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The Role of Solvent in Protein Folding and in Aggregation

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Abstract. We discuss features of the effect of solvent on protein folding and aggregation, highlighting the physics related to the particulate nature and the peculiar structure of the aqueous solvent, and the biological significance of interactions between solvent and proteins. To this purpose we use a generalized energy landscape of extended dimensionality. A closer look at the properties of solvent induced interactions and forces proves useful for understanding the physical grounds of 'ad hoc' interactions and for devising realistic ways of accounting for solvent effects. The solvent has long been known to be a crucially important part of biological systems, and times appear mature for it to be adequately accounted for in the protein folding problem. Use of the extended dimensionality energy landscape helps eliciting the possibility of coupling among conformational changes and aggregation, such as proved by experimental data in the literature.

Key words: Computational modeling, energy landscapes, hydration, hydrophobic interactions, protein aggregation, protein conformational changes, protein folding, protein-solvent interactions, spinodal and coexistence

1. Introduction

Proteins are in a way very simple and in a way very complex systems, their complexity coming from the large number of interactions of comparable size among their components. As discussed elsewhere in this volume, the convenience of viewing protein folding in terms of an energy landscape is widely appreciated [1–9]. The substantial uniqueness of the native functional state, and the rapid folding process, distinguish proteins from truly frustrated systems. In the energy landscape perspective this is accounted for by a funnel-like landscape. This provides a thermodynamically stable ground state considered unique in first approximation, and virtually accessible from all reasonable starting configurations. A relatively smooth funnel surface enables rapid folding. Simple but progressively refined models have been used to approach this problem, and have proved very useful.

The solvent has long been known to be a crucially important part of biological systems. In the protein folding problem, it is typically accounted for by including in the effective potentials 'polar' or 'hydrophobic' interaction terms. Though this

characterization is actually based on the particulate nature of the solvent (going thus beyond the continuous dielectric approximation), times appear mature for a necessary closer look. To this purpose it is useful to consider the overall landscape of the protein+water system. That is, a landscape of potential energy in the multi-dimensional space including all degrees of freedom of both protein and water. Such extention appears natural considering that the energy landscape perspective has also been used for understanding liquid water in terms of its inherent structures [10, 11]. As we shall see in the following, using the protein+water energy landscape helps looking at the various aspects of the problem from a unified point of view and extracting useful conclusions.

The rigorous statistical mechanical treatment of solvent induced interactions was given by Kirkwood long ago in terms of the so-called potential of mean force, or PMF, an effective potential containing direct as well as solvent induced interactions [12, 13]. In order to make his treatment analytically manageable, he introduced the so called Kirkwood's superposition approximation (KSA), implying pair additivity of solvent induced interactions and related forces. This approximation, subsequently used almost universally, has encouraged the mentioned categorization in terms of pairwise 'polar' and 'hydrophobic' interactions. Such categorization, however, requires great caution because, in the case of aqueous solutions, solvent induced interactions and forces turn out to be highly non-additive [14, 15], with consequences relevant to both protein folding and aggregation. It must be appreciated that KSA is largely valid in the case of hard-sphere [16, 17] and to some extent even in the case of Lennard-Jones (LJ) solvents [18]. Consequently, taking into account non-additivity of solvent induced interactions is not mandatory in those cases. It is the very special geometry and structure of the water molecule that invalidates KSA in the case of aqueous solutions. This is true even without taking into account polarizability of the water molecule, that would cause even larger non-additivity [14].

Indeed, it has been shown by molecular dynamics (MD) simulations that non-additivity of solvent induced interactions and forces is large enough to blur the character of 'hydrophobicity' or 'polarity' at single residue resolution [15, 19, 20]. Further, non additivity of solvent induced interactions brings to their high specificity and context dependence, and introduces long-range correlations that may span the entire size of a protein [21]. On a larger scale instead, a mean-field approximation holds frequently [22–26].

These features of solvent induced interactions are highly relevant to the understanding of protein folding and protein aggregation, and must be taken into account in realistic modeling. This is easily appreciated by remarking that solvent induced interactions cause typical forces, on individual residues, in the 20–200 pN range or higher, and that the work of a 70 pN force across 1Å corresponds to 1 Kcal/mol. Such energy scale is quite relevant to folding, recognition and conformational changes. Even stronger, more non-additive and sign-dependent, are the effects of charges on solvent induced interactions [27, 28]. This can have direct implica-

tions on protein conformational changes caused by charges, signal transduction and energy conversion.

We shall see in the following how it is possible to get practical conclusions from a problem that, at first sight, shows discouraging complications and seems to be treatable only through drastic approximations.

2. Interactions in the Protein+Water System

It was remarked long ago by Anfinsen that, by plain thermodynamics, protein folding must lead to a final state where the free energy of the entire system (protein+solvent) has reached its minimum [29]. The pathway to such equilibrium state is conveniently viewed in terms of the global energy landscape of the protein+solvent system, in the P+nN dimensional space (where P is the number of coordinates of the protein, n is the number of coordinates describing a single water molecule and N is the number of water molecules). It is useful to consider first the individual subsystems. Note that, as to the dynamics of the whole system, we are only interested in the time scale set by the protein motions relevant to folding and function, i.e. of 10^{-6} sec or longer. Consequently, effects of faster motions can conveniently be thermodynamically averaged.

2.1. ENERGY LANDSCAPE OF A SINGLE PROTEIN IN VACUO

For a protein in vacuo the overall potential energy of the system is a function of the P coordinates of the protein. If one is interested in the behavior of the system with respect to a given time scale, a thermodynamic average should be operated over those variables having characteristic times distinctly shorter than those of interest (e.g. intra-residue motions). This reduces the dimensionality of the configurational space. The landscape so obtained no longer represents a pure potential energy, since the thermodynamic averaging has given it a partial character of free energy. This introduces a temperature dependence of the landscape.

2.2. ENERGY LANDSCAPE OF WATER ALONE

For N deformable atomic molecules, a 9N dimensional space must be considered. At variance with the case of proteins, the potential energy landscape of water alone is characterized by a very large number of relative minima of comparable potential energy. It is useful to look at it in terms of 'basins' surrounding each minimum. Water configurations corresponding to these minima are usually referred to as inherent structures [10, 11] and can be viewed in terms of a hydrogen bond network. Since the energy of H-bonds is of the order of kT, the system undergoes frequent structural rearrangements, corresponding to the hopping of its representative point among basins. These reconfigurational events occur on time scales of the order of picoseconds. In addition, vibrational motions occur within each basin

on time scales 10 to 100 times shorter. Thermodynamic averaging over such fast modes gives a partial free energy character to the energy landscape. Corresponding average geometric configurations are the so called 'V-structures' [30, 31].

Time scales which interest us are 10^{-6} sec or longer, that is much longer than reconfiguration timescales. Therefore thermodynamic averaging must be extended in our case over all water configurations. The total free energy can now be easily expressed. For given thermodynamic conditions (e.g. N,V,T) only a small subset of basins, having essentially the same depth, is populated with overwhelming probability. The configurational free energy of the system, proportional to N, is a function of the depth (ϕ) and of the logarithmic multiplicity (σ) of basins and contains a vibrational contribution (G_{vibr}) due to intra-basin motions [11]:

$$G(N, V, T) = \phi - kT\sigma(\phi) + G_{vibr}(\phi, T) \tag{1}$$

The statistically more populated basins in this landscape identify the statistically relevant configurations of the solvent in geometrical space.

2.3. ENERGY LANDSCAPE OF WATER PERTURBED BY SIMPLE SOLUTES

We first consider the effect of perturbations due to a simple rigid solute on the landscape of the water subsystem. Due to interactions with the solvent and to excluded volume, a solute alters the solvent potential energy landscape and consequently the set of statistically populated basins, their depth, multiplicity and dynamical contribution. From (1) we see that this causes a variation of the free energy of the system ΔG_{SW}, referred to as hydration free energy. This free energy variation is due to the change in statistically relevant configurations of water, that is in the properties of the H-bond network. The water involved in these configurational changes is referred to as hydration water.

In the case of n rigid solutes close enough so that their hydration regions overlap [32], perturbations generated in the landscape are in general interdependent [14, 15, 19]. Thus, the overall free energy variation due to the presence of n solutes $\Delta G_{SW}(1, \ldots, n)$ is not the sum of the $\Delta G_{SW}(i)$ terms due to single solutes and is generally written as:

$$\Delta G_{SW} = \sum_i \Delta G_{SW}(i) + \sum_{i,j} \Delta G_{SW}(i, j) + \sum_{i,j,k} \Delta G_{SW}(i, j, k) + \ldots \tag{2}$$

Consequently, there is an effective force acting on each solute in water, exclusively due to the presence of the solvent (solvent induced force, or SIF), and attempting to rearrange the configuration of all solute elements so as to lower the overall free energy of hydration. This can be written as:

$$SIF = -grad\Delta G_{SW} \tag{3}$$

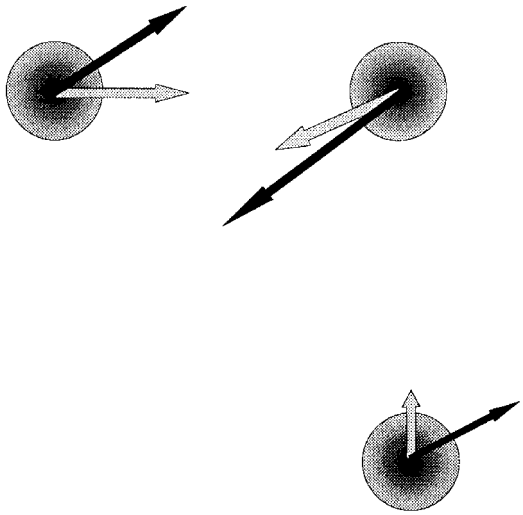

Figure 1. Non pair additivity of SIFs. Grey arrows represent SIFs acting on a given solute element, calculated from pair additivity, that is calculated as the vectorial sum of the two SIFs obtained in the presence of each of the other solute elements in the absence of the third. Black arrows represent actual SIFs obtained in the presence of all three solutes. Non additivity changes both direction and magnitude of SIFs. Direct interactions not shown. (Cartoon drawn from results in Bruge' et al., 1996 [14]).

where the gradient is calculated with respect to the solute coordinates. (Note, however, that in the case of direct interactions among elements of a complex solute the quantity to be minimized at equilibrium will be the overall free energy. Accordingly, in the equilibrium configuration the total force, and not SIFs, on each solute element will be zero). From a computational point of view, SIFs on each solute element in a given solute configuration can be obtained as the long time average of the sum of all forces exerted on it by each water molecule [12, 33].

Actually, contrary to KSA, third and higher order terms in (2) are comparable to second order ones. This causes high non-additivity of SIFs [14, 15, 19–21], visualized in Figure 1. Given the size of SIFs (often comparable to, or even larger than direct interaction forces), these non additive contributions are highly relevant to folding, recognition and conformational changes.

2.4. ENERGY LANDSCAPE OF THE PROTEIN+WATER SYSTEM

The global energy landscape of the protein+solvent system is a very complex one in a P+nN dimensional space. Nevertheless, the foregoing considerations concerning the case of the individual subsystems are of help in simplifying considerably this otherwise unmanageable problem. The relation to the case of simple solutes is given by the fact that 'solute elements' are now protein residues. Also, characteristic times of functionally relevant protein dynamics are (as already seen) far

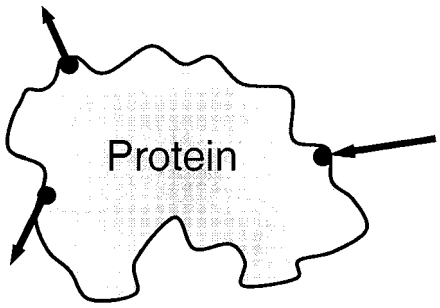

Figure 2. SIFs acting on three identical residues at different sites of a protein. A residue of a given type can behave as hydrophilic or hydrophobic, depending on its environment. Direct interactions not shown. (Cartoon drawn from results in Martorana et al., 1998 [20]).

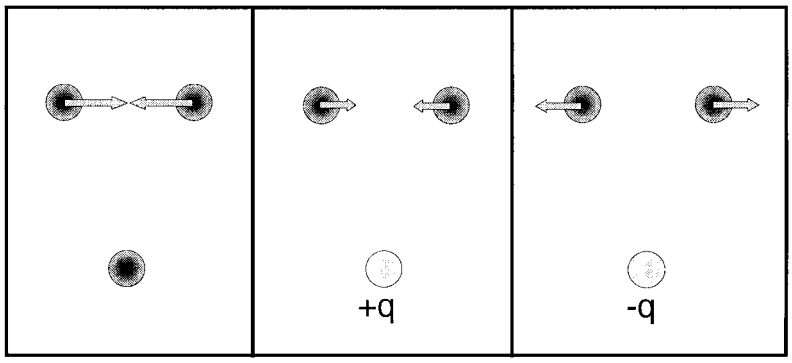

Figure 3. The cartoon, from results in Bulone et al. [28], shows how a charge sign dependent disruption of the hydrophobic attraction between two apolar solute elements is possible. Apolar elements of course have no direct interaction with a charge.

larger than those of water. It is therefore meaningful and necessary to average over solvent variables. This considerably reduces the dimensionality of the configurational space. As before, averaging changes the potential energy landscape (function of the nN+P variables) into a 'partial free energy' landscape (function of just the P protein variables), containing the inter-residue direct interaction potential (which can be taken to a large extent as pairwise additive), as well as the highly non additive solvent induced interactions ΔG_{SW}. This term is again expressed by (2), where i,j... now indicate different residues, or parts of them, and the implied thermodynamic average is of course computed for each protein conformation. Non pair-additivity of solvent induced interactions, expressed by higher order terms in (2) and illustrated in Figure 1, may now cause unexpected features such as shown in Figure 2. This illustrates that the effect of the context, that is of the configuration of all residues of the entire protein, can even cause a sign-reversal of the SIF acting on a particular residue, respect to that expected on the grounds of its hydrophobic/hydrophilic character [20, 15]. Strong effects of this type have

been shown to occur, already in simple cases, when electric charges are present [15, 27, 28]. Indeed, a charge sign dependent disruption of the so called hydrophobic bond is possible [28], as illustrated in Figure 3. A further consequence of the non pair-additive character of solvent induced interactions is a propagation of effects of local changes of solute elements on the overall hydration of the protein and on the related free energy [19]. This may link to the sizeable non-additivity of the functional effects of even distant point mutations [19, 34]. Another consequence of the collective nature and propagation of hydration effects is the time correlation of instantaneous SIFs acting on distinct and distant sites of a protein [21]. Finally, the role of solvent in the configurational energy landscape and related solute-solvent dynamical coupling is evidenced by recent computer simulations, showing that a strikingly protein-like energy landscape can emerge from interactions with the solvent in a very simple composite solute, having *in vacuo* a flat configurational energy landscape [35, 36].

In conclusion, on the small scale (single residue, or higher, resolution) solvent induced interactions are highly specific. This means that they cannot generally be predicted by cut-and-paste methods based on the individual character of surrounding residues. Rather, their inherently strong non-additivity makes them highly dependent upon the given, specific arrangement of all solute elements. In view of the high quantitative relevance of solvent induced interactions, such strong specificity would suggest that a correct theoretical approach to the protein folding problem is too complex to be manageable. The situation, however, improves considerably if we look at it on longer length scales, that is on scales encompassing many residues or proteins. When this is done, solvent induced interactions can be described in terms of their average values. Therefore, mean-field descriptions may become valid, as shown in the case of the R-T transition of hemoglobin [22, 37] and in other cases described below. The case of the R-T transition of hemoglobin covers special interest for at least two reasons: i) it has offered the possibility of a quantitative evaluation of the functional role of solvent induced interactions. Specifically, experiments have shown that in this case solvent induced interactions actually reverse the sign of the free energy change at the transition. Without this sign reversal, hemoglobin could not perform its oxygen transport function [22, 37]. ii) A further interest of this case lies in the fact that it concerns a number of protein residues sufficiently large to make mean-field approximation meaningful. In this approximation, concepts such as hydrophobic collapse [38–40] or folding with partially pre-formed secondary structure elements maintain their usefulness and meaning, except in unusual cases such as so-called chameleon proteins [41]. Proceeding down to shorter-scale details, again requires full consideration of solvent induced interactions and their strong non additivity and specificity. At this level, some insight can be gained from paradigmatic cases of model composite solutes such as those we have referred to as ‘morphemes’ [14]. For detailed refinements, however, resorting to detailed computations using explicit solvent appears inescapable.

3. Aggregation and Interactions of Proteins in the Solvent

We now consider the case of p proteins simultaneously present in the solvent. This would require considering the energy landscape of the system in the complete nN+pP configurational space. It is convenient to take such space as composed of independent inter and intra-protein coordinates. Again we may go from a purely potential energy landscape to a 'partial free energy' landscape, by averaging over fast variables, including solvent configurations.

In this multi-protein landscape each point corresponds to a given configuration of the overall system, including both intra-protein configurations (microscopic scale) and inter-protein configurations (mesoscopic scale), averaged over all configurations of the solvent and other fast variables. Minima in this space correspond to protein configurations that would be mechanically stable. Gradients of this 'partial free energy' with respect to the two sub-sets of variables (intra-protein and inter-protein) represent forces driving the system towards equilibrium states on the two length scales. The (nN averaged) partial free energy landscape in the pP dimensions makes clear how we can expect interactions between processes which occur on the two different length scales, such as folding-unfolding-misfolding and inter-protein cross-linking and, on a larger scale, solution demixing and supramolecular aggregation. The following example illustrates this possibility. Consider the p subspaces relative to each individual protein and suppose that a parameter change (e.g. a temperature rise) induces a conformational change of individual proteins away from their native state. In consequence of this change, the representative point of the individual protein will occupy a new region of its P-dimensional subspace, and the free energy will be expressed by the equivalent of (1). Extending our view so as to include inter-protein coordinates, however, may show that in this extended-dimensionality space the system is no longer in a free energy minimum, so that equilibrium can be reached for a different set of inter-protein coordinates. When this is allowed the situation can be such that a further decrease of free energy can be obtained by further displacements of the representative point of the system within each of the p subspaces. Such displacements will represent individual conformational changes of the p proteins. If so, of course, the process can continue so as to reach an even deeper minimum through a self-consistent interaction of intra and inter-protein readjustments. The simplest situation of this type is that of a protein conformational change giving rise (as sketched in Figure 4.a) to thermodynamic instability and demixing of the solution. The latter is a decomposition of the solution in mesoscopic regions of lower and higher concentration [42]. In higher concentration regions, a deeper free energy minimum can be reached by inter-protein cross-linking/ gelation/ aggregation/ coacervation, as well as by further conformational changes, as sketched in Figure 4.b. Early experiments already showed the occurrence of processes of this type [43–45], as confirmed by extended work on several systems [23–26, 46–49]. Such experimental data of aggregation prompted by demixing obey, in the part that concerns demixing, the mean-field

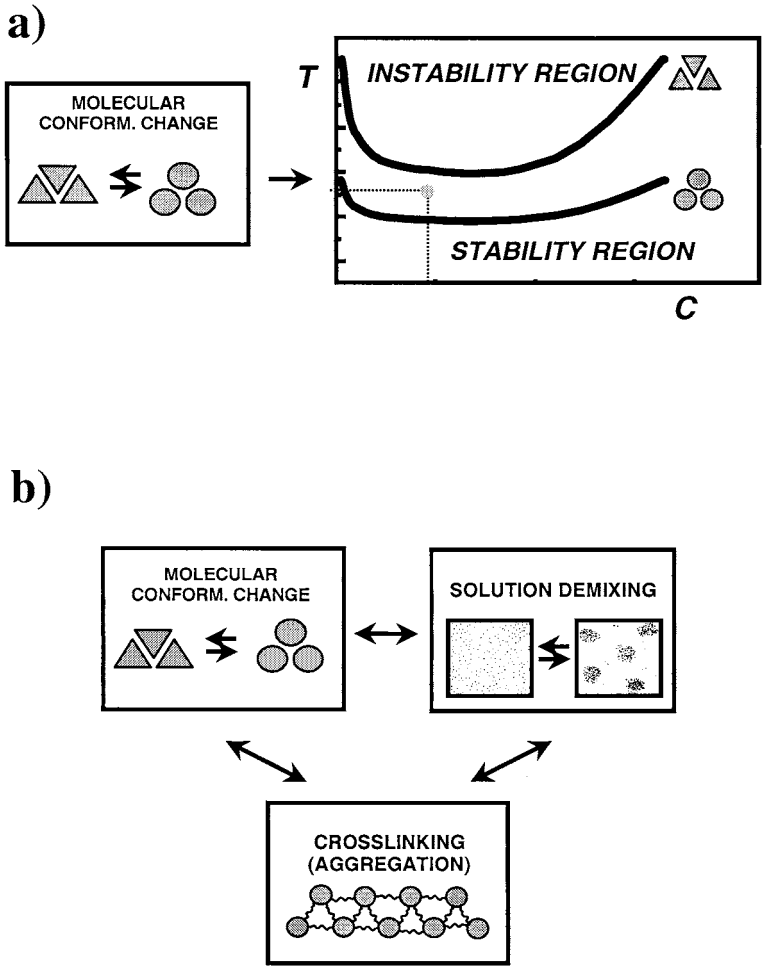

Figure 4. **a)** A protein conformational change (from triangles to circles in the cartoon) can shift towards lower (or higher) temperature the region of thermodynamic stability, where the system is stable as a homogeneous solution; consequently, due to the sole change of protein conformation, the representative point of the system may now be in the instability (demixing) region, although both concentration and temperature have remained unchanged. **b)** Inter-protein aggregation is due to the interaction of different processes. For example, a conformational change of individual proteins can lead to instability and demixing on the mesoscopic scale. Crowding of proteins, due to demixing, causes crosslinking which, in turn affects single protein conformation.

theory that describes the interplay between solute-solute vs. solute-solvent interactions. Fitting of experimental data allows thus sorting out the role of solvent on this mesoscopic scale. On the other hand, details of further conformational changes induced by demixing and cross-linking/ aggregation involve the high specificity and context dependence of SIFs, which of course adds to that of direct interactions.

Solvent induced interactions are therefore highly relevant to processes concurring in protein aggregation both in their mean-field aspects and in their high-specificity and context dependent aspects, according to the length scale. For a comprehensive view of phenomena over the different length scales it is important to keep in mind that the landscape considered is obtained by adding to an energy term a free energy term coming from thermodynamic averaging over all solvent configurations, for each protein conformation. In this averaging there is no distinction between intra and inter-protein configurations and therefore the size and relevance of solvent induced interactions on the two scales is expected to be the same. This is confirmed by experiments, as recalled above.

4. Conclusions

We have sketched a description of intra and inter-protein interactions in the solvent in terms of a comprehensive energy landscape in the nN+pP dimensions, including those relative to proteins and to the solvent. Thermodynamic averaging over fast variables somewhat simplifies this otherwise unmanageable problem. As a consequence of thermodynamic averaging, a free energy term adds to the potential energy, and brings from the nN+pP dimensional space to the reduced pP dimensional space. This additional solvent induced free energy arises from the overlap of perturbations of the statistically relevant solvent configurations by solutes. The size of such term is comparable to or even larger than that of direct interactions, that is of interactions occurring also *in vacuo*. Such solvent induced interactions and related forces (SIFs) exhibit strong non-pair additivity and consequent strong specificity in their context dependence. These features are exquisitely related to the peculiar structure of the water molecule and to the associated great variety of statistically relevant configurations of the water H-bond network, and would be considerably less relevant with simple solvents.

Strictly, on the small scale (single residue, or higher, resolution) full consideration of the solvent molecular structure and of the strong non-additivity and specificity of solvent induced interactions is required. However, a sufficiently long MD simulation of a protein, in a full-atom and explicit solvent model appears so far as a kind of 'holy grail' [50]. Yet, simplifications are possible if we look at longer length scales, encompassing many residues or proteins. In this case, solvent induced interactions can often be described in terms of their average values, and mean-field treatment becomes valid. In this approximation, concepts such as hydrophobic/hydrophilic interactions, hydrophobic collapse, folding with partially preformed secondary structure elements and similar ones maintain their validity and meaning, with only some *caveats*. At single residue (or all-atom) resolution, insights may be gained from the study of simple, yet meaningful models (or 'morphemes' [14]), capable of capturing essential features of solvent induced interactions and related SIFs in a given solute configuration. A particularly useful result with such types of models is the large charge sign-dependent modulation of hydrophobic interac-

tions by electric charges, where effects are particularly strong and of high potential relevance to biological processes such as protein folding, chaperonin action, signal transduction, and energy conversion.

Finally, the fact that solvent induced interactions act on both microscopic and mesoscopic length scales (as defined above) brings to the interaction of processes concurring in aggregation on the two length scales. This interaction is easily visualized in the extended nN+pP dimensional space that we have considered, and can have far reaching consequences.

Summing up, on one hand taking into consideration interaction with explicit solvent brings to further complexity; on the other hand the conceptual tool of thermodynamic averaging over fast variables somewhat simplifies the situation. On the detailed microscopic scale the high specificity of solvent induced interactions and related SIFs, and their long range effects cannot be neglected. In fact, they are apt to elicit features and interactions of potentially high biological interest, unpredictable on the simple pair-additivity basis. We may wait for the 'holy grail' [50] of sufficiently long full-atom, explicit solvent simulation studies of protein folding. In the meanwhile 'morphemes' of solvent induced interactions in simpler model systems can be inspiring for devising realistic models, perhaps through the use of intermediate approximations, or of some 'knowledge based' non-additivity feature.

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