Trends in Peptide and Protein Sciences
Volume 4 (2019): e1

DOI: http://dx.doi.org/10.22037/tpps.v4i0.24579

Review Article

Some Clues on the Conformational Stability of Globular Proteins

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Article history:
Received: 27 February 2019
Accepted: 6 April 2019

Keywords:
Antifreeze protein from snow flea
Conformational stability
Cooperativity
H-bonds
Hydrophobic effect
Solvent-excluded volume
Water accessible surface area

HIGHLIGHTS

- The stability and folding characteristics of antifreeze protein from snow flea, sfAFP, are challenging.
- The globular shape of sfAFP is not dictated by nonpolar side chains - water contact.
- The balance between the translational entropy of water molecules and the conformational entropy of the chain is important in protein folding.
- The balance between the formation of intramolecular peptide-peptide H-bonds and the breaking of intermolecular peptide-water H-bonds is important in protein folding.

ABSTRACT

The native structure of the glycine-rich antifreeze protein from snow flea, sfAFP, its conformational stability and cooperative folding-unfolding transitions represent a challenge for the current understanding and rationalization of protein folding and stability. The hydrophobic effect, the main stabilizing factor of folded structures, is redefined in terms of the solvent-excluded volume associated with the insertion of a given structure in water to arrive at a different molecular mechanism. The need to minimize the solvent-excluded volume in order to maximize the translational entropy gain of water molecules drives protein folding and determines the globular shape of folded structures. The burial of nonpolar side chains from water contact, as emphasized by the sfAFP folded structure, is not a necessary condition. In fact, a large fraction of nonpolar surface is water-accessible in the folded structures of globular proteins. The significant reduction in water accessible surface area upon chain folding is the necessary and fundamental condition, and rationalizes the conformational stability and cooperativity of sfAFP native structure.

Introduction

Recently, interesting experimental data on the structure and stability of the snow flea antifreeze protein, sfAFP, indicated that the current understanding of the factors driving the folding of globular proteins is far from complete (Chan and Dill, 1993; Shakhnovich, 2006; Dill et al., 2008). sfAFP consists of 81 residues, 37 of which are glycines (i.e., 46% of the residues do not possess side chains; usually the Gly percentage is around 6-7%), 11 are alanines, and 4 are cysteines involved in two disulphide bridges (Graham and Davies, 2005). This protein assumes an unusual and novel structure, characterized by a bundle of six short polyproline type II helices, PPII, organized in two sheets of three helices, parallel to each other in the sheet, and antiparallel to each other between the two sheets (see Fig. 1, PDB ID code 2PNE). The six PPII helices are bound together by an extended network of backbone-backbone H-bonds, so that the central helix in each sheet is H-bonded to other four helices (Pentelute et al., 2008; Gates et al., 2017). These H-bonds prove to be stronger than the usual ones, according to DFT quantum

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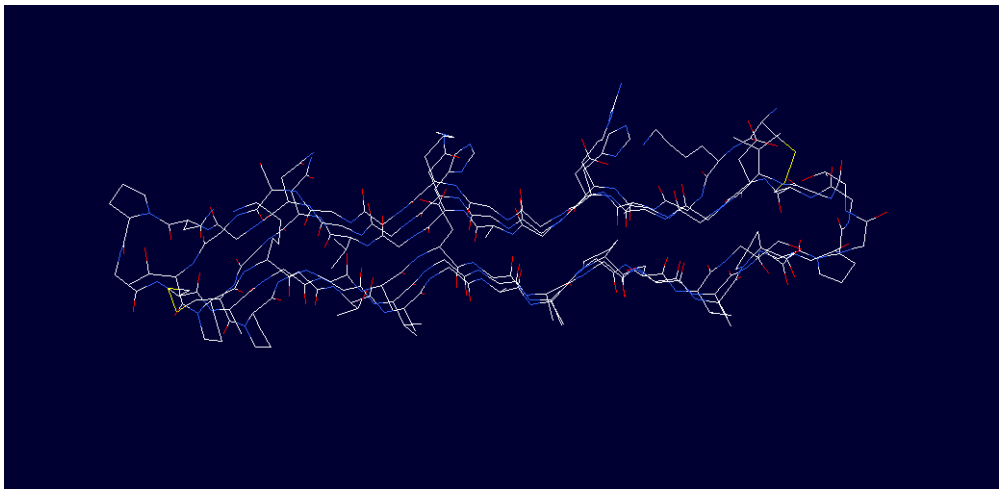

Figure 1. Picture of the sfAFP native structure; PDB ID code 2PNE.

chemical calculations, because they are very short, due to the absence of side chains in the protein interior (Gates et al., 2017). Therefore, the sfAFP native structure is characterized by an elongated shape and the lack of both a hydrophobic core and canonical secondary structure elements, such as α-helices and β-sheets. Nevertheless, the core of sfAFP proves to be very well packed due to the structural features of the special arrangement of the six PPII helices.

Circular dichroism, CD, and fluorescence measurements have shown that the temperature-induced denaturation of sfAFP is a reversible, cooperative, two-state process with a denaturation temperature of 32.5 °C (Gates et al., 2017). This value may appear low, but it should be noted that the nonpolar water accessible surface area (Lee and Richards, 1971), WASA, buried in the sfAFP native structure amounts to 20 $Å^2$ per residue, a value that is markedly smaller than the average one, amounting to about 50 $Å^2$ per residue (this is the consequence of the absence of side chains in the protein interior). MD simulations showed that the conformational entropy of the sfAFP native structure is large, as a consequence of the occurrence of significant structural fluctuations (Gates et al., 2017). This finding suggested that the gain in conformational entropy upon denaturation should be small, stabilizing the native structure. In fact, the denaturation Gibbs energy change amounts to 20 kJ mol^{-1} at 4 °C, a value in line with those of common globular proteins (Rees and Robertson, 2000). Kinetic data, obtained by means of CD and fluorescence measurements at room temperature using GdmCl as a perturbing agent, confirmed that the folding process of sfAFP can be described as a cooperative two-state transition (Gates et al., 2017). In conclusion, the results of Sosnick and colleagues unequivocally indicate that conformational stability and cooperative folding-unfolding of globular proteins do not need the presence of a sizeable hydrophobic core with optimized packing of side chains (Gates et al., 2017). This does appear strange, because there is a wide consensus in the scientific community that the hydrophobic effect plays the pivotal role in stabilizing the native structure (Blokzijl and Engberts, 1993; Dill et al., 2008), and is produced by the aversion between nonpolar side chains and water molecules with the formation of a nonpolar protein interior (Karandur et al., 2016). This view is challenged by the sfAFP native structure, its stability and cooperative behaviour.

In the present work, I would like to show that the hydrophobic effect has to be redefined in its geometric and molecular ground to fully understand why it is the actual driving force of protein folding and stability, also in the special and strange case of sfAFP.

General structural features of globular proteins

In biochemistry textbooks, it is generally written that the surface of globular proteins is rich in polar and charged groups to have attractive interactions with water molecules and to increase the protein solubility, whereas the interior is rich of nonpolar side chains to stabilize the folded structure, thanks to the hydrophobic effect. This simplistic structural view cannot be considered correct. More than thirty years ago, an interesting structural analysis (Miller et al., 1987) concluded that: ″The average water-accessible surface is found to be 57% nonpolar, 24% polar and 19% charged, with 5% root-mean-square variations. The molecular surface buried inside the protein

is 58% nonpolar, 39% polar and 4% charged." These results are supported by the analysis of 178 ultra-high resolution structures of monomeric proteins deposited in the Protein Data Bank (Merlino et al., 2017). The average WASA proves to be: (59 ± 4)% nonpolar, (23 ± 5)% polar and (19 ± 5)% charged. These values demonstrate that it is not true that nonpolar residues stay in the protein interior: 59% of native state WASA is nonpolar. This structural datum implies that there is no segregation of nonpolar side chains in the protein interior, and this cannot be the right description of the hydrophobic effect. It has to be recognized that globular proteins are much more complex than micelles, they are heteropolymers; the covalent bonds building up the polypeptide chain are the main factors determining the solvent exposure versus burial distribution of both backbone and side chains.

In fact, most of the peptide groups are not water-accessible and participate in intramolecular H-bonds (Miller et al., 1987). The formation of intramolecular H-bonds is a fundamental requirement to be satisfied, because the same peptide groups form H-bonds with water molecules in unfolded conformations. This explains the widespread occurrence of secondary structures, such as α-helices and β-sheets, which were discovered exactly with the criterion to satisfy the formation of peptide-peptide H-bonds (Pauling et al., 1951a and 1951b). If a polypeptide chain were not able to form a sufficient number of intramolecular H-bonds, it would be incapable to construct a stable and folded structure. Note, however, that the energetics of intramolecular H-bonds cannot be the driving force of protein folding, because it mainly serves to compensate the loss of intermolecular peptide-water H-bonds. Nevertheless, it is important in order to arrive at a unique folded and stable structure.

The hydrophobic effect in globular proteins

The hydrophobic effect is related to the thermodynamic features of the solubility in water of nonpolar molecules: the low solubility of the latter is ruled by a large negative entropy change around room temperature (Blokzijl and Engberts, 1993). A negative entropy change is not necessarily associated with an increase of structural order. In liquids, it can be associated with a decrease in the configurational space accessible to liquid molecules, as a consequence of the insertion of a solute molecule. The latter, in fact, causes a solvent-excluded volume effect in all liquids (Lee, 1985; Madan and Lee, 1994; Graziano, 2006). The solvent-excluded volume effect can be quantified by means of a theoretical concept: the creation in the liquid of a cavity suitable to host the solute molecule. If the cavity is created by keeping fixed the pressure, the liquid volume increases by a quantity equal to the partial molar volume of the cavity itself; this means that the solvent-excluded volume is not the cavity van der Waals volume, V_{vdW}, but is represented by the shell existing between the cavity WASA and its van der Waals surface. This shell region cannot be occupied by the centres of solvent molecules because the cavity V_{vdW} has to be empty to host the solute molecule. The solvent-excluded volume occurrence leads to a reduction in the configurational space accessible to liquid molecules, and so to a translational entropy loss for liquid molecules. This entropy loss causes the low solubility of nonpolar species in water, because it is exaggerated in water with respect to the other common liquids, because of the small size of water molecules and the consequent large number density (Lee, 1985; Graziano, 2006; Graziano, 2016).

When the solute is a flexible polymer, it can assume very different conformations that, even keeping fixed the chain V_{vdW}, correspond to different solvent-excluded volumes. The latter can be described by the WASA values of the different conformations of the polymeric chain. Globular proteins are heteropolymers and their folding, at constant temperature and pressure, is ruled by the solvent-excluded volume minimization (i.e., WASA minimization), with the aim of rendering maximal the translational entropy of water molecules in the presence of the chain (Graziano, 2014; Grimaldi and Graziano, 2018). As a consequence, the native structure of a globular protein should have the smallest WASA and this would be the criterion to sample the conformational space of a given polypeptide chain. The magnitude of the solvent-excluded volume effect is measured by the values of the reversible work of cavity creation, ΔGc, and the latter scales linearly with cavity WASA (Lee, 1985; Madan and Lee, 1994; Graziano, 2006; Graziano, 2015a). In the case of globular proteins, it is not necessary to distinguish the nonpolar WASA from the polar one, because both of them cause a decrease in the configurational space accessible to water molecules. Moreover, it is important to recognize that the solvent-excluded volume effect is a multi-particle effect, not additive by definition.

Since it is well known that, if V_{vdW} is fixed, the sphere is the 3D object with the smallest surface area, the native structure can be modelled as a sphere, whereas the unfolded conformations can be modelled as a set of prolate spherocylinders, all possessing the same V_{vdW} of the sphere representing the native structure (Graziano, 2014; Pica and Graziano, 2015a). It is worth noting that: (a) it is correct to assume that the volume does not change on passing from the unfolded ensemble to the native structure, because the volume change upon denaturation proves to be a very small and usually negative quantity (Royer, 2002; Chalikian, 2003); (b) the choice of prolate spherocylinders is qualitatively correct, because the denatured state of several globular proteins has a shape resembling a prolate ellipsoid (Tran and Pappu, 2006). The prolate spherocylinders have a WASA larger than that of the corresponding sphere and the difference increases

Table 1. Classic SPT-ΔG_c estimates for the creation of prolate spherocylindrical cavities at 20 °C and 1 atm in a hard sphere fluid having the experimental density of water and particle diameter $\sigma(H_2O)$ = 2.8 Å. The cavity V_{vdw} has been fixed at the V_{vdw} value of the prolate spherocylinder with a = 8.0 Å and l = 40.9 Å, that represents the N-state of sfAFP. The values in the last column are given by the difference ΔG_c(N-state) - ΔG_c(prolate spherocylinder).

a Å	l Å	V_{vdw} Å^3	WASA Å^2	R_g Å	ΔG_c kJ mol^{-1}	$\Delta\Delta G_c$ kJ mol^{-1}
5.0	125.3	10365	5553	36.3	1636	-555
5.5	101.8	10371	5012	29.6	1493	-412
6.0	83.7	10371	4580	24.5	1376	-295
6.5	69.4	10361	4229	20.5	1280	-199
7.0	58.0	10365	3948	17.5	1201	-120
7.5	48.7	10372	3719	15.0	1136	-55
8.0	40.9	10368	3526	13.1	1081	- -

The values of the radius of gyration R_g for prolate spherocylinders have been calculated by means of the following relationship: $R_g = [(a^2/2) + (l^2/12)]^{1/2}$.

on lengthening the spherocylinder. The magnitude of the solvent-excluded volume effect increases with cavity WASA, and so the ΔGc magnitude increases with the cylindrical length of the prolate spherocylinder (Graziano, 2015a).

The theoretical approach developed along these lines has provided a rationalization of the general occurrence of cold denaturation (Graziano, 2014; Grimaldi and Graziano, 2018), the effect of stabilizing and destabilizing agents (Graziano, 2011a and 2011b; Vigorita et al., 2018), the effect of heavy water (Pica and Graziano, 2018), the pressure-induced denaturation (Graziano, 2015b), some aspects of the extra-stability of thermophilic globular proteins (Pica and Graziano, 2016c), and some peculiarities of the collapse transition of poly(N-isopropylacrylamide), PNIPAM (Pica and Graziano, 2015b, 2016a and 2016b). In other words, the hydrophobic effect is mainly geometric in origin, and has little to do with the burial of nonpolar side chains.

Rationalization of sfAFP conformational stability

The native structure of sfAFP has the shape of a rectangle parallelepiped with sides of 47 Å, 17 Å and 13 Å, whose V_{vdw} = 10387 Å^3; it does not have a globular shape and cannot be represented as a sphere (see Fig. 1). It can be modelled as a prolate spherocylinder of radius a = 8.0 Å, cylindrical length l = 40.9 Å, V_{vdw} = 10368 Å^3, WASA = 3526 Å^2, and radius of gyration R_g = 13.1 Å, close to the value calculated from the sfAFP crystal structure, 13.3 Å (Gates et al., 2017). This prolate spherocylinder is the model of the N-state of sfAFP. Some prolate spherocylinders with the same V_{vdw}, but significantly larger WASA, can model unfolded conformations of sfAFP; their geometric sizes are reported in Table 1. According to SAXS data, the unfolded ensemble of sfAFP can be described as a self-avoiding-random-walk with an average radius of gyration $R_g \approx 25$ Å (Gates et al., 2017). Since the prolate spherocylinder with radius a = 6 Å and cylindrical length l = 83.7 Å has R_g = 24.5 Å, it should be a reliable, even though crude, model of the D-state of sfAFP.

The trend of ΔGc values versus WASA for the considered cavities is shown in Fig. 2: a linear relationship is evident. The present calculations have been performed by means of classic scaled particle theory (Pierotti, 1976; Graziano, 2011a and 2015a), SPT, which provides analytical formulas for both spherical and prolate spherocylindrical cavities, using the experimental density of water at 20 °C and 1 atm. The use of experimental density is very important because the latter carries information on the actual interactions existing in liquid water. It is worth noting that qualitatively similar results (i.e., the ΔGc increase caused by a rise of cavity WASA, on keeping fixed the cavity V_{vdw}) have been obtained by means of MD simulations in detailed water models (Wallqvist and Berne, 1995; Patel et al., 2010). The trend in Fig. 2 resembles a side of the funnel that is considered to be the right description of the energy landscape for protein folding (Dill et al., 2008). The decrease in solvent-excluded volume drives the process; for sfAFP, the Gibbs energy decreases by 295 kJ mol^{-1} on passing from the D-state (i.e., spherocylinder with l = 83.7 Å) to the N-state (i.e., spherocylinder with l = 40.9 Å), because water molecules gain translational entropy as the chain becomes more compact. The geometric model is too

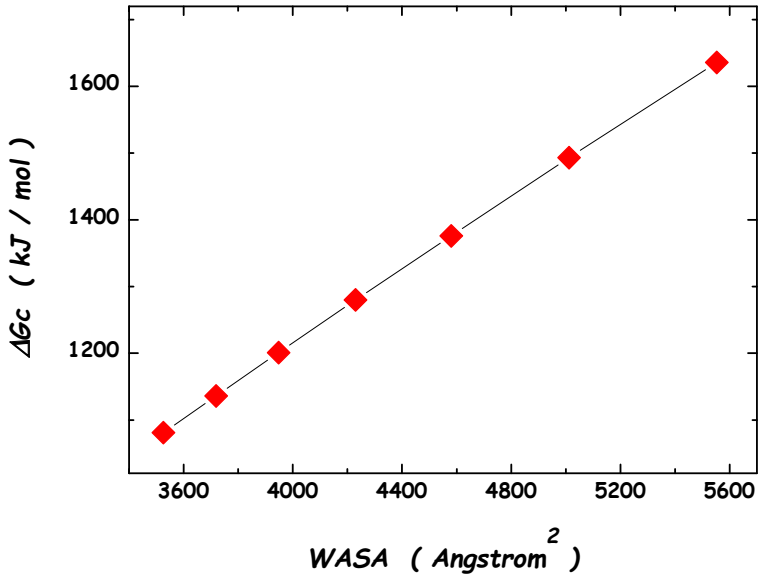

Figure 2. Trend of ΔGc versus WASA, according to classic SPT calculations, at 20 °C and 1 atm, for prolate spherocylindrical cavities, all possessing the same van der Waals volume of 10368 Å^3, using the experimental density of liquid water (see Table 1).

simple to account for all the details of protein folding, but its physical robustness should allow one to single out what the actual driving force of the folding process is.
The Gibbs energy gain given by ΔGc(N-state) - ΔGc(D-state) is only one of the contributions governing the conformational stability of sfAFP. The folding Gibbs energy change is given by (Graziano, 2011a, 2011b and 2014):

ΔG(folding) = ΔΔGc - TΔSconf + ΔEa

where ΔSconf = Sconf(N-state) - Sconf(D-state) is the conformational entropy loss associated with folding, a term that largely favours unfolded conformations. This conformational entropy loss has been calculated by different authors in the last years, and, even though it belongs to the whole protein, it is usually normalized to the number of residues in the chain. Sosnick and co-workers obtained that ΔSconf is largely determined by the protein backbone and ΔSconf ≈ -16 J K^{-1}molres^{-1} in the case of Gly residues (Baxa et al., 2014), a value close to that arrived at by another group following a different route (Sharp et al., 2015). Since MD simulations found that the sfAFP native structure is characterized by large fluctuations (Gates et al., 2017), ΔSconf should be smaller; reliable values could be -12 or -14 J K^{-1}molres^{-1}. Using the latter numbers, the -TΔSconf contribution in Eq. (1) would amount to 285 or 332 kJ mol^{-1} at 20 °C for sfAFP folding. It is clear that the conformational entropy provides a large positive quantity, opposing polypeptide chain folding.
The ΔEa term in Eq. (1) consists of two contributions: (a) the difference in energetic interactions with water between the native structure and the unfolded ensemble; (b) the difference in intramolecular energetic interactions between the native structure and the unfolded ensemble (Graziano, 2011a, 2011b and 2014). It is not simple to calculate reliable values for the ΔEa term, because the calculations depend on the knowledge of precise structural features of the unfolded ensemble. In the case of sfAFP around room temperature, T = 20 °C, it should be:

ΔG(folding) = ΔΔGc - TΔSconf + ΔEa = -295 + (285 or 332) + ΔEa ≈ -10 kJ mol^{-1}

This implies that ΔEa = 0 kJ mol^{-1} or -27 kJ mol^{-1}, depending on the value assigned to the conformational entropy term. These numbers, even though approximate, indicate that the ΔEa contribution should stabilize the native structure by a small amount. This result may appear strange considering that the H-bonds occurring among the six PPII helices in the native structure sfAFP are stronger than the usual ones. However, it should be recognized that: (a) the number of such strong backbone-backbone H-bonds is not large, exactly 29 (Gates et al., 2017); (b) the latter number should be compared with the larger number of H-bonds that the same peptide groups can form with water molecules in unfolded conformations.

This implies that the ΔEa term is expected to be small because it is necessary to look at the energetic interactions of both the native structure and the unfolded ensemble. An almost perfect balance for the energetic interactions between the native structure and the unfolded ensemble is not a stupid situation because, by recognizing the large number of H-bonds that an unfolded polypeptide chain forms with water molecules and the need to break several of them upon folding (Graziano, 2014; Pica and Graziano, 2016), it dictates the need to have a significant content of secondary structure elements in the N-state to allow the formation of a significant number of intramolecular H-bonds. In fact, the sfAFP native structure possesses a special arrangement of six PPII helices with a tight network of strong backbone-backbone H-bonds.

Last but not least, a very important property of small globular proteins is the strong cooperativity of their temperature-induced denaturation (Privalov, 1979; Dill and Stigter, 1995). This property holds also for sfAFP. In the present theoretical approach, the strong cooperativity is due to the tight coupling existing between the ΔΔGc term, the ΔEa term, and the TΔSconf one. A change in the geometric arrangement of the chain produces a change in the solvent-excluded volume effect, in the intramolecular and intermolecular energetic attractions, and in the number of conformations accessible to side chains. Moreover, the change in the geometric arrangement of the chain causes a modification of the first and second hydration shells whose size is ruled by the geometric shape adopted by the polypeptide chain. A huge number of protein atoms and water molecules are coupled together, leading to a cooperative behaviour. Indeed the temperature-induced denaturation can be treated as the melting (i.e., a first-order phase transition) of a ″crystal molecule″ (Liquori, 1969).

Conclusion

In conclusion, it has been pointed out that the sfAFP native structure, its conformational stability and cooperative behaviour represent a challenge for the widely accepted views of protein folding and stability. A redefinition of the hydrophobic effect in terms of the solvent-excluded volume associated with the insertion of a given structure in water leads to a different molecular understanding. The globular shape of folded structures is dictated by the need to minimize the solvent-excluded volume in order to maximize the gain in translational entropy of water molecules with the constraint that a given chain has to be hosted in water. The globular shape is not dictated by the need to bury nonpolar side chains from water contact, as emphasized by the sfAFP folded structure (Gates et al., 2017). In fact, a large fraction of nonpolar surface is in contact with water molecules in the folded structures of globular proteins (Miller et al., 1987; Merlino et al., 2017). What is really important is the large WASA decrease associated with chain folding that occurs also in the special case of sfAFP. The analysis implies that protein folding is ruled by a delicate balance between the gain in translational entropy of water molecules and the loss in conformational entropy of the chain, and between the formation of intramolecular peptide-peptide H-bonds and the breaking of intermolecular peptide-water H-bonds.

Competing Interests

There are no conflicts of interest to declare.

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