Cold and Warm Denaturation of Proteins

G. CALDARELLI¹ and P. DE LOS RIOS²

¹*INFM Sezione di Roma1 and Dipartimento di Fisica, Università 'La Sapienza', P.le A. Moro 2, 00185, Roma, Italy, e-mail: gcalda@pil.phys.uniroma1.it*

²*Institut de Physique Théorique, Université de Lausanne, 1015 Lausanne, Switzerland, e-mail: Paolo.DeLosRios@ipt.unil.ch*

Abstract. We introduce a simplified protein model where the water degrees of freedom appear explicitly (although in an extremely simplified fashion). Using this model we are able to recover both the warm and the cold protein denaturation within a single framework, while addressing important issues about the structure of model proteins.

Key words: Cold denaturation, hydrophobic forces, proteins

1. Introduction

Proteins are extremely complex structures: they are long heteropolymers made of up to 20 different amino-acids species, each of them with its own chemical, electrostatic and steric properties; the physiological solvent, an aqueous solution, and its characteristics play a fundamental role both in the dynamics and in the thermodynamics of folding. It is therefore not surprising that only in recent times statistical physicists have begun working on this problem, mainly after the introduction of the so-called HP model [1], where the above mentioned richness has been reduced to a manageable level. In the HP model, proteins are modeled as self-avoiding polymers on a lattice (two or three dimensional), greatly reducing the number of accessible conformations [2]. The chemical and electrostatic properties of amino-acids have also been simplified: indeed, it has been recognized since long that the main force stabilizing the native conformations of globular proteins is the *hydrophobicity* of non-polar amino-acids [3]. Consequently, the important properties of amino-acids are reduced to two: they are either polar (ions or dipoles, labeled with P) or non-polar (H).

Hydrophobicity can be described as the tendency of hydrophobic molecules to reduce as much as possible their surface of contact with water: two hydrophobic molecules try to stick together in order to hide from water their mutual surface of contact. Consequently, hydrophobicity has been introduced in the HP model as an effective attractive interaction between H amino-acids. Then, the solvent degrees of freedom can be neglected. Here we show that such a simplification can be removed, and water can be taken into account, keeping the complexity of the

model at a still manageable level: the benefits are a better description of the protein phenomenology (namely, *cold* destabilization and eventually denaturation [4, 5]) and some insights on the structure of the protein core.

In the last fifteen years there has been a growing body of evidence for the so called *cold destabilisation* of proteins: the free energy difference ΔF_D^N between denature and native conformations of proteins has parabolic shape, with a maximum at temperatures of the order of 15–25 °C, or lower, implying that at lower temperatures the native conformation is less and less stable. In some cases, even the *cold denaturation* of proteins has been obtained [6].

There are at least two reasons to believe that a good description of cold destabilisation and denaturation is relevant to protein folding.

In order to describe protein folding with a simple model, it is important to capture the essential physics of the process, at the temperatures at which it takes place. If the stability of native conformations of proteins begins to decrease below 15–25 °C, it is unlikely, at least *a priori*, that the physics responsible for such a behavior is not important around the maximal stability temperature, in a range relevant for *in vivo* protein folding. A further reason to believe that a good model for protein folding should also agree with the cold destabilisation phenomenology is that, actually, there is no clear-cut distinction between the physics that stabilizes proteins, and the one that destabilizes them. In both cases a re-analysis of the concept of hydrophobicity and of hydrophobic hydration is necessary.

Already Frank and Evans [7] identified the origin of hydrophobicity in the partial ordering of water around non-polar molecules (such as, for example, pentane, benzene and some amino-acids). Water molecules tend to build ice-like cages around non-polar molecules. Although a detailed analysis of these structures is, to our knowledge, still lacking (actually recently some better understanding and consensus are emerging [8–11]), we can guess their energetic and entropic properties. Indeed, water molecules forming these cages are highly hydrogen bonded, much as in ice; consequently, their formation is energetically favorable with respect to bulk liquid water. Yet, the possible molecular arrangements in the cages are a small number compared to all the disordered molecular conformations typical of liquid water. The latter are energetically unfavorable with respect to bulk water because water molecules fail to form hydrogen bonds with hydrophobic amino-acids. Therefore the free energy of formation of a cage ($F_{cage} - F_{no\ cage} = \Delta F$) is a balance between an enthalpy gain/loss and an entropy loss/gain: ordered cages give an enthalpy gain ($\Delta H < 0$) and an entropy loss ($\Delta S < 0$); the scenario is the opposite for disordered states. All of the above arguments call for a model able to reproduce (at least qualitatively) such a rich phenomenology.

2. The HP-Water Model

The model we propose here borrows two of the simplifications from the HP model: proteins are still modeled as heteropolymers on a lattice, made of just two different

Figure 1. Bimodal effective models. Panel (a): MLG model, with bimodal energy distributions both for bulk and shell water molecules. The lower levels represent ordered group of water molecules, the higher levels disordered ones. The order of energies and of degeneracies, as obtained from experiments, is $E_{ds} > E_{db} > E_{ob} > E_{os}$ and $q_{ds} > q_{db} > q_{ob} > q_{os}$ (ds = disordered shell, os = ordered shell, db = disordered bulk, ob = ordered bulk). Panel (b): the simplified bimodal energy distribution, with just two free parameters, K and q , since we can take J as energy scale.

amino-acid species: polar (P) and non-polar (H). Every site of the lattice that is not occupied by the polymer is occupied by water (in general, by a group of water molecules that can be arranged in q states).

2.1. TWO-STATE MODELS FOR WATER

Water is described using the Muller-Lee-Graziano (MLG) two-states model (Figure 1a) [8, 9]. The energy of each H amino-acid depends on the states of the water molecules it is in contact with (the water molecules in the hydration shell). As mentioned above, hydration water can build ordered cages around the molecule, that are energetically favorable with respect to the possible disordered configurations, hence $E_{ds} > E_{os}$. Yet, the disordered configurations outnumber the ordered ones: $q_{ds} > q_{os}$. Water molecules that are not in contact with non-polar molecules (*bulk* water) are described by a two-state model as well: water molecules can build networks of hydrogen bonds that are energetically favorable with respect

to disordered configurations ($E_{db} > E_{ob}$) even if there are far more disordered configurations than ordered ones ($q_{db} > q_{ob}$). The above arguments hold separately for bulk and shell molecules. In order to understand the order of the energies and of the degeneracies we need to describe the effects of the transfer of water molecules from the bulk to the hydration shell. Indeed, hydrogen bonds between hydration shell water molecules on the average are stronger than hydrogen bonds in the bulk ($E_{ob} > E_{os}$); conversely, the number of hydrogen bonded configurations in the hydration shell is smaller than in the bulk ($q_{ob} > q_{os}$). Actually, the two inequalities are mutually consistent: the greater the number of equivalent configurations, the higher the probability to switch from one to the other; therefore, the persistence time of the bulk hydrogen bonds is shorter than the persistence time in the hydration shell, whence the energy inequality. The ordered bulk orientations that do not contribute to ordered shell configurations can be counted among the disordered shell configurations, that are therefore more abundant than the disordered bulk configurations. Moreover, such configurations are energetically less favorable than bulk disordered configurations because every time a water molecule points one of its bonding directions toward the non-polar molecule, it loses energy: therefore $E_{ds} > E_{db}$ and $q_{ds} > q_{db}$. Such a hand-waving picture has been recently confirmed by Silverstein et al., who derived the double two-state MLG model using a molecular model of the water-amino acid system [11].

The simple MLG model is extremely effective in describing the thermodynamics of solvation of hydrocarbons (that, as recalled above, are strongly hydrophobic: indeed the residues of hydrophobic amino-acids are essentially hydrocarbons, *e.g.*, the residue of leucine is an isopropyl group) [9]. The degeneracies and energy differences can be fitted to experiments, in order to get the detailed values. The MLG model has six free parameters (one degeneracy and one energy can be chosen as reference): too many for a *simple* theoretical model. We therefore introduce the Bimodal model (BM): as a simplifying assumption (see Figure 1b), we say that out of the q possible states of a water molecule, one can be singled out to be a cage conformations (labeled $s = 0$), energetically favorable with energy $-J$ ($J > 0$), and the remaining $q - 1$ ($s = 1, \dots, q - 1$) states are energetically unfavorable with energy $K > 0$ (they represent the disordered states of reduced hydrogen-bond coordination). We stress that the term (*un*)*favorable* is always with respect to bulk liquid water. Bulk water molecules that are not in contact with H amino-acids do not contribute to the energy. Such a model is much simpler than the MLG one, yet it bears qualitatively similar results, as we shall show.

2.2. THE MODEL

As we mentioned above, we model proteins as polymers on a lattice. Monomers can be either hydrophobic or polar. Sites that are not occupied by any amino-acid, are occupied by water. The energy of the model is given by the energy of the water sites. Every water site is occupied by *some* water molecules, so that its available

energy states should be given by a suitable convolution of several MLG or BM models, as given in the previous section. As a simplifying assumption, we describe the energetics of a water site using a two-state model, choosing the bulk or shell parametrisation depending on whether the site is in contact with a H monomer or not. P amino-acids do not interact with water so that their energy is always 0: such a crude approximation is made with the idea that hydrophobicity is the leading effect stabilizing the native conformation of proteins. Some better description of the water- P interaction would be welcome, but such ingredient is unnecessary for our present purposes.

Indeed, in the original formulation of the HP model, only interactions between hydrophobic amino-acids were considered. Although this is clearly a strong assumption, it has the advantage to keep the model as simple as possible and to clearly address the effect of the sole hydrophobicity. On the other hand, the various approximations of the model imply that the questions we can answer are somehow limited. In this paper we look only at the thermodynamic behavior of proteins, and at the simplest of the structural features, that is the segregation of hydrophobic residues in the core of the protein. Other important problems can be addressed already within the HP-model scheme, such as the relation between sequences and structures, and in particular the designability of the latter. We will tackle such issues in future works.

In what follows we will make explicit use of the BM model: formulas for the MLG model are similar and can be easily derived. Given a protein of N amino-acids, with the sequence $a_1, a_2, \dots, a_N$ ($a_i = P$ or H), the energy of the protein is then

$$E = \sum_{\langle j, H \rangle} (-J\delta_{Sj,0} + K(1 - \delta_{Sj,0})) \quad (1)$$

where the sum is over the water sites that are nearest neighbors of some H amino-acid. Starting from (1) we can write the partition function of the system as

$$Z_N = \sum_C Z_N(C) \quad (2)$$

where $Z_N(C)$ is the partition function associated to a single conformation C :

$$Z_N(C) = q^{n_0(C)} ((q-1)e^{\beta K} + e^{\beta J})^{n_1(C)} \quad (3)$$

and the dependence on the water degrees of freedom has been explicitly calculated. $n_1(C)$ is the number of water sites nearest neighbors of some H amino-acid, n_0 is the number of water sites in the bulk or in contact only with P amino-acids. We also keep the description of water as simple as possible, neglecting any interactions between different water sites.

We deal with model proteins of length up to $N = 17$ on the square lattice, and compute the partition function, and all the thermodynamic quantities and averages

Figure 2. Specific heat, monomer-monomer contacts and number of water sites in an excited state for the protein shown in the inset; $K = 2$, $J = 1$, $q = 1000$.

by exact enumeration of the 2155667 different conformations. We show the results for the particular sequence *PHPPHPPHPPHPPHH*. Such a sequence has been chosen to have a compact state with all the hydrophobic amino-acids in the core. That such a state is the native one (most stable and unique) is what we must check with the statistical mechanical treatment. We choose $J = 1$ (actually, both K and the temperature T can be normalized with respect to J), $K = 2$ and $q = 105$ (a better determination of these values could come from molecular dynamics and structural studies). We take the Boltzmann constant $k_B = 1$.

3. Results

In Figure 2 the specific heat C_v , and the average number of monomer-monomer contacts, n_c , are shown. The low-temperature peak in the specific heat coincides with a jump of n_c : at lower temperatures the protein is swollen, and maximizes the number of water-H contacts, in agreement with cold denaturation. The number of contacts, n_c , begins decreasing coinciding with the high-temperature peak

Figure 3. Same as in Figure 2, for a full implementation of the MLG model, with $E_{os} = -1.4$, $E_{ds} = 1.8$, $E_{ob} = -1$, $E_{db} = 1$, $q_{os} = 1$, $q_{ds} = 999$, $q_{ob} = 50$, $q_{db} = 950$.

of the specific heat, that therefore coincides with the usual warm denaturation phenomenon.

Between T_c , and T_w , there is a region where the most probable conformation is the one represented in the inset of Figure 2: as it can be seen, it is compact with a hydrophobic core, out of reach for water (we also checked that this *native* state is unique, in that its Boltzmann weight is the largest above T_c). We have analyzed the behavior of different protein lengths and of different sequences, and we have always found the same qualitative behavior of C_v and n_c . Our model is therefore able to describe, within a single framework, both cold and warm denaturation. Moreover, it shows a native state with a mostly hydrophobic core.

Although the ratio between $T_w/T_v \sim 10$ in Figure 2 is unphysical (from experiments $T_w/T_c \sim 1.5$), using the full MLG model it is possible to come closer to real values: in Figure 3 the ratio is $T_w/T_c \sim 3$, and going toward more and more refined models of water and of water/amino-acids interactions it is surely possible to get physical values of the ratio. Of course the price to be paid is the larger number of parameters to adjust. In this work we address the physical principles responsible for the thermodynamic behavior of proteins on a broad range of temperatures: we

Figure 4. Free energy, enthalpy and entropy (times T) differences between denatured conformations and the native one (shown in the inset of Figure 2), for the same parameter values as in Figure 2. Since $T\Delta S$ grows linearly at high temperatures, ΔS saturates.

believe that the differences between the bimodal model and the MLG model (and other possible more refined models) govern the details of the behavior more than the essential features.

We next compare the free energy, enthalpy and entropy variations of folding of our model with those from the literature [4, 5]. Indeed, such a comparison is a difficult one, since it is hard to define what a denatured state is in our theoretical calculations, and even the experiments have not yet been conclusive on such issue. Therefore, as a simple approximation, we consider as denaturate those conformations with at most 4 monomer-monomer contacts (a polymer of 17 monomers over a square lattice has at most 9 monomer-monomer contacts). The native state has 8 monomer-monomer contacts.

In Figure 4 we show $F_{Denaturate} - F_{Native} = \Delta F_N^D$, ΔH_N^D and $T\Delta S_N^D$. They coincide qualitatively with the ones from experiments [4, 5]. We point out the presence of two temperatures below and above which $\Delta F_N^D < 0$: the denatured state of our model protein is more stable than the native state. Between these two temperatures, instead, $\Delta F_N^D > 0$, and the native state is the most stable. In the same temperature range where $\Delta F_N^D > 0$, ΔH_N^D and $T\Delta S_N^D$ have a strong tem-

Figure 5. Two different conformations of the same sequence differing only for a reorganization of the core amino-acids.

perature dependence: they even change sign, a signature of the rich physics behind the water-protein system. At high temperatures we find that both ΔH_N^D and ΔS_N^D saturate ($T \Delta S$ grows linearly, therefore ΔS saturates), as experimentally observed [5]. Some particular care should be paid to the low temperature behavior of ΔH_N^D and $T \Delta S_N^D$. Indeed, ΔH_N^D goes to a constant value, which is consistent with a lower bound for the energies, and $T \Delta S_N^D$ tends to 0 with T . Experiments should be made below T_c to assess such a behavior (although a recent model suggests such scenario [10]). We find therefore that our model reproduces qualitatively the known calorimetric data of protein denaturation over a broad range of temperatures.

4. Effective Interaction

4.1. TWO-BODY INTERACTIONS

The hydrophobic effect is often modeled through attractive effective HH interactions. Within our framework (and on a square lattice, for simplicity), we consider a system of two H amino-acids in solution and we compare the partition function of the system when the two amino-acids are in contact,

$$Z_c = q^2(e^{\beta J} + (q-1)e^{\beta K})^6, \quad (4)$$

with the one when the distance between the two amino-acids is infinite,

$$Z_0 = (e^{\beta J} + (q-1)e^{\beta K})^8. \quad (5)$$

The effective interaction is defined as

$$\epsilon_{HH} = T \ln \frac{Z_c}{Z_0} = 2T \ln \frac{q}{e^{\beta J} + (q-1)e^{\beta K}} \quad (6)$$

Figure 6. Angle configurations used to compute the three-body effective energies: a) three H particles and b) two H and one P particles.

(ϵ_{HH} is positive if attractive, with this definition). The $T \rightarrow \infty$ limit is

$$\epsilon_{HH}(T \rightarrow \infty) = 2K - \frac{2}{q}(K + J) \quad (7)$$

and is attractive for large values of q : it is the usual hydrophobic effective interaction. Yet, the $T = 0$ limit is $\epsilon_{HH} = -2J$, repulsive. A meaningful effective interaction should at least include such a temperature dependence. Actually, the strong temperature dependence of ϵ_{HH} is not the only limitation to a definition of an attractive HH interaction. Indeed, such an interaction can be meaningful only for amino-acids surrounded by water molecules, but it cannot be defined in the core of proteins, where water is absent. As a consequence, in the absence of some true interactions between amino-acids, the hydrophobic interaction alone is not able to favor thermodynamically the native state against different compact states obtained by reordering only the core of the protein. As an example, the two conformations in Figure 5, corresponding to the sequence $PPHPPPPPPPPHHHP$ have the same probability to occur in our model, since they hide and expose to water the same number of H amino-acids.

4.2. MANY-BODY INTERACTIONS

When a protein is folding, its amino-acids find an *ever changing* environment that depends on water and on the other amino-acids. Even the reliability of two-body effective interactions vs. many-body ones is an open issue still to be settled. In our model we can compute some many body effective interactions. First, we observe that next-nearest-neighbor interactions are equal to nearest-neighbor interactions, $\epsilon_{nnn} = \epsilon_{HH}$, since there are again six water sites in contact with the hydrophobic molecules. Then we consider three H particles in an *angle* configurations (see Figure 6a). The effective energy can be computed as

$$\epsilon_{HHH} = 5T \ln \frac{q}{e^{\beta J} + (q-1)e^{-\beta K}} \neq 2\epsilon_{HH} + \epsilon_{nnn}. \quad (8)$$

Already this simple situation shows that integrating away the solvent degrees of freedom cannot be reduced to simple two-body effective interactions: the intrinsic three-body contribution to the free energy amounts to roughly 20% of the total. Many-body effects can be pronounced also if the corner amino-acid is polar ((see Figure 6b). In that case the effective energy is

$$\epsilon_{HPH} = 7T \ln \frac{q}{e^{\beta J} + (q-1)e^{-\beta K}} \neq 2\epsilon_{HP} + \epsilon_{nnn} \quad (9)$$

with $\epsilon_{HP} = T \ln[(q/(e^{\beta J} + (q-1)e^{-\beta K}))]$. Actually, the non additivity and more generally the context-dependence of water mediated effective interactions has also been recently pointed out [16].

4.3. VALIDITY OF EFFECTIVE INTERACTIONS

This model suggests that it is improper to define interactions of hydrophobic origin inside proteins, and that the detailed structure of the cores of proteins should be stabilized by other mechanisms. Recently, many methods have been devised to derive effective potentials able to stabilize protein structures: some of them [12] are of a statistical nature and have been shown to be intrinsically flawed [13]; some other methods that have a more rigorous physical basis have also been proposed [14]. Still, no matter the physical soundness of the method, the presence of intrinsic many-body effective potentials casts a shadow over any simplistic two-body description of amino-acid/amino-acid interactions.

Moreover, effective interactions can be safely defined whenever they substitute some non-changing environment [15]. It is therefore intrinsically difficult to define effective potentials of some general validity between amino-acids: our model points out such a problem for hydrophobic interactions.

5. Conclusions

In conclusion, we have introduced a model of proteins in water that is able to reproduce the known features of proteins (namely, cold destabilisation and warm

denaturation, a native state with a mostly hydrophobic core, and the correct free energy, enthalpy and entropy of folding). We also checked our results for different protein lengths, sequences, parameter values and even implementing the full MLG model for the description of water. Although some details may change, the overall behavior is consistent and robust. Moreover, lattice models are intended to be only qualitatively instructive, whereas a quantitative description can be given only by more detailed off-lattice models.

Our model is a first step in an interpolation between fully microscopic models, where water is dealt with at a molecular level, and fully effective models, where water is accounted for by effective potentials between amino-acids. As we have shown, the reliability of simple two-body, context-independent effective potentials is, at least as a matter of principle, questionable (very recently, Park et al., proposed at least a distinction between surface and core two-body potentials [17]): within our scheme such a problem emerges clearly. Although our model is an extremely simplified one, we believe that moving toward more realistic descriptions of the water/amino-acid system would complicate the structure of the effective potentials, rather than simplifying it, and that therefore a much better understanding is needed.

Acknowledgements

The authors thank E.I. Shakhnovich and S. Vaiana for useful suggestions. This work has been partially supported by the Swiss National Research Foundation under grant FNRS 21-61397.00 and by the European Network Contract FMRXCT980183.

References

1. Lau, K.F. and Dill, K.A.: *Macromolecules* **22** (1989), 3986; Chan, H.S. and Dill, K.A.: *Physics Today* **46** (1993), 24.
2. Vanderzande, C.: *Lattice Models of Polymers*, Cambridge University Press, Cambridge, 1998.
3. Kauzmann, W.: *Adv. Protein Chem.* **14** (1959), 1.
4. Murphy, K.P., Privalov, P.L. and Gill, S.J.: *Science* **247** (1999), 559.
5. Privalov, P.L. and Gill, S.J.: *Adv. Protein Chem.* **39** (1988), 191; Makhatadze, G.I. and Privalov, P.L.: *J. Mol. Biol.* **213** (199), 375; Makhatadze, G.I. and Privalov, P.L.: *J. Mol. Biol.* **232** (1993), 639; Privalov, P.L. and Makhatadze, G.I.: *J. Mol. Biol.* **232** (1993), 660; Makhatadze, G.I. and Privalov, P.L.: *Adv. Protein Chem.* **47** (1995), 307 and references therein.
6. See e.g. Pace, N.C. and Tanford, C.: *Biochemistry* **7** (1968), 198; Privalov, P.L., Griko, Yu.V., Venyaminov, S.Yu. and Kutysheko, V.P.: *J. Mol. Biol.* **190** (1986), 487; Griko, Y.V. and Privalov, P.L.: *Biochemistry* **31** (1992), 8810; Zhang, J., Peng, X., Jonas, A. and Jonas, J.: *Biochemistry* **34** (1995), 8631; Nicholson, E.M. and Scholtz, J.M.: *Biochemistry* **35** (1996), 11369; Graziano, G., Catanzano, F., Riccio, A. and Barone, G.: *J. Biochem.* **122** (1997), 395; for a general review see Privalov, P.L.: *CRC Crit. Rev. Biochem. Mol. Biol.* **25** (1990), 181, and references therein.
7. Franks, H.S. and Evans, M.W.: *J. Chem. Phys.* **13** (1945), 507; see also Creighton, T.E.: *Proteins, Structures and Molecular Properties*, W.H. Freeman & Company, New York, 1993, pp. 157–161, and references therein.
8. Muller, N.: *Acc. Chem. Res.* **23** (1990), 23.

9. Lee, B. and Graziano, G.: *J. Am. Chem. Soc.* **22** (1996), 5163.
10. Silverstein, K.A.T., Haymet, A.D.J. and Dill, K.A.: *J. Am. Chem. Soc.* **120** (1998), 3166.
11. Silverstein, K.A.T., Haymet, A.D.J. and Dill, K.A.: *J. Chem. Phys.* **111** (1999), 8000.
12. Many methods to predict the native state of proteins have been recently developed, based on residue-residue interactions of a statistical origin, without any specific assumption on the 'real', microscopic, nature of such potentials. Their derivation from first principles, when possible, is still an open issue; see e.g. Miyazawa, S. and Jernigan, R.L.: *J. Mol. Biol.* **256** (1996), 623; Skolnick, J., Jaroszewski, L., Kolinski, A. and Godzik, A.: *Protein Sci.* **6** (1997), 676.
13. Vendruscolo, M. and Domany, E.: *J. Chem. Phys.* **109** (1998), 11101.
14. Seno, F., Micheletti, C., Maritan, A. and Banavar, J.R.: *Phys. Rev. Lett.* **81** (1998), 2172; Micheletti, C., Seno, F., Banavar, J.R. and Maritan, A.: *Proteins* **42** (2001), 422.
15. Banavar, J.R. and Maritan, A.: *Proteins* **42** (2001), 433.
16. San Biagio, P.L., Bulone, D., Martorana, V., Palma-Vittorelli, D.B. and Palma, M.U.: *Eur. Biophys. J.* **27** (1998), 183.
17. Park, K., Vendruscolo, M. and Domany, E.: *Proteins* **40** (2000), 237.