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Stochastic strategy to analyze protein folding

M.A. Moret[a,b,*], P.M. Bisch[c], E. Nogueira Jr.[d], P.G. Pascutti[c]

[a]*CEPPEV - Fundação Visconde de Cairu, 40226-900, Salvador, Bahia, Brazil*
[b]*Departamento de Física, Universidade Estadual de Feira de Santana, 44031-460 Feira de Santana, Bahia, Brazil*
[c]*Instituto de Biofísica, Universidade Federal do Rio de Janeiro, 21949-900 Rio de Janeiro, RJ, Brazil*
[d]*Instituto de de Física, Universidade Federal da Bahia, 40210-340 Salvador, Bahia, Brazil*

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Abstract

We propose a stochastic approach to combine methods from computational physics and Tsallis statistics in order to analyze the potential energy hypersurface of the proteins. This approach enables us to study protein folding by the topology of the energy hypersurface close to the native structures. As a bonus, this stochastic procedure allows us to obtain some of the possible intermediate states of protein folding. In particular, we describe results which suggest that the enthalpy drives the process of protein folding close to the native state. Finally, we propose an alternative view of the process of protein folding.
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1. Introduction

Several empirical rules of protein folding can be deduced from information obtained from studies of protein in vitro [1]. Folding of small proteins in vitro is often complete within a few minutes [2]. Spectroscopic methods allow one to detect

*Corresponding author.
E-mail address: moret@cairu.br (M.A. Moret).

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events occurring within the first few milliseconds of folding. In such a process the formation of intermediate states occurs in many cases [2–6]. In order to understand the process of protein folding, the knowledge about the stable partially folded states [2,7,8] and the folding theories [9–14] is crucial. To study protein folding through molecular simulations, it is necessary to take into account that a molecular system has a great number of minima in the energy hypersurface (molecular conformations) [1,14,15] that increases with the number of the degrees of freedom in the molecular system. To solve these kinds of problems, a stochastic molecular optimization [16] was recently proposed, which encompasses both the evaluation of the classical force field and a generalization of the Simulated Annealing [17], that is based on Tsallis statistics [19]. The Generalized Simulated Annealing (GSA) [18] has been applied to a variety of problems such as genetic algorithm [20], molecule optimization using classical methods [16], or semi-empirical methods [21], geophysical problem [22], material science [23–26], Traveling Salesman Problem [27] and numerical data fitting [28]. GSA shows to be the most effective simulated annealing method [16,27].

In this paper, our interest is exploring the energy hypersurface of the polyalanines, the 85-102 Barnase fragment (a β-hairpin) and small proteins. We mainly show the behavior of the Insect Defensin A, that is an interesting molecular system because this is a small protein where three disulfide bridges stabilized the secondary structures (α-helix and β-sheet) [29]. We recall that the results obtained are equivalent quantitatively for the studied systems.

Our strategy amounts to couple a GSA routine [16] with the THOR package. This computational code is a tool to investigate structures of biological interest [30,31] and it is based on the GROMOS classical force field [32]. The GSA approach enables one to scan the conformational space, mainly close to local minima. The regions where the energy value is greater than the local minima may be discarded in the stochastic procedure, so that the not-allowed regions of the Ramachandran Map [33] may be rejected. Furthermore, the conformational energy of the molecule is made up of a sum of bonded and nonbonded terms. In this approach, only hydrogen atoms covalently bonded to oxygen or to nitrogen are considered explicitly, whereas CH_1, CH_2, and CH_3 groups are assumed to be an atomic unit. The energy hypersurface is rather complex, and approximations in the force field are required to make such a study tractable within our current numerical capabilities. Then, we use a simplified version of the conformational energy, which maintains fixed bond lengths and bond angles within their ideal values. We focus our search on the dihedral angle space [16]. Therefore, we analyze the changes of the following energy function:

$$E = \frac{1}{2}\sum_{m} K_{S_m}(r_m + r_0)^2 + \sum_{n} K_{\varphi_n}(1 + \cos(m\varphi_n + \varphi_0)) + \sum_{i<j}\left[\frac{C_{12}(i,j)}{r_{i,j}^{12}} - \frac{C_6(i,j)}{r_{i,j}^{6}}\right] + \frac{1}{4\pi\varepsilon_0\varepsilon_r}\sum_{i<j}\frac{q_i q_j}{r_{i,j}}, \quad (1)$$

where the first term represents the sulfur bond potential. This term is considered because this kind of bond stabilizes the potential energy of the Insect Defensin A close to the native structure. The backbone bond and the angular bond potentials

have their ideal values calculated [34], because these potentials were maintained close to these values. Although these approximations smooth the hypersurface [35], these potentials do not contribute substantially to the amino acid sequence obtaining the tertiary structure (native structure).

The search of the energy minima, or the scanning of the energy hypersurface consists in comparing the geometry obtained by GSA routine with the energy measured by THOR, as showed in Refs. [16,36,37]. Recently, GSA has been applied to study helix folding [36], and in Ref. [37] was applied to study the multifractal behavior on various molecular systems. In the next we discuss the results of the polypeptides that have secondary structures.

2. Secondary structures

Experimental and theoretical evidences suggest that protein folding initiates by small structures that quickly adopt conformations close to the native one. The knowledge of those initial stages is of highest importance for the understanding of the process of protein folding. In this sense, we applied the stochastic methodology in order to study polypeptides that are good formers of the secondary structure (polyalanines) and we applied it to study a β-hairpin (85-102 Barnase fragment).

2.1. Polyalanines

Helices are the most prevalent secondary structural motif observed in proteins with known structure. Several recent theoretical studies of the helix–coil transitions have shown the relation between the unfolded state, in random coil conformations, and the helical conformational states, by using molecular dynamics, Monte Carlo, or other theoretical approaches [36]. Both standard molecular dynamics and Monte Carlo simulation methods require extended computational time to obtain helical secondary structures. We applied the GSA procedure to study the critical number of amino acids necessary to stabilize the α-helix structure, and an upper limit may also be imposed by the entropy effect. Isolated α-helix structures, in fact, would have to be longer than 13 residues to be stabilized by the attractive interactions [38,39].

Different amino acids have been found to present weak though definite preference in favor of or against being in α-helix structure, and the intrinsic helical propensity of some amino acids has been demonstrated to be position dependent [40]. In this sense, alanine (ALA) is considered to be a good α-helix promoter. Such preferences were the main considerations in all early attempts to predict secondary structures from amino acid sequences, but they were not strong enough to obtain accurate predictions.

The electrostatic interactions between adjacent dipoles in the peptide backbone are repulsive; so a critical number of residues is needed to be counterbalanced by the attractive interactions, such as H-bonds. Then, short peptides of less than seven residues are flexible, and they show no conformational preference. When the number of residues of the chain increases (8–13 residues), the structures tend to collapse into

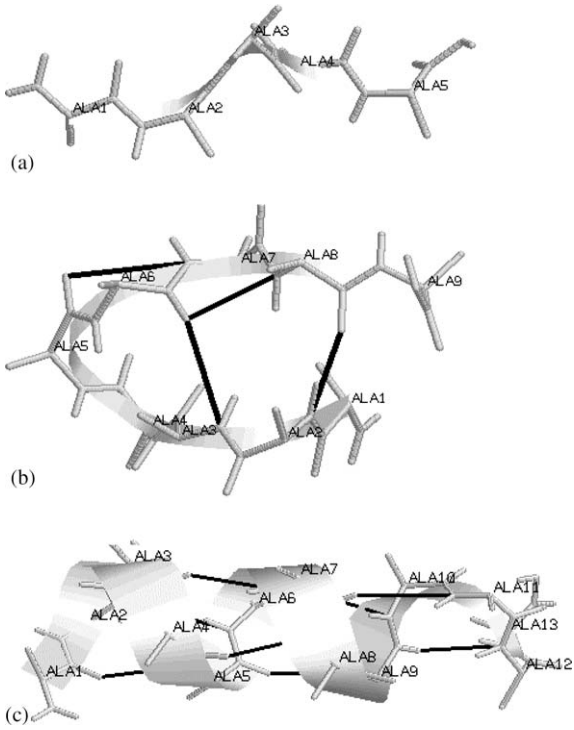

Fig. 1. The global-minima structures of the polyalanines with 5(a), 9(b) and 13(c) residues.

