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Inversion of the Hydrophobic/Hydrophilic Paradigm Demystifies the Protein Folding and Self-Assembly of Problems

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Abstract The idea that the hydrophobic effect is the major driving force for processes such as protein folding and protein-protein association has prevailed in the biochemical literature for over half a century. It has recently become clear that the evidence in favor of the hydrophobic paradigm has totally dissipated. The dominance of the hydrophobic effect has been reduced into nothing but a myth. On the other hand, the new paradigm based on a host of hydrophilic effects has emerged. This new paradigm offers simple and straightforward answers to the long sought problems of protein folding and protein-protein association.

Keywords: *proteins, hydrophobic, hydrophilic, solvation, folding, association*

1. Introduction

Open any textbook of biochemistry, biophysics or molecular biology, look at the index for the entry "hydrophobic effect," and you are likely to find a statement claiming that the hydrophobic effect (or bond, or interaction) is the most important driving force in some biochemical processes such as protein folding and protein-protein association. Here are two quotations from recent textbooks:[1,2]

"An important non-covalent force that causes a polypeptide to fold into its native conformation are the hydrophobic interaction forces…Hydrogen bonds…although they contribute to the thermodynamic stability of protein's confirmation, their formation may not be a major driving force for folding." Delvin (2006)

"The hydrophobic effect, which causes non-polar substances to minimize their contacts with water, is the major determinant of native protein structure…hydrogen bonds, which are central features of protein's structures, make only minor contributions to protein stability." Voet et al. (2008)

Most authors will not bother to present the evidence for such extraordinary statements. Instead, they will refer the reader to the relevant literature. Most readers will not bother to look at the relevant literature, and if they do, they will find arguments that they cannot comprehend and will accept the textbook's statements on faith.

A few authors will present the "evidence" for the importance of the hydrophobic effect. Briefly, this evidence can be summarized in two sentences;

(i) The Gibbs energy of transferring non-polar molecules from water to an organic liquid is known to be large and negative.

(ii) It is known that most non-polar groups of the protein are found in the interior of the native structure.

These two experimental facts are more than enough to convince the reader that in the process of protein folding the "burial" of the hydrophobic groups in the interior of the protein provides the "driving force" for the process of folding.

A few authors will also venture in "explaining" the molecular origin of the hydrophobic effect. Citing the classical paper by Frank and Evans [3], the following two statements are made:

(iii) The Gibbs energy of transferring a non-polar molecule from an organic liquid to water is "entropy driven."

(iv) The large negative entropy change in the process of solvation of a non-polar molecule in water is due to "ordering" or to "iceberg formation" around the non-polar solute.

From the last two statements one can easily conclude that the "ordering" of water molecules by a non-polar molecule *explains* the large positive Gibbs energy of solvation, hence also the source of the large "driving force" for the process of protein folding.[4,5].

The purpose of this article is to show that although statements (i), (ii) and (iii) are correct [4], and statement (iv) may be justified theoretically [4], the conclusions derived from these statements are not warranted. This finding is sufficient to demolish the myth that the hydrophobic (HϕO) effect is the most important factor in biochemical processes. Instead, a new paradigm, based on a rich repertoire of hydrophilic (HϕI) effects has emerged. The HϕI effects provide both *strong interactions* as well as *strong forces* (though the *forces* are derived from the *interactions*, these two are different concepts and should be used in different context). The HϕI forces offer a simple and straightforward answer to the long sought

problem of how protein folds into a precise 3-D structure in a relatively short period of time. The HϕI *interactions* provide a sound explanation of the stability of the 3-D structure of the proteins as well as the stability of aggregates of macromolecules.

2. The Fall of the Hydrogen Bond (HB) Dominance and the Rise of the of the Hydrophobic Effect (HϕO) in Protein Folding

A convenient point to begin with is Pauling's book "The Nature of the Chemical Bond [6,7]. In the first two editions of the book, Pauling discussed the HB, without mentioning proteins or nucleic acids. In the second edition, the chapter on HBs ends with some estimates of the HB energies and HB distances. The third edition contains two new sections on HBs in proteins and HBs in nucleic acids.

Following the works of Pauling and his collaborators, the role of HBs in stabilizing the native form of proteins became well established. The HBs, with bond energies of the order of 24 kJ/mol, which provided explanation for many anomalous properties of water, also took over the main "cohesive forces needed for the organization of native proteins" [8].

The first blow to the HB dominance came from the realization that though the HB *energy* is of the order of 24 kJ/mol, its formation in aqueous solution must have a negligible effect on the "driving force" for the process of protein folding. The argument apparently started with the work of Schellman [9,10], summarized by Kauzmann [11] and eventually was encapsulated in Fersht's HB-inventory argument [12]. The argument is simple, straightforward and convincing (and as we shall soon see, it is also wrong). Write the stoichiometric reaction between a donor and an acceptor of a HB in the form

$$E-C=O \quad w+w \quad HN-S \rightarrow \\ E-C=O \quad HN-S+w...w \qquad (1)$$

where ***E*** and ***S*** stand for an enzyme and a substrate, respectively, but can be any two molecules or parts of the same molecule, and *w* is a water molecule. Equation (1) suggests that in the process of formation of a *direct* hydrogen bond between a donor (here amine group) and an acceptor (here a carbonyl group), *two* HBs are *broken* on the lhs of the equation and *two* HBs are *formed* on the rhs of the equation. Therefore, ignoring the differences in the various HB energies between the various pairs, we can conclude, by simply *counting*, that the net effect of the formation of a direct HB is negligibly small. As Kauzmann summarized this argument:[11]

"Hydrogen bonds, taken by themselves, give marginal stability to ordered structures, which may be enhanced or disrupted by interactions of side chains."

This conclusion has been reiterated by numerous authors including Delvin [4] and Voet *et al.* [2] as quoted in the introduction.

If HBs do not provide the main "driving force" for protein folding, what factors do provide those "driving force?" [4,5] This apparent conceptual "vacuum" was filled by the HϕO effect in 1959 [11]. The HϕO effect was known long before Kauzmann applied it to the problem of protein folding [8]. It was applied successfully to explain surface tension of certain aqueous solutions of organic molecules, micelle formation and membranes. All these phenomena involve molecules having two moieties; a hydrophobic part, which "fears" water and tries to avoid it, and a second, which "loves" water and mingles with water comfortably. As Tanford and Reynolds quoted from a personal communication with Kauzmann, the idea of the HϕO effect was hovering "in the air" for long a long time [8].

In his review article "Some Factors in the Interpretation of Protein Denaturation," Kauzmann applied the idea of the HϕO effect to protein folding [11]. For this purpose he coined the term HϕO-bond, and speculated that this "bond" could be a major factor in the stabilization of the native structure of protein. (At that time the concept of HϕO effect was vague, and not well defined. It was only later that a systematic definition and distinction between different HϕO effects was suggested [13]).

Kauzmann's idea was very simple and convincing. It was known that the Gibbs energy of transferring a small non-polar solute such as methane or ethane, from water into an organic liquid involves a large negative change in Gibbs energy. This is the same "driving force" that drives the formation of micelles and membranes in aqueous solutions. Kauzmann also noticed that about one third of the side-chains of a typical protein are HϕO, and most of these find themselves buried in the interior of the folded protein. If the process of transfer, Figure 1a, is used to represent the process of transfer of a side chain, Figure 1b, then we can estimate that a protein of about 150 amino acid has about 50 HϕO groups, and if each of these contributes between -12 and -16kJ/mol, then we get a very large "driving force" for the folding process.

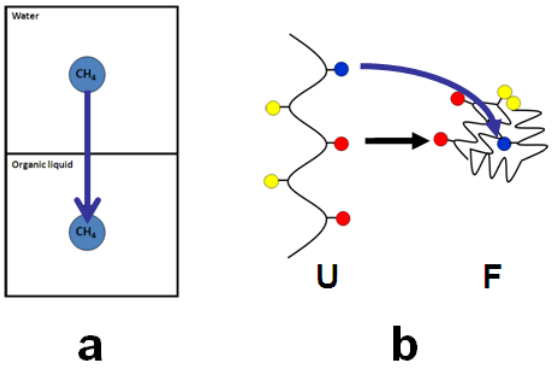

Figure 1. (a)The process of transferring a methane molecule from water into an organic liquid. (b) The process of transferring a methyl group (blue disk) attached to a protein from water into the interior of the protein

Kauzmann's idea was bold and brilliant and had captured the imagination of many scientists. It is not surprising therefore, that the dominance of the HϕO effect has prevailed for over half a century. The fact that HϕO groups are in the *interior* of the *protein*, and the fact that the transfer of HϕO molecules from water to an organic liquid is large and negative are undeniable. The former lends credibility to Kauzmann's model, while the latter provides the large negative Gibbs energy change.

It is not surprising that the usage of the HϕO effect has flourished in explaining many biochemical processes such as protein folding, protein-protein association and molecular recognition. Very few have questioned the validity of Kauzmann's model for the process of protein folding, or the validity of its explanation based on Frank and Evans' ideas of iceberg formation. The HϕO paradigm seemed to have been unassailable, and became the central dogma in the biochemical literature [5,13].

3. The Fall of the Hydrophobic (HϕO) Effect and the Rise of the Hydrophilic (HϕI) Effects

The title of this section might seem to most biochemists to be misplaced and overly exaggerated. After no recent textbook even mentions that there are cracks in the HϕO dogma, let alone its "fall." And what are the HϕI effects? These are unheard of. Everyone had already been convinced that hydrogen bonds (HBs) contribute insignificantly to the stability of the native structure of the protein. Nevertheless, some murmurs of doubts have been heard during the past twenty years.

In 1980, in the preface of my book "Hydrophobic Interactions," I wrote: [13]

"In spite of my researches in this field over almost 10 years, I cannot confirm that there is at present either theoretical or experimental evidence that unequivocally demonstrates the relative importance of the HϕO interactions over other types of interactions in aqueous solutions."

My doubts were based on *lack* of *evidence* in favor of the contention that the HϕO effect is the *most important* effect in the "driving force" for protein folding. How can one claim that one factor is more important, or most important when one does not have a full *inventory* of *all* the factors involved in protein folding? Remