Exploring proteins multi-funnel energy landscape.

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Abstract. - An all-atom model of proteins is used to show that the same sequence of amino acids can have many alternative structures, that are very distant from, and that can be as stable as, the corresponding native structure. Such alternative structures are not easily rationalized as belonging to the native basin and indicate instead that the free energy landscape of proteins is multi-funnel-shaped and that Anfinsen's thermodynamic hypothesis alone cannot explain protein folding. An alternative two-step process for folding is proposed and its consistency with the experimental evidence available is discussed.

Introduction. – An outstanding question in Biology and Medicine, known as the protein folding problem, is how a given sequence of amino acids, in cells, most of the times assumes the native structure [1, 2]. An important concept is that of the free energy landscape and the current working hypothesis is that this landscape is funnel-shaped [3–6] and that the native structure corresponds to its global minimum [2–6]. However, a question arises about the consistency between the funnel hypothesis and the interactions that stabilize protein structure. These interactions are reasonably well represented by potentials such as these [7]:

\[
V = \sum_{\text{bonds}} K_{r}(r - r_{eq})^2 + \sum_{\text{angles}} K_{\theta}(\theta - \theta_{eq})^2 + (1) \\
+ \sum_{\text{dihedrals}} \frac{V_n}{2}[1 + \cos(n\phi - \gamma)] + \\
+ \sum_{i<j} \left[ \frac{A_{ij}}{R_{ij}^2} - \frac{B_{ij}}{R_{ij}^6} + \frac{q_i q_j}{\epsilon R_{ij}} \right]
\]

where bond stretching and bond bending (the first two sums) are harmonic, rotations around a bond are described by a truncated Fourier series (third sum) and nonbonded interactions are modelled by the Lennard-Jones potential and Coulomb interactions due to the partial charges on each atom (the last sum). A few systematic studies of the shapes of the energy landscape of small polypeptides and water clusters using these kind of potentials have been attempted, which show both funnelled and multi-funnelled landscapes [8–11], with the local topography of the energy landscape being related to the conformation of the molecule [9]. Furthermore, a 4 µs study of the free energy landscape of a 16 amino acid beta-hairpin led to three well defined non-native basins with free energies comparable to that of the native basin [12]. On the other hand, the conformational space of proteins, albeit small, remains too large to be probed in a systematic manner, even with the most powerful computers. Instead, here a cursory study of the energy landscape of four proteins is made, taking their native basin as the reference.

Using the nomenclature of the Protein Data Bank (PDB) [13], the four proteins are: 1QLX (104 amino acids) [14], 1I0S (161 amino acids) [15], 1AAP (56 amino acids) [16] and 1IGD (61 amino acids) [17]. These proteins have different sizes, different biological origins and different functions. While the first is a fragment of the human prion [14], the second is an oxireductase from archae [15], the third is the protease inhibitor domain of Alzheimer’s amyloid β-protein [16] and the fourth is a immunoglobulin binding domain of streptococcal protein G [17]. The main criterion for their selection was to have one representative of each of the four main classes of proteins identified in the CATH hierarchical structural classification scheme [18]: mainly α (1QLX), mainly β (1I0S), essentially structureless (1AAP) and α/β (1IGD). To probe the energy landscape of these four proteins, for each one, three alternative structures were built by forcing it to assume the fold, or part of the
fold, of each of the other three, as explained in detail below, and the stability of the resulting structures was compared with that of the corresponding native structure.

The coordinates for the atoms in the native structures of the four proteins selected were taken from the PDB [13] and their structures were energy minimized with the AMBER force field [7], to relieve any steric or otherwise strongly unfavorable interactions. Alternative structures for each protein were then built using the energy minimized native structures as templates, by forcing the sequence of each protein to have the backbone fold, or part of the backbone fold, of each of the other three proteins. For example, the initial coordinates for the structure in the first row, second column of figure 1 were obtained by imposing the backbone fold of the first 104 amino acids of 1I0S onto the backbone of the 104 amino acids of 1QLX and the initial coordinates for the second row, first column were obtained by imposing the backbone fold of the 104 amino acids of 1QLX on to the backbone of the first 104 amino acids of 1I0S. These and the other alternative structures thus generated were first relaxed, in order to eliminate all the steric interactions such a procedure leads to, and, after relaxation, they were energy minimized [19]. All 16 energy minimized structures were solvated in water using the box option of the leap program of AMBER [7] and the resulting systems were energy minimized, keeping the protein fixed. Then the NAMD program [20], with the AMBER force field, was used to heat each of the systems to 298 K and to equilibrate them at that temperature for 0.6 ns. A representative statistical ensemble, at 298 K, for each of the 16 systems thus constructed, was obtained by storing snapshots every picosecond from a further 0.2 ns molecular dynamics (MD) run. It should be noted that the smallest system in these simulations has 12234 atoms and the largest has 33451 atoms.

Results. – Figure 1, which was made with the program VMD [21], shows the native folds of the four proteins and also the twelve alternative folds, at the end of the MD sampling run. The data in tables 1 and 2 was calculated from the same statistical sample. The native structures for the four proteins are found along the diagonal of figure 1 and each row includes the native fold plus its three alternative structures, all in the same colour. All proteins in the same column were generated to have at least part of the fold of the native structure in that column. Inspection of figure 1 shows that, even after heating and equilibration at 298 K, the alternative structures retain most of the backbone folds that were imposed on them initially, even if these lead to very unnatural protein structures, particularly for the amino acids sequences concerned.

The average energies of all sixteen structures are displayed in table 1, in which the data is organized in the same manner as in figure 1. All systems in the same row of table 1 are exactly the same, i.e., not only do they have the same protein and ions but also the same number of water molecules, namely, the N water molecules closest to the protein. The number N was chosen as that for which the interaction energy between the protein and water reached an average saturation value. For the 1QLX and 1I0S proteins N is 4000 and for the smaller proteins 1AAP and 1IGD N is 2000. In each cell of table 1, the first number is the total energy of the system constituted by the protein plus ions plus N water molecules and is dominated by the water-water interactions. The second number in each cell is the total energy of the protein, including the intra-protein interactions (third number), the protein-ions interactions (fourth number) and the protein-water interactions (fifth number). Inspection of table 1 shows that some of the alternative structures have equivalent, or even lower, potential energies than the native structure. For example, imposing part of β-fold of 1I0S on to the first 56 amino acids of 1I0S leads to a structure with an average potential energy lower than the native structure of 1QLX and imposing the essentially disordered structure of the native fold of 1AAP on to the first 56 amino acids of 1I0S leads to a structure with an average energy approximately equal to that of the corresponding native structure.
mainly

-38773
-1904
-1562
-591
-3413
-924
-815
23
-97
-18281
-572
-18140
-6709
-3452
138
18353
7088
3299
597
249
3252
6242
6333
3584
2285
1854
7438
18493
3060
443
671
38089
466
38404
18663

Str 1AAP

α/β

-598
-18140
-6709
-3452
138
18353
7088
3299
597
249
3252
6242
6333
3584
2285
1854
7438
18493
3060
443
671
38089
466
38404
18663

Str 1IGD

-4492
-323
-38650
-2619
-5270
7437
Str 1I0S

-38796
-6428
-2485
-5278
2385
1534
38173
535
disordered

of the protein, including

c
structures have a very similar behaviour to their corre-
equilibration at 298 K. However, some of the alternative
eral trend is that native structures take longer to deviate
each plot, the solid line is for the native structure. A gen-
same order as in the previous figure and tables, and, in
structure. The plots are organized per sequence, in the
RMSD per atom of each structure during this extra heat-
et of 2 K per ps. Figure 2 shows the variation of the
structural stability of the structures, all were heated from the
structures mentioned above.

that all structures show the same average structural sta-
bility until 50 ps (when the temperature has increased to
398 K) and that the greatest divergence takes place at
100 ps for the smaller proteins 1AAP and 1IGD (when
the temperature has increased to 498 K) and at 150 ps,
when the temperature is 598 K, for the larger proteins
1QLX and 1I0S. Thus, this qualitative measure of activa-
tional energy for conformational changes shows that, given
a certain amino acid sequence, it is possible to find struc-
tures that are very different from the native and yet have
a similar structural stability.

Discussion. – The native plus the three alternative
structures studied here provide a mere glimpse into the
conformational space of each of the four proteins selected.
They were built by taking proteins whose sequences,
in cells, lead them a particular structural class and by
making them assume structures of other, very different,
classes of proteins. This protocol was applied to proteins
of different sizes and without any regard as to how best
to fit the alternative folds to each particular protein,
i.e., one could argue that the alternative structures
studied here constitute examples of the most unnatural
alternative conformations that we can obtain for the four
proteins selected. Nevertheless, we find that some of them
have energies that are at least comparable, and in some
cases, even more favorable, than the native structures.
Furthermore, the alternative structures generated are
sufficiently separated from the native configuration to
make transitions to it improbable in normal conditions

Table 1: Average Energies (kcal/mol)

| Seq | mainly α Str 1QLX | mainly β Str 1I0S | disordered α Str 1AAP | α/β Str 1IGD |
|-----|-----------------|-----------------|----------------------|-------------|
| I   | -38773±150      | -38067±168      | -38650±140           | -38224±148  |
| Q   | -6424±65        | -6709±73        | -6333±78             | -6242±88    |
| L   | -2619±50        | -1854±47        | -2573±56             | -2531±58    |
| X   | -97±32          | -363±57         | -177±25              | -138±41     |
|     | -3712±74        | -4492±76        | -3584±80             | -3573±118   |
| I   | -38404±101      | -38796±152      | -38173±164           | -38089±147  |
| I   | -7332±80        | -7438±85        | -7437±87             | -7088±90    |
| O   | -1534±67        | -1948±72        | -1562±58             | -1533±80    |
| S   | -443±36         | -220±42         | -597±40              | 3±35        |
|     | -5355±83        | -5270±113       | -5278±101            | -5558±110   |
| I   | -18493±128      | -18663±113      | -18140±135           | -18305±137  |
| A   | -333±71         | -3452±51        | -3078±54             | -3413±52    |
| A   | -671±53         | -535±40         | -924±29              | -622±40     |
| P   | -523±28         | -775±42         | -249±48              | -815±35     |
|     | -2140±93        | -2141±51        | -1904±57             | -1975±51    |
| I   | -18353±128      | -18478±103      | -18281±122           | -18315±109  |
| G   | -3252±58        | -3299±53        | -2943±55             | -3060±58    |
| D   | -466±35         | -323±37         | -572±46              | -598±38     |
|     | -202±45         | -591±24         | -86±24               | 29±36       |
|     | -2584±65        | -2385±56        | -2285±84             | -2485±66    |

- Total energy of the system including protein, ions and the N closest water molecules (see text).
- Total energy of the protein, including all the atom-atom interactions in the protein plus the ion-protein interactions and the protein-water interactions.

In order to have an insight into the entropy associated with each structure, the root mean square deviations (RMSD) per atom of each of the structures with respect to its thermal equilibrium average structure are presented in table 2 (only non-hydrogen backbone atoms are used). The data indicate that the native structures fluctuate less than the alternative structures in all cases, something that reinforces the thermodynamic viability of the alternative structures mentioned above.

As a further qualitative measure of the relative structural stability of the structures, all were heated from the equilibrium value of 298 K to a final value of 698 K, at the rate of 2 K per ps. Figure 2 shows the variation of the RMSD per atom of each structure during this extra heating procedure, with respect to the corresponding initial structure. The plots are organized per sequence, in the same order as in the previous figure and tables, and, in each plot, the solid line is for the native structure. A general trend is that native structures take longer to deviate from the initial structure, something that is in agreement with the fact that they fluctuate less while at thermal equilibrium at 298 K. However, some of the alternative structures have a very similar behaviour to their corresponding native structure. Also, other general trends are
One limitation of the simulations reported here is their time length, which is short when compared with experimental times for conformational changes. While no transitions from the non-native folds to the native folds were observed, it cannot be ruled out that such transitions might occur at longer times. It should be noted, however, that the fluctuations observed for the 16 structures are between 0.85 and 1.26 Å, approximately the same as in the structures determined in NMR measurements [22]. This is due to a coupling of the thermal bath that is much stronger in the simulations and one consequence is that the computational time for conformational changes to take place is shorter. Also, an indication of the relative size of the energy barriers for conformational changes can be obtained by heating the systems and, in this respect, figure 2 shows that, although the barriers for the native structures are higher, they do not seem to be significantly different from those of the non-native structures. Thus, while the results presented here cannot be said to prove it they do suggest that the free energy landscape of proteins has the shape of a multi-funnel, in which each funnel is associated with an average structure that can be very different from the native, and yet be as thermodynamically viable as the native structure.

Although the current theoretical framework for protein folding is based on a funnel-shaped free energy landscape [3–6], experimental evidence for a multi-funnel free energy landscape in the case of proteins was first obtained in 1968 by Levinthal who found two forms of an alkaline phosphatase at 317 K, one active and the other inactive, synthesized at different temperatures, in mutants of E. Coli [1]. More recently, other cases have been found of proteins that can assume more than one structure in the same thermodynamical conditions [23–27]. While a funnel free energy landscape has difficulty in explaining why protein misfolding happens, a multi-funnel free energy landscape can readily rationalize it as a case in which a non-native funnel was selected. On the other hand, a multi-funnel free energy landscape cannot explain protein folding just by a principle of free energy minimization. Indeed, in a multi-funnel, the difficulty in determining the native structure from a given amino acid sequence is not just due to the size of the conformational space and the lack of computer power, but, more essentially, to the fact that the native structure is not a well-defined global free energy minimum.

Table 2: Average fluctuations (Å)

| Seq     | mainly α | mainly β | disordered | α/β |
|---------|----------|----------|------------|-----|
| 1I0S    | 1.26 ± 0.17 | 0.89 ± 0.09 | 1.26 ± 0.22 | 1.16 ± 0.16 |
| 1IAP    | 1.20 ± 0.13 | 0.84 ± 0.07 | 1.26 ± 0.15 | 0.92 ± 0.12 |
| 1IGD    | 1.24 ± 0.20 | 1.16 ± 0.19 | 1.08 ± 0.16 | 1.26 ± 0.15 |

Fig. 2: Variation of the RMS deviation of each snapshot with respect to the corresponding initial structure. The temperature increased throughout the simulation at the rate of 2 K per ps. Each plot is for a different protein and the order from top to bottom is as in Figure 1. In each plot, the solid lines are for the native structures and the remaining three lines are for the proteins indicated by the keys. The values are in Å.
second step in which the structure relaxes as its energy is minimized within that funnel [28]. The first step is a kinetic mechanism for which there is direct experimental evidence in a few cases [25–27] and that was already proposed by Levinthal who suggested that there are specific pathways for folding [1]. Considering that such pathways can be characterized by intermediates one can say that the experimental evidence for a kinetic mechanism is indeed substantial [29, 30] and may even include proteins that apparently follow a two-state process [30, 31]. The second step is an energy minimization mechanism, as first proposed by Anfinsen [2] and incorporated in the funnel models [3–6]. Within this two-step picture of folding, proteins denature reversibly as long as heating does not make them diffuse away from the native funnel and denature irreversibly otherwise. Chemical unfolding, on the other hand, cannot be described within a single free energy landscape picture because as the denaturant concentration varies, so does the system and consequently the associated free energy landscape.

It is curious to note that experimental evidence also points to the existence of two steps in protein folding, one in which a compact structure forms, in the dead time of the experiments [31] and another, which takes much longer, from microseconds to milliseconds or more, at the end of which proteins become active. The suggestion here is that the first step is related to the selection of the funnel, while the second step is due to the relaxation down the funnel selected. In this two-step picture, the second step is the rate-limiting step, i.e. the rates of folding are dependent on this (slow) relaxation down the funnel selected, but the definition of the structure is accomplished in the first step, when a particular funnel is selected. Thus, within a multi-funnel free energy landscape, to understand how a given amino acid sequence leads to a specific three dimensional structure one must understand the kinetic mechanism by which a specific funnel is selected.

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