H-bonded turns. Above a critical number of 13 amino acid residues the enhancement of the hydrogen bond number stabilizes the polyalanine in an α-helix structure, Fig. 1. In fact, for long-chain peptides, most of the possible H-bonds of the backbone tend to be formed. Then, an energy gap arises from the lowest-energy conformation to the next low-energy conformation. This energy gap enlarges with the number of residues [36].

2.2. 85-102 Barnase fragment

The second system studied, Barnase, is a member of the microbial ribonucrease family. We study the same protein fragment analyzed in Ref. [41] that is formed by the residues SER85 to HIS102 of Barnase. This fragment folds in a β-hairpin, and was studied by annealing of the molecular dynamics [41]. The annealing technique of the molecular dynamics consists of heating the system and later cooling it until room temperature. The idea of heating a molecular system until great temperatures ($T > 500$ K), unreal from the biological point of view, is because the kinetic energy of the atoms stays above the rotational barriers of the molecule in study. On the other hand, the process of cooling to the room temperature obtaining the dynamic properties of the analyzed system. From this methodology three different unfolded

conformations were obtained. We emphasize that there are both experimental and theoretical indications that this small peptide (or the homologes in other RNases) is the folding initiator in the RNases family [41].

These three different structures, obtained during the heating of the system, were used in the simulation of the re-folding by the cooling of the system. Of those three simulations of the molecular dynamics two were capable of recovering part of the information of β-hairpin, re-folding for conformations close to the native one. However, the third structure lost the information of β-hairpin completely, being far away from the native structure. We use these three conformations as initial ones in the performed simulations.

During the conformational search on the dihedral angles of this protein fragment it is observed that a tendency exists of a β-turn being formed around the SER91-TRP94 region, as observed experimentally, independent of the conformations of the rest of the fragment. This fact corroborates that this small site is a strong candidate to be a conserved structure from the initial stages of protein folding. However, there is no proof in the literature if the folding of β-hairpins is begun by local interactions (β-turn) or if it is given by the interaction among the β-strands. Both conceptions were proven recently experimentally [41].

In these simulations, for each initial conformation, the procedure was executed 32 times, using 32 different GSA parameters ($q_A = 1.1$, q_v varying from 2.0 to 2.7 and $T_0 = 1$, 5, 10 and 100), showing a non-dependence on initial conformation to reach the anti-parallel β-strands, which characterizes the β-hairpin conformation, at the global minimum. It is noticed that several amino acids have the tendency to fold to a conformation, on average, close to the native one. We observed that most of the

Table 1
The mean values of the dihedral angles obtained from the stochastic simulations

Residue	Φ	Ψ	χ_1	χ_2	χ_3	χ_4
SER85	−113	124	178			
ASP86	−113	120	−86	140		
ARG87	−106	121	146	−156	178	−135
ILE88	−98	−175	145	−107		
LEU89	−112	−172	−150	92		
TYR90	−119	153	−168	−140		
SER91	−99	163	163	105		
SER92	−146	141	−132	120		
ASP93	−78	−105	−105	−133		
TRP94	−135	−170	−131	162		
LEU95	−89	−152	−139	106		
ILE96	−117	129	180	179		
TYR97	−92	113	−88	151		
LYS98	−105	161	−123	179	147	−165
THR99	−114	92	147	−117		
THR100	−97	−92	−174	140		
ASP101	−101	119	119	121		
HIS102	−148	−179	138	179		

amino acids tend to the β-region independent of a correct representation of the solvent-molecule hydrophobic force, as shown in Table 1. The values shown in Table 1 characterize the mean values obtained from all the simulations performed. Most of the Φ and Ψ values correspond to the β-hairpin conformation.

In the next section we show the results of the secondary structure and tertiary structure analysis of a small protein.

3. Insect Defensin A

To study characteristics of the possible folding pathways in silico as well as to obtain information about conformations around the native structure, we have used as initial conformation an optimized structure close to the native one. In order to analyze the possible conformations we have investigated two different situations: in the first one we study the native structure, i.e., under the influence of the three disulfide bridges, and in the second one we apply the same methodology to study the case where all disulfide bridges are broken. Ref. [37] shows the $f(\alpha)$ spectrum [42,43] of the Insect Defensin A that presents a characteristic multifractal behavior of this small protein in the two analyzed situations. In this sense, it was observed that this multifractal behavior for other different molecular systems is obtained from the $f(\alpha)$ spectra [37]. The analysis shows that the dimension of the phase space of the problem influences the accessibility to different parts of the energy hypersurface. Besides, in relation to the $f(\alpha)$ spectra as a function of the polypeptide size, it was shown that the proteins adopt conformations in the energy hypersurface only in allowed regions from the $f(\alpha)$ spectrum. Then, the energy hypersurface presents allowed (and not-allowed) regions that depend on the protein size. This result provides an alternative explanation about the Levinthal paradox [44], since a great number of possible configurations must be in the not-allowed regions, making the number of allowed configurations decrease and restricting the protein to navigate over the folding routes only.

Table 2 shows the values of the C_α root-mean-square deviation (C_αRMSD) in eight local energy minima around the global one, and the correspondent conformational energy of the Insect Defensin A restrained by its three disulfide bridges. From Table 2 values we observed that a small perturbation in the backbone of the native structure produces very high changes at the molecular energy, when this molecule is under the influence of the disulfide bridges. These results are justified because the native structure is very stable. Moreover, we observe that the energy hypersurface of the Insect Defensin A exhibits an energy gap of circa 9 kcal/mol between the conformations in global minimum and in the first local one. The energy differences between local minima are small at unfolded conformational states ($\mathrm{RMSD} > 3.0\,\mathring{\mathrm{A}}$) and large around the folded conformation in global minimum ($\mathrm{RMSD} < 1.0\,\mathring{\mathrm{A}}$), making the energy hypersurface like a funnel, in partial accordance with the funnel model for protein energy surfaces [14,45]. We observed this behavior in the polyalanine helix folding too [36], where the difference of conformational energy tends to increase as a function of polypeptide size in relation to the global minimum.

Table 2
Values of the conformational energy and the C_α root-mean-square deviation (C_αRMSD) of the Insect Defensin A. A small perturbation in the native structure produce very high changes at the molecular energy

Conformational energy (kcal/mol)	C_αRMSD (Å)
25.67	0.65
75.75	0.80
87.83	0.91
175.85	1.11
176.29	0.77
182.07	1.20
183.73	0.87
188.30	0.91

Another interesting result is that close to the native structure, despite the large energy differences, this protein presents different stable conformations, i.e., local minima in the energy hypersurface. These structures present C_αRMSD values around 1.0 Å or lower, and hence the conformational entropy can be considered the same for these structures. Actually, for this protein an energy funnel that maintains its structure close to the native one is observed.

Inspired by the analysis of the Nuclear Magnetic Resonance (NMR) spectra, where C_αRMSD is an essential parameter to define a structure and in the conformational entropy behavior, we propose a simplification on the thermodynamical analysis of the folding process. Hence, evaluating the free energy (ΔG_i) is

$$\Delta G_i = \Delta H_i + T\Delta S_i \,, \tag{2}$$

where ΔH_i is the enthalpy, T is the temperature and ΔS_i is the conformational entropy term to the stable structure i [15]. Furthermore, near the global minimum the main chain conformational entropy for any state i is close to the one for state j ($\Delta S_i \approx \Delta S_j$), when structures have C_αRMSD values around or lower than 1.0 Å. Then, the results suggest

$$\Delta\Delta G = (\Delta H_i - \Delta H_j) \,, \tag{3}$$

i.e., the enthalpy drives the protein folding when the molecule is close to the native state.

To obtain information about the secondary structure stability of the Insect Defensin A we analyzed the energy hypersurface of the α-helix and β-sheet structures, without the influence of the disulfide bridges. Furthermore, we have searched the possible conformations to the amino acids that compound each of the two secondary structures, maintaining all others amino acids fixed on their native conformations. From the results of the α-helix (HIS13-ARG23) we observe that a stable unfolded conformation (a random coil) increases $\sim$10 kcal/mol the conformational potential energy in relation to the global minimum, an α-helix.

When we perform searching in the region of the β-sheet (ARG26-ARG39), we note that a stable unfolded conformation (a random coil) has the conformational potential energy close to the β-sheet. However, the global minimum is a random coil, but the β-sheet energy increases the conformational energy $\sim$0.8 kcal/mol, only. This value is close to the KT; therefore, transitions between both conformations are possible at room temperature. From the moment the disulfide bridges are formed, the random coil starts to have a very high energy, then there is not a possible minimum and it turning the β-sheet as the global minimum.

To analyze the stability of the tertiary structure we performed a conformational search over the complete energy hypersurface of the Insect Defensin A, without the influence of the disulfide bridges. We note that breaking the disulfide bridges the mobility (number of conformational states) of this protein increases, as shown from the standard deviation values of the Φ and Ψ angles in Fig. 2. Also, the simulations indicate that part of the α-helix and parts of the β-strands are very stable, because these structures are conserved in all simulations performed. As expected, when we evaluate the standard deviation of Φ and Ψ dihedral angles (Fig. 2), simulations show that breaking the disulfide bridges the flexibility of the structure increases. The results show that as ARG23, ASN25, ARG26, LYS33 and CYS36 increase their mobility, the number of allowed states increases when the disulfide bridges are broken. On the other hand, some parts of this protein are conserved maintaining their conformations close to the native structure. Therefore, these parts are stable and independent of the disulfide bridges formation. Typical standard deviation values from ALA17 to LEU22 (part of the α-helix), from GLY27 to GLY32 (part of the β-strand) and from VAL37 to ARG39 (part of the second β-strand) confirm this scenario. Thus, evaluating the conformational entropy for these unfolded states, we found that the dihedral Φ and Ψ RMSD values ($10-20^\circ$ for the salt bridge constrained conformations and $10-100^\circ$ for unconstrained ones) are greater than the one close to the native structure.

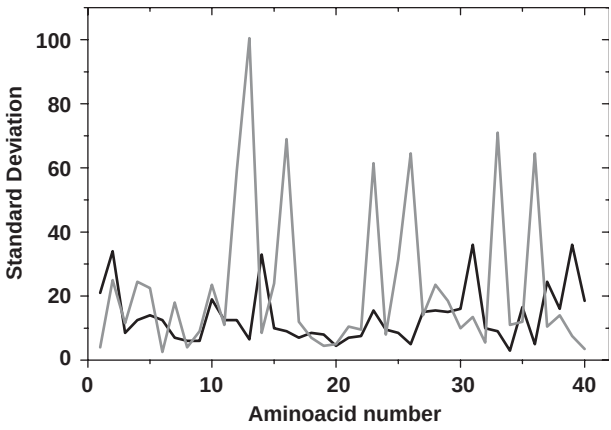

Fig. 2. Standard deviation values of the Φ and Ψ angles, maintaining the influence of the disulfide bridges (black) and without it (gray).

The results suggest us to propose the structures shown in Fig. 3 as intermediate structures of this protein. A possible folding route for Insect Defensin A—starting from the native structure, several intermediate conformational states of similar energy are found. An α-helix segment is suggested as early folding nucleation (a) followed by a β-turn (b) and the β-sheet (c, d) formation, prior to protein folding. Further arrangements are done to disulfide bonds formation. These results show that the conformations, proposed in silico, have the same secondary structures. We recall that when the ARG23 goes out to the α-region this amino acid allows a great mobility to some amino acids that compound the original β-sheet structure (ARG26-ARG39).

Therefore, we argued that in the early/intermediate stages of folding parts of the polypeptide chain generate microstructures, which can associate and coalesce to form substructures with a native conformation. On the other hand, the majority of the secondary structures are conserved, because of the big energy gap around these secondary structures. In the last stage of protein folding the native structure is close to the bottom of the energy funnel. It is in partial accordance with the folding funnel model [14] that describes the thermodynamics and kinetic behavior of the transformation of unfolded molecule to the native state. In this case, any polypeptide chain with biological function explores the folding routes toward the native structure through intermediates consisting of a population of partially folded species whose number decreases as the protein navigates down to the minimum of the energy landscape [14,45]. To compare our results with the funnel theory we consider that the

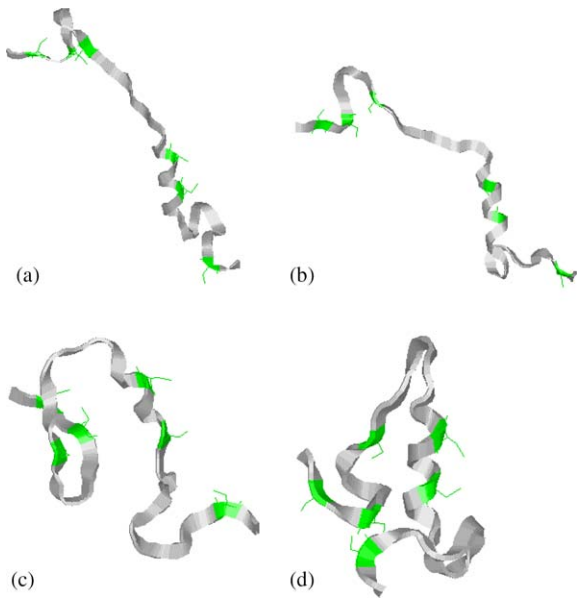

Fig. 3. (a–d). Several intermediate states of similar energy present in this possible folding route for Insect Defensin A.

number of local minima of the protein backbone decreases as the protein navigates down to the global minimum of the energy landscape independent of the conformational entropy, when its structure is very close to the native one. The access to a minor number of conformational states, corresponding to the local energy minima, occurs because the annealing procedure depends on the decreasing temperature [16]. Microstructure formation is observed due the local interactions, as van der Waals and H-bonds, which stabilize local conformations on few amino acid residues decreasing degrees of freedom of the protein backbone. At high temperatures a great number of local minima are accessible and no microstructures are stabilized.

Then, the results point out that it is not possible to only decrease the conformational entropy for driving the folding process and obtain the native structure. Furthermore, the enthalpic contribution is important in driving the folding routes, when the structure is close to the native one.

Also, in an unfolding–refolding transition experiment the free energy of small protein varies between 5 and 15 kcal/mol [1]. From the simulations it was noted that an unfolded structure increased the conformational energy (enthalpic contribution) by $\sim$9 kcal/mol. Therefore, the result was in qualitative accordance to the unfolding–refolding transition energies.

4. Conclusions

In this paper, we study secondary and tertiary structures of the polypeptides and proteins by using a stochastic approach, i.e., by the coupling between a classical force field and GSA.

For α-helices we note that above a critical number of 13 amino acid residues the enhancement of the hydrogen bond number stabilizes the polypeptide in an α-helix structure. In fact, for long-chain peptide, most of the possible H-bonds of the backbone tend to be formed. Then, an energy gap arises from the lowest-energy conformation to the next low-energy conformation. This energy gap enlarges with the number of residues [36].

In relation to β-sheets we observe that the β-turns fold independently and the β-strands tend to remain in the β-region without interactions among the β-strands.

For the Insect Defensin A we observe for this small protein a strong influence of the disulfide bridges in the stabilization of this structure. By breaking the disulfide bridges structures were found, which were proposed for competing in the stages of intermediate folding.

For protein folding, in particular, we have demonstrated that correctly folded structures can be attained in thousands stochastic molecular optimization steps, while using standard Simulated Annealing method, it can take several millions of steps [16,36]. Besides, it was shown that the protein energy hypersurface has a multifractal behavior [37]. These results show that proteins can find their native structure without sampling all the configurational space, pointing out to an alternative solution for the Levinthal paradox [44].

Concluding, GSA enables us to study small proteins close to the native structure. We applied this strategy to a kind of molecule. Moreover, we have found in these investigations that once the protein folding pathway is multifractal pathway, then amino acids lose mobility when the protein goes to the correct folded conformation. Therefore, we argued that in the last stage of protein folding, the molecule does not have mobility and the enthalpy drives the protein folding process.

Acknowledgements

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