while our model shows that this is a generic property of neutral evolution with structural conservation. Our model also predicts that the substitution rate can strongly fluctuate from one branch to another of the evolutionary tree. The average sequence similarity within a neutral network is close to the threshold of randomness, as observed for families of sequences sharing the same fold. Nevertheless, some positions are more difficult to mutate than others. We compare such structurally conserved positions to positions conserved in protein evolution, suggesting that our model can be a valuable tool to distinguish structural from functional conservation in databases of protein families. These results indicate that a synergy between database analysis and structurally-based computational studies can increase our understanding of protein evolution.

Keywords: Neutral evolution, Non-Poissonian substitution process, Conserved protein residues
INTRODUCTION

The advent of large scale genome projects is transforming the field of molecular evolution (Koonin et al., 2000). The molecular mechanisms of evolution are becoming increasingly amenable to direct observation (Henikoff et al., 1997; Gerstein, 1998; Thornton et al., 1999). The advent of large scale genome projects, however, the structural tension of the neutral theory, the fluctuating neutral space model (Takahata, 1987), accounts for the non-Poissonian statistics of substitutions. One of the goals of this study is to investigate the consequences of structural conservation on the properties of neutral networks and on the substitution process associated to them. We show that neutral evolution does not lead to a Poissonian substitution process. This result complements the fluctuating neutral space model by Takahata (1987), and suggests that arguments against the neutral theory based on the fact that the substitution statistics is non-Poissonian (Gillespie, 1991) are not conclusive. A deeper understanding of the mechanism of neutral evolution will help to single out the perhaps less common but more interesting cases of positive selection as, for instance, functional changes and responses to changes in the environment. It can also be useful for calibrating the molecular clocks used to reconstruct phylogenetic trees, whose reliability is severely limited by the fluctuations of the substitution rate (Ayala, 1997).

Another interesting application of the SCN model is the possibility to distinguish between the genome of a species not because they bring a selective advantage but due to random genetic drift acting on alleles of equivalent selective value. Kimura’s neutral model predicts that the rate of amino acid substitution of a given protein is approximately constant for different species within major evolutionary groups, independent of the number of individuals and ecology of the species. This was in agreement with earlier observations on protein evolution which lead to postulate a kind of “molecular clock” (Zuckerkandl & Pauling, 1962). According to Kimura’s theory, the fraction of amino acids neutrally substituted in a time $T$ is a Poissonian variable of expectation value $kT$ where $k$, the substitution rate, is different for different proteins but does not change for different species. A subsequent study by Ohta and Kimura (1971) measured the variance of the substitution process acting on different species, finding that it is larger than the mean, i.e. the process is non-Poissonian. Such a result was confirmed by the more sophisticated analyses by Langley and Fitch (1973) and Gillespie (1989). This and other observations lead first Ohta and then Kimura to adopt, in place of the original neutral model, a model based on slightly deleterious mutations (Ohta, 1976), and Gillespie to reject in toto the neutral theory favouring the hypothesis that most substitutions in protein sequences are fixed by positive selection (Gillespie, 1991). Takahata, however, showed that an extension of the neutral theory, the fluctuating neutral space model (Takahata, 1987), accounts for the non-Poissonian statistics of substitutions.

In this work we apply to seven of the most studied protein folds the structurally constrained neutral model (SCN) which three of us recently introduced in the context of lattice models (Bastolla et al., 1999; Bastolla et al., 2000b). We compare qualitatively the substitution process obtained from the SCN model and that observed in protein sequence evolution. The SCN model is based on the observation that evolution conserves protein structure much more than protein sequence (Holm & Sander, 1996; Rost, 1997). We assume that all mutations conserving the structure have the same probability of being fixed, thus resulting in a neutral model. The main reason to introduce structure conservation as a working hypothesis is the experimental observation that many mutations do not modify significantly the activity of a protein and its thermodynamic stability, while mutations substantially improving protein functionality are rare (Orencia et al., 2001).

The neutral theory of molecular evolution was introduced in the late 60s by Kimura (1968) and by King and Jukes (1969) to explain the high substitution rates observed in vertebrates as well as the large amount of intra-specific genetic variation observed in most species. According to neutralism, most amino acid substitutions are fixed in
functional and structural conservation. By simulating neutral evolution we identify the key positions which are more difficult to mutate. We identify them as structurally conserved positions, and those positions conserved in actual evolution but not in the SCN model as functionally conserved ones. Practically all residues whose functional role is known belong to this class. Most positions are not conserved in the SCN model, as similarly observed in actual evolution data. We identify them as neutrally evolving positions, and argue that their preeminence is an evidence of the importance of neutral evolution. Finally, a small number of positions appear structurally important in the SCN model but are not significantly conserved. This could be due to a limitation either of the SCN model or of the protein database, but it could also be a clue of structural changes, possibly positively selected. Other methods to identify computationally structurally important positions have been proposed recently (Kannan & Vishveshwara, 1999; Cecconi et al., 2001). In particular, another method based on simulated evolution has appeared in a recent preprint after this work had been completed (Dokholyan & Shakhnovich, 2001).

As most computational studies of protein evolution, the SCN model is based on an approximate stability criterion relying on the Z-score (Bowie et al., 1991; Goldstein et al., 1992) and on a folding parameter measuring the degree of correlation of the energy landscape (Bastolla et al., 1999). While these parameters can not predict precisely the thermodynamic stability of a specific sequence, our previous studies show that they correlate with the observed stability. Thus, we expect that the statistical properties derived from the analysis of a large number of sequences capture real features of protein evolution.

STRUCTURALLY CONSTRAINED NEUTRAL MODEL

Following Kimura, we divide mutations in two classes: those which result in inactivating the protein, which are regarded as lethal and can not spread in the population, and those after which the protein remains active, which are regarded as selectively neutral. This mutational spectrum implies that protein sequences evolve on a neutral network, i.e. a set of sequences where the protein is active and which can be connected through point mutations. Under this mutational spectrum, fixation of slightly deleterious mutations can not take place, since these are not included in the model, as well as advantageous mutations. This is of course an important limitation of neutral models.

Kimura’s neutral model assumes that the rate of appearence of neutral mutations is constant throughout evolution. In a paper of 1977, however, Kimura comments that rate constancy may not hold exactly (Kimura, 1977). Our model is not based on any assumption on the neutral mutation rate. Rather, we compute the effect of mutations on protein stability using an effective model of protein folding (Bastolla et al., 2000a) which provides us with a genotype to phenotype mapping. In this respect, the rate of occurrence of neutral mutations is an outcome of the model. It turns out that this rate shows very broad fluctuations throughout evolution. As we shall see below, the variance in evolutionary rates predicted by our model is in qualitative agreement with observations of protein evolution (Gillespie, 1991).

The model does not take into account population dynamics. This is based on the fact that, within Kimura’s model, the substitution rate is not influenced by population size. An extension of Kimura’s model to take into account small variations in the neutral mutation rate confirmed this result (Bastolla & Peliti, 1991). However, population size might influence the substitution rate if the rate of neutral mutations shows broad fluctuations, as observed here. The explicit inclusion of population genetics into the model would be needed to investigate this interesting possibility.

A neutral network is defined starting from a protein sequence in the Protein Data Bank (PDB). The corresponding protein structure has to remain thermodynamically stable during evolution. Thermodynamic stability is evaluated through an effective model of protein folding. The folding parameters of the native structure, computed through the model (see Materials and Methods), must be above 98.5 percent of the value they have in the PDB sequence. Sequences where this condition is met are named viable sequences. A neutral network is a set of viable sequences which can be connected to the starting sequence through point mutations passing on other viable sequences. Thus sequences on a neutral network share the same protein fold and are evolutionarily connected. For every amino acid sequence A in the neutral network we can measure the fraction of neutral neighbors $x(A)$, which is the fraction of its possible point mutations which are viable.

We model protein evolution at the level of a single sequence. During evolution, the sequence
moves on the neutral network generating an evolutionary trajectory, i.e. a list of subsequently visited sequences belonging to the neutral network. In the present context, the only relevant quantity is their fraction of neutral neighbors \( x(A) \in (0,1] \). Thus an evolutionary trajectory can be represented through a very long list \( x = \{x_1,x_2,\cdots\} \).

The rate of occurrence of a neutral mutation starting from sequence \( A \) is the product of a constant mutation rate times the probability \( x(A) \) that the mutation is viable. Thus the neutral mutation rate is not constant in the framework of our model, and can be explicitly computed by computing \( x(A) \) for all sequences in an evolutionary trajectory. The statistics of the substitution process can then be obtained by coupling the evolutionary trajectory \( T \), i.e. a list of subsequently visited sequences belonging to the neutral network.

Two kinds of random variables must be distinguished. We indicate by angular brackets the average over evolutionary trajectories. The variance of the substitution rate with respect to different evolutionary trajectories. This term, which is not present in the standard neutral model, explains why the variance of the number of substitution is typically larger than its mean value, contrasting with a Poissonian process.

If all sequences have the same fraction of neutral neighbors \( x(A) \equiv x \), the number of substitutions in a branch of length \( t \) is Poissonian with mean \( \mu T x \) and the substitution rate is equal to \( \mu x \) as in Kimura’s model. If \( V_x \) is not zero, the substitution distribution is more complicated and has to be computed numerically using the evolutionary trajectories simulated.

**NUMERICAL RESULTS**

**Folding of random sequences**

As a preliminary analysis, we measured the distribution of the folding parameters \( \alpha \) and \( -Z' \) (see Materials and methods) of the native structures considered in this work for random sequences of the same length of the corresponding PDB sequences. On over 20,000 attempts, we always found folding parameters much lower than for PDB sequences. The only exception was the smallest protein, the 53-residues rubredoxin, for which a single random sequence had one of the stability parameters comparable to that of the PDB sequence, even if still smaller than it. This result is consistent with the work of Keefe and Szostak who were recently able to select ATP binding proteins from a random library of more than \( 10^8 \) sequences (Keefe & Szostak, 2001). We note that it is possible to evaluate the size of the neutral network from the joint distribution of \( \alpha \) and \( Z' \), but to this end better statistics are needed than those obtained here.

**Connectivity of neutral networks**

For sequences \( A \) belonging to the neutral network, the fraction of neutral neighbors \( x(A) \) counts the fraction of all possible point mutations of \( A \) which still fall into the neutral network. We measured this quantity for at least 20,000 sequences for each fold, finding that it has a broad distribution (see Fig. 1). The shape of the distribution is qualitatively similar for all of the studied proteins, but in the case of cytochrome c, the distribution is shifted to lower connectivities. Results for all seven folds are summarized in Table 1.
The connectivity landscape $x(A)$ is locally correlated for distances in sequence space of the order of at least ten substitutions. The correlation function $C(t) = \frac{\langle x(A_{t_0})x(A_{t_0+t}) \rangle - \langle x(A) \rangle^2}{\sigma_x^2}$ between connectivities of two sequences at distance of $t$ steps decays similarly for all proteins and can be fitted to a stretched exponential $C(t) \approx \exp\left(-\frac{t}{\tau_\eta}\right)$, with exponents $\eta$ ranging from 0.60 to 2.8. Thus correlations decay to one tenth after about ten substitutions (data not shown).

**Substitution process**

The broadness of the connectivity distribution directly implies that the substitutions process fluctuates more than a Poissonian process, i.e. it is overdispersed. We computed average and variance of the substitution process numerically, using the evolutionary trajectories generated in our simulations. Results for myoglobin are shown in Fig. 2. Notice that the substitution rate $\langle S_t \rangle / t$ is roughly constant in time, and the total dispersion index $R(t) = \frac{V(S_t)}{\langle S_t \rangle}$ takes values between 1.0 at small $t$ and 1.9 at large $t$, consistent with the value $R = 1.7$ estimated by Kimura for myoglobin.

**FIG. 1.** Distribution of the fraction of neutral neighbors for four protein folds.

**FIG. 2.** Moments of the substitution process for myoglobin versus mutational time $\mu t$. Solid line: average substitution rate divided by $\mu$, $\langle S_t \rangle / \mu t$. Black circles: mutational variance $\langle S_t^2 \rangle - \langle S_t \rangle^2$ divided by the mean $\langle S_t \rangle$. White circles: total dispersion index, $R_u + R_x$, where $R_u$ is the trajectory variance $\langle S_t \rangle^2 - \langle S_t \rangle^2$ divided by $\langle S_t \rangle$.

Due to local correlations in sequence space, different evolutionary trajectories $x(A_1) \cdots x(A_n)$, representing different populations, give different mean and variance of the substitution process over short time scales. This phenomenon produces new lineage effects, i.e. apparently varying substitution rates in different branches of the phylogenetic tree. To illustrate them, we show in Fig. 3 the mean $\langle S_t(x) \rangle$ and the variance $V(S_t, \{x\})$ for three realizations of the evolutionary trajectory $\{x\}$. Such an effect could overshadow the generation time effect for replacement substitutions (Britten, 1986; Li et al., 1987; Gillespie, 1991). It could also been responsible for the wide fluctuations in the substitution rate for different lineages observed by Ayala (1997).
FIG. 3. Substitution process for three realizations of the evolutionary trajectories of myoglobin. Black circles represent variances, no symbols represent mean values.

Sequence similarity

One may ask whether sequences sharing the same fold must have a high level of similarity. To investigate this question, we measured the distribution of sequence similarity for sequences in the neutral network obtained through simulation of our model as well as for homologous sequences in public databases. Similarity between two aligned sequences of the same length is defined as the fraction of positions where the same amino acid appears. Results are showed in Fig. 4 for the globin fold. Similar results have been obtained for all other folds.

Sequences in the neutral network (solid line) have an average similarity only slightly larger than random sequences, for which a Gaussian-like distribution of average value $1/20$ is expected. This confirms a previous finding of three of us for neutral networks of lattice structures (Bastolla et al., 1999). As previously observed by Rost (1997), the same result holds also for sequences in the FSSP family of sequences sharing the same structure (Holm & Sander, 1996), which are showed as dotted line. Low similarity for sequences with the same fold has also been found in a recent computational study based on sequence optimization for native protein structures (Dokholyan & Shaknovich, 2001). The dashed line shows the similarity distribution for sequences in the PFAM database of similar sequences (Bateman et al., 2000). The PFAM family has on the average a much larger similarity. This is in part due to the fact that in this case sequence similarity must be large enough for the homology to be detected and in part to the fact that proteins in a PFAM family are subject to stronger functional conservation than proteins in the FSSP family. For the globin family, which has been intensively studied, even the PFAM similarity is very low.

Residue conservation

The SCN model identifies structurally conserved residues. In this respect, the present results can be compared to the model of Shaknovich and coworkers (Shakhnovich et al., 1996; Dokholyan & Shaknovich, 2001) based on sequence optimization (Shakhnovich & Gutin, 1993; Shakhnovich, 1994), and to the bioinformatic studies of Ptitsyn’s group (Ptitsyn, 1998; Ptitsyn & Ting, 1999).

We evaluate the conservation of each position measuring its rigidity (see Materials and Methods). This was done for each fold using three different sets of sequences: (i) Sequences obtained from simulations of our neutral model; (ii) Homologous sequences in the PFAM database (Bateman et al., 2000); (iii) Sequences with the same structure in the FSSP database (Holm & Sander, 1996). The PFAM family often contains orthologous proteins performing the same function while in the FSSP family different functions may be present and function conservation plays a less important role. Nevertheless, there is usually a good correlation between the rigidity of a given position evaluated through the PFAM and FSSP databases. An exception is the TIM barrel family, one of the most common folds, used to perform different functions, each approximately associated to a different PFAM
family. In this case, the two sets of rigidities show no correlation. For this fold, using a bioinformatic analysis, Mirny and Shakhnovich (1999) found evidence of functional conservation (the same functional positions tend to be conserved in all functional families, although with different residues in each functional family) but could not find evidence of structural conservation.

Before turning to the analysis of conserved positions, we observe that there are three reasons why sequence databases may tend to overestimate structurally based conservation. The first one is the small size of databases. The second one is the fact that many sequences are evolutionarily related: databases usually provide biased samples of the tree of life. To reduce these effects, we limit our analysis to sequences that do not have similarity larger than a threshold $q$ which we choose equal to 0.85 in order not to reduce too much database size, and try to estimate the maximal conservation that one would observe with a database of similar size and correlations, in the null hypothesis that all positions are equivalent. The third reason is that many residues are conserved on functional grounds, sometimes even in the FSSP database, and it may be difficult to distinguish them from structurally conserved residues.

Conservation in the neutral network only expresses structural conservation, thus the comparison between rigidities predicted by the SCN model and observed in evolution may allow to single out functionally conserved positions or positions involved in interactions with cofactors, which are not represented in our model. We tested this for two well studied protein families: the globin family and the cytochrome c family. In both cases structurally conserved positions identified by the SCN model coincide with those identified in previous bioinformatic studies as part of the folding nucleus (Ptitsyn, 1998; Ptitsyn & Ting, 1999), and additional structurally conserved positions are found. For other protein families less is known about functional residues, but the few ones which are identified in the SwissProt file are recognized as such by the SCN model. In our analysis of the globin family, positions in contact with the heme are not regarded as structural, even if the heme plays also an important stabilizing role, since interactions between amino acids and cofactors are not considered in the model, and they are much more specific than interactions between amino acids.

In Fig. 5 we compare the rigidities obtained from our model to those measured in the FSSP family for the myoglobin fold. Each point represents a position on the native structure. The dotted lines in the figure are rough estimates of the maximal rigidity expected in a random situation, i.e. all equivalent residues and same distribution of similarity as in the set examined. Only residues more rigid than that are considered significantly conserved. Many of the most conserved residues are in contact with the heme group (large circles). A notable exception is Pro37, which is strongly conserved and not in contact with the Heme group. Although the conservation of this residue has not been fully explained so far, Ptitsyn and Ting report that it may be due to functional reasons (Ptitsyn & Ting, 1999). The three positions most conserved according to the SCN model coincide with structural positions identified in the bioinformatic analysis by Ptitsyn and Ting. They are, in order of rigidity, Leu115, Trp14, Met131. Val10 is rather conserved both in our model and in the bioinformatic study. The remaining two positions identified by Ptitsyn, Ile111 and Leu135, are not among the most conserved in our model, although they are above the average. In addition, there are eight more positions significantly conserved in the SCN, whose evolutionary conservation is somewhat lower (but above the average in all cases except His119). They are: Val13, Val17, His24, Leu69, Leu76, His119, Phe123, Ala134. Interestingly, structurally conserved residues form a cluster, so that it has been proposed that they play the role of “folding nuclei” (Shakhnovich et al., 1996; Ptitsyn 1998; Mirny & Shakhnovich, 2001). A similar situation applies also for the case of cytochrome c: two of the positions identified in (Ptitsyn, 1998) are the most conserved in our model (Phe7, Leu74), another one is significantly conserved (Trp77) and the fourth one is not present in the structure we choose as reference (PDB code 451c). Moreover, there are three positions significantly conserved in our model and in the FSSP alignment (Tyr27, Ile48, Val66) and one conserved in our model but not in the alignment (Gly36).

* Residues are labeled in the order in which they are listed in the PDB file of the structure \textit{1a6g}
FIG. 5. Rigidity in the FSSP family versus rigidity in the neutral network for the myoglobin fold.

FIG. 6. Structure of myoglobin with the Heme group. The structurally most conserved residues are represented in color together with their side chains. These are: Leu115, Trp14, Met131, Val10, Val13, Val17, His24, Leu69, Leu76, His119, Phe123, Ala134. The PDB code of the structure is 1a6g. The colour code represents temperature increasing from blue to red.

We show a similar plot also for rubredoxin, a small bacterial protein involved in electron transport. In this case, we studied two homologous proteins, one from a mesophylic and one from a thermophylic bacterium. Their sequences have 57% similarity and belong to the same PFAM and FSSP classes. Although the structures are rather similar, the stability of the thermophylic protein, as measured by the $Z'$ and $\alpha$ parameters, is higher than the stability of the mesophylic protein, as it should be. This result supports our choice of the stability criteria. We compare the rigidities obtained from the SCN model for the two structures in Fig. 7 (upper panel). There is a remarkable correlation, despite the fact that results are obtained from independent evolutionary runs with different selection parameters. In Fig. 7 (lower panel) we compare the rigidity observed in the SCN model (for mesophylic rubredoxin) with the rigidities observed in the PFAM and FSSP databases.

FIG. 7. Comparison of rigidities between the two simulated neutral networks of mesophylic and thermophylic rubredoxin (top) and between simulated and observed rigidities for mesophylic rubredoxin (bottom). Full circles refer to the PFAM family, open diamonds to the FSSP family.

Finally, we show in Fig. 8 a scatter plot of rigidities for the protein showing the worst correlation between predicted and observed rigidities: the TIM barrel, one of the most common folds, used for several enzymatic functions. The one that we studied is a triose phosphate isomerase functioning in the glycolysis. In this case, there is also no observable correlation between rigidities in the PFAM and FSSP databases, and rigidities in the
FSSP class are very low, in particular because several residues are deleted in many sequences.

FIG. 8. Rigidity in the FSSP database versus rigidity in the neutral network for the TIM barrel fold. The circles identify two positions of known functional role for one of the enzymes of the TIM barrel family.

Unlike for other folds, in the case of ubiquitin the structurally conserved positions are distributed along the main axis of the protein. There is also a conserved polar position on one loop. For lysozyme as well the most conserved residues form a non compact core. In all other cases the structurally conserved residues form a hydrophobic cluster which is rather compact. There is some correlation between conservation and burial in the interior of the protein as measured by the number of contacts, but burial alone does not explain all of the conservation. We notice that also in our previous lattice simulation the most conserved positions were those in the interior of the lattice structure (Bastolla et al., 1999).

DISCUSSION

In this work we studied a neutral model of protein evolution based on structure conservation. For all of the examined folds, local connectivities of neutral networks are broadly distributed. This result implies that even in neutral evolution the number of substitutions fluctuates more than a Poissonian variable, i.e. it is overdispersed. Therefore overdispersion can not by itself be used as a test for positive selection, as argued for instance by Gillespie (1991). Our results, nevertheless, show that the dispersion index of the SCN substitution process is typically small, rarely overcoming four. Thus, proteins with extremely high dispersion index, as some of those studied by Gillespie (1989) or Ayala (1997) are not likely to have evolved in this way. The average substitution rate is almost constant in time, but it may vary considerably for different evolutionary trajectories, corresponding to different branches of the phylogenetic tree. This fact should be taken into account when studying lineage effects such as the generation time effect (Britten, 1986; Li et al., 1987; Gillespie, 1991).

By simulating neutral evolution, we identified structurally conserved positions and compared them to evolutionary conserved positions in known protein families. The comparison is favorable for myoglobin, cytochrome c, lysozyme, ribonuclease and rubredoxin, while for ubiquitin and the TIM barrel correlation between predicted and observed conservation is almost absent. We note, however, that the TIM barrel shows very little structural conservation, and the small size of the ubiquitin family makes the comparison not conclusive. The plots comparing conservation in simulated evolution (on the abscissa) to conservation in real evolution (on the ordinate) can be divided in four parts. In the upper left quadrant there are positions not conserved in the SCN model but conserved in evolution. We suggest that most of them are conserved for functional reasons or because of interactions with cofactors, which are not taken into account in our protein model. Positions of known functional importance belong to this class, but not enough is known on protein function to prove our interpretation in all cases. In the upper right quadrant there are positions conserved both in the SCN model and in the databases, whose conservation is likely to have a structural ground. For this small subset the rigidities that we predict are correlated to the observed ones. Interestingly, those positions form spatial clusters which have been identified with folding nuclei (Shakhnovich et al., 1996; Ptitsyn, 1998; Mirny & Shakhnovich, 2001). Although we can not discuss such interpretation, since our evolutionary algorithm does not take into account folding kinetics, it is to be expected that positions important for stability also play an important kinetic role. In the bottom left quadrant there are positions not conserved neither in the SCN model nor in the databases. These positions are likely to be the main actors in neutral evolution. Last, in the bottom right quadrant there are few positions conserved according to the SCN model which do not appear to be evolutionarily conserved. Barring artifacts due to the SCN model, we should consider the possibility of
conservation with low rigidity (but typically much higher than random). In order to verify whether this is the case, we need larger and less correlated protein classes. Another possibility is that these positions are frequently substituted because they can produce structural changes, possibly positively selected. Although this possibility is rather speculative, it would be interesting to investigate it in more detail.

Our results are based on an approximate stability criterion relying on the Z-score (Bowie et al., 1991; Goldstein et al., 1992) and on a parameter measuring the degree of correlation of the energy landscape (Bastolla et al., 1999). While such criterion may not be suitable for the quantitative prediction of the thermodynamic stability of particular proteins, we believe that the statistical properties of the SCN model reflect those of actual protein evolution. This confidence has several grounds. First, in the study of a lattice model, three of us have previously applied a rigorous criterion of stability, and compared it to a criterion obtained from the Z-score (Bastolla et al., 2000b). Although the two criteria give different responses for specific sequences, it is possible to choose a threshold such that most sequences selected with the Z-score criterion are also selected with the rigorous criterion. Second, the present results are robust with respect to changes in the selection thresholds and stability criteria. Third, we tested our stability parameters on a large number of sequences obtained from mutations of a TIM barrel enzyme, whose phenotypic effect has been experimentally measured in a recent paper (Silverman et al., 2001). We found that, even if our criterion cannot predict the effect of individual mutations, the latter is correlated to the parameter with correlation coefficient 0.4.

The present results can be used in the rational approach to directed evolution of biocatalysts (Altamirano et al., 2000; Voigt et al., 2001) since we identify sites that are more tolerant to mutations and therefore likely targets for evolutionary improvement. This is a remarkable possibility, since it indicates how results based on the assumption of neutral evolution can be used to search for positive substitutions.

MATERIALS AND METHODS

Protein model

We represent a protein structure by its contact matrix $C_{ij}$, where $C_{ij} = 1$ if residues $i$ and $j$ are in contact and $C_{ij} = 0$ otherwise. Two residues are considered in contact if any two of their heavy atoms are closer than 4.5 Å. The effective free energy associated to a sequence of amino acids $A$ in the configuration $C$ is approximated as a sum of pairwise contact interactions,

$$E(A, C) = \sum_{i<j} C_{ij} U(A_i, A_j),$$  \hspace{1cm} (3)

where $A_i$ labels one of the twenty amino acid types and $U(a, b)$ is a $20 \times 20$ symmetric interaction matrix. Here we use the matrix derived by Bastolla et al. (2000a), which describes accurately the thermodynamic stability of a large set of monomeric proteins (Bastolla et al., 2001).

Three remarks are needed: First, the effective energy parameters implicitly take into account the effect of the solvent and depend on temperature. They express free energy rather than energy. Second, the effective energy of a structure is defined with respect to a completely extended reference structure where no contacts are formed and which sets the zero of the energy scale. Third, one can derive from the database not the parameters $U(a, b)$ themselves but the dimensionless quantities $U(a, b)/k_B T$. It is thus important to use dimensionless parameters to evaluate the stability of the protein model.

Candidate structures

We generate candidate structures for a protein sequence of $N$ residues by generating all possible gapless alignments of the sequence with structures in the Protein Data Bank. This procedure is called threading. In this way, we typically generate several hundreds of thousands of protein-like structures per sequence. In the present context, threading is directly used to produce the contact maps of the candidate structures. In order to speed up the computations, we use a non redundant subset of the PDB excluding proteins with homologous sequences, selected by Hobohm & Sander (1994).
The folding parameter $\alpha$

For a given sequence $A$, the energy landscape is well correlated if all configurations of low energy are very similar to the configuration of minimal effective energy, $C^*(A)$. Structure similarity is measured by the overlap $q(C, C^*)$, counting the number of contacts that two structures have in common and normalizing it through the maximal number of contacts, so that $q$ is comprised between zero and one. In a well correlated energy landscape, the inequality holds

$$\frac{E(A, C) - E(A, C^*)}{|E(A, C^*)|} \geq \alpha(A) (1 - q(C, C^*)),$$

(stating that the energy gap between each alternative structure $C$ and the ground state $C^*$, measured in units of the ground state energy, is larger than a quantity $\alpha(A)$ times the structural distance $1 - q(C, C^*)$). The dimensionless quantity $\alpha(A)$, which is the largest quantity for which the above inequality holds, can be used to evaluate the folding properties of sequence $A$. For random sequences, many different configurations have quite similar energy and $\alpha(A) \approx 0$. In this case the energy landscape is rugged, the folding kinetics is very slow and the thermodynamic stability with respect to variations in the solvent is very low. In contrast, computer simulations of well designed sequences have shown that, when $\alpha(A)$ is finite, the folding kinetics is fast and the stability with respect to changes in the energy parameters as well as mutations in the sequence is very high.

Our algorithm computes the parameter $\alpha(A)$ for a fixed target configuration $C^*$ and a large number of sequences $A$. We thus indicate this parameter as $\alpha(A, C^*)$, since we do not know a priori that $C^*$ has lowest energy. Notice however that, if $\alpha(A, C^*)$ is positive, all alternative structures have higher energy than $C^*$. We impose that $\alpha(A, C^*)$ is larger than a positive threshold $\alpha_{thr}$ for sequences $A$ belonging to the neutral network.

$Z$-score

The $Z$-score $Z(A, C^*)$ (Bowie et al., 1991; Goldstein et al., 1992) is a measure of the compatibility between a sequence $A$ and a structure $C^*$, widely used in structure prediction. It depends on an effective energy function, and measures the difference between the energy of sequence $A$ in configuration $C^*$ and its average energy in a set of alternative configurations, $\{C\}$, in units of the standard deviation of the energy:

$$Z(A, C^*) = \frac{E(A, C^*) - \langle E(A, C) \rangle}{\sqrt{\langle E(A, C)^2 \rangle - \langle E(A, C) \rangle^2}}.$$  

When sequence $A$ folds in structure $C^*$ their corresponding $Z$ score is very negative.

Given the above definition, one has still to specify how to select alternative structures. A possibility, often used for lattice models (Mirny & Shakhnovich, 1996) is to assume that alternative structures are maximally compact, randomly chosen structures, whose average energy can be estimated as $\langle E(A, C) \rangle_C = N_{c_{\text{max}}} \langle e(A) \rangle$. Here, $N_{c_{\text{max}}}$ is the maximal number of contacts of candidate structures and $\langle e(A) \rangle$ is the average energy of a contact, averaged over all possible contacts formed by sequence $A$. This leads to introduce the parameter

$$Z' = \frac{E(A, C^*)/N_{c_{\text{max}}} - \langle e(A) \rangle}{\sqrt{\langle e(A)^2 \rangle - \langle e(A) \rangle^2}}.$$  

The use of $Z'$ has two main advantages: First, it makes the value of the Z-score much less sensitive to chain length $N$ and to the particular set of alternative structures used; second, the evaluation of $Z'$ is much faster than that of the $Z$-score. This is necessary in order to explore efficiently sequence space.

Sampling the neutral network

Our algorithm explores the neutral network of a given protein starting from its PDB sequence $A_0$ and iterating the following procedure: At time step $t$, (i) The number of viable neighbors of sequence $A_t$ is computed; (ii) The sequence $A_{t+1}$ is extracted at random among all the viable neighbors of $A_t$. In this way we generate a stochastic process along the neutral network which simulate neutral evolution and loses memory of the initial sequence very fast.

Sequence $A$ is regarded as viable if both parameters $\alpha(A, C^*)$ and $-Z'(A, C^*)$ are above predetermined thresholds, chosen as 98.5 percent of the values of those parameters for the sequence in the PDB. This enforces conservation of the thermodynamic stability and folding capability of the native structure $C^*$. We verified that the observed behavior does not change qualitatively for thresholds between 95% and about 100% of the PDB values.
We impose strict conservation of the cysteine residues in the PDB sequence, and do not allow any residue to mutate to cysteine, since a mutation changing the number of cysteine residues would leave the protein with a very reactive impaired cysteine that would probably affect its functionality. Accordingly, the total number of neighbors tested is \( X_{\text{tot}} = 18(N - N_{\text{cys}}) \), where \( N \) is the number of residues and \( N_{\text{cys}} \) is the number of cysteine residues in the starting sequence.

The total number of viable point mutations, \( X(\mathbf{A}) \), expresses the local connectivity of the neutral network. We normalize it by the total number of neighbors, \( X_{\text{tot}} \), getting the fraction of neutral neighbors, \( x(\mathbf{A}) = X(\mathbf{A})/X_{\text{tot}} \in (0, 1] \).

To compute \( x(\mathbf{A}) \), we have to evaluate the \( \alpha \) parameter for all sequences \( \mathbf{A}' \) obtained through a point mutation of sequence \( \mathbf{A} \). From Eq. [1] we note that the \( \alpha \) parameter can be obtained from the configuration with the highest destabilizing power, i.e. the highest value of the energy gap divided by the structural distance from the native configuration. These change from sequence to sequence, but it is expected not to change very much for neighboring sequences. Thus, in order to speed up the computation of \( \alpha(\mathbf{A}') \), instead of considering all candidate configurations we consider only the 50 configurations with the highest destabilizing power (i.e. the energy gap divided by the structural distance from the native configuration) for sequence \( \mathbf{A} \) and compute their mutated destabilizing power using sequence \( \mathbf{A}' \). The \( \alpha \) parameter is then obtained from the configuration with the highest destabilizing power. This procedure could slightly overestimate \( \alpha(\mathbf{A}') \) since not all configurations are used, but we have checked that the error introduced in the \( x \) value is in all cases below 0.1%.

**Substitution process**

Given an evolutionary trajectory \( \{x_1, x_2, \ldots\} \), the distribution of the number of substitutions taking place in a time \( T \) can be computed by considering Eq. [1], where \( k \), the number of attempted mutations, is a Poissonian variable of average value \( \mu T \).

In order to handle the computation, we divide all values of \( x_i \) in \( M \) classes, choosing \( X_a \) as representative value of all \( x_i \)'s belonging to class \( a \). The number of operations needed to evaluate the substitution probability increases exponentially with the number of classes \( M \). At the same time, the evaluation becomes more and more accurate as \( M \) increases. We chose \( M = 6 \) in our numerical computations as a reasonable compromise between accuracy and rapidity, checking that larger values of \( M \) introduce only small changes.

**Rigidity**

A measure of the conservation profile for a set of evolutionarily related sequences can be obtained measuring the rigidity of each position \( i \),

\[
R(i) = \sum_{a} f_i(a)^2,
\]

where \( f_i(a) = 1/20 \) is the frequency with which amino acid \( a \) is observed at position \( i \), normalized so that \( \sum_a f_i(a) = 1 \). Deletion of position \( i \) in a sequence is regarded formally as a 21st amino acid. A large rigidity \( R(i) \) means that position \( i \) is highly conserved. For unconstrained positions and in absence of deletions, \( f_i(a) = 1/M \), where \( M \) is the number of amino acids, and \( R(i) = 1/M \). In general, rigidities are larger than \( 1/M \) because of the finite size of the sequence set and because sequences in the set are correlated due to common evolutionary origin. Since cysteine residues are strictly conserved, we always get \( R(i) = 1 \) for them. Thus we omit these residues from the analysis of conservation.

**PFAM and FSSP databases**

We compare the rigidity measured in the set of neutral sequences generated with the present method with the rigidity obtained from two databases: the PFAM database (Bateman et al., 2000) and the FSSP database (Holm & Sander, 1996). The PFAM database is a collection of families of homologous sequences obtained by multiple alignment. Since multiple alignment methods work only for sufficiently high similarity, there are no sequences of low similarity in this database. The FSSP database is a collection of protein classes sharing the same fold (as determined by the program of structural alignment DALI (Holm & Sander, 1996)). Since the structures must be experimentally known, the FSSP database is usually smaller than the PFAM database. However it includes in the same class distant homologs whose evolutionary relationship can not be detected by means of sequence comparison alone. Due to database biases, many sequences in the PFAM database are strictly conserving, we always get \( R(i) = 1 \) for them. Thus we omit these residues from the analysis of conservation.
and FSSP databases are highly similar. To reduce this effect, proteins with similarity higher than a threshold $q_{thr} = 0.85$ to any other protein have been eliminated.

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Table I. Summary of the seven neutral networks studied. For rubredoxin, m. and th. stand for the mesophylic and thermophylic form respectively. $x$ indicates the fraction of neutral neighbors and $\tau$ is the correlation length of $x$ along an evolutionary trajectory obtained from the stretched exponential decay of the correlation function.

**Abbreviations:** PDB Protein Data Bank, SNC Structurally Constrained Neutral Model, FSSP Fold classification based on Structure-Structure alignment of Proteins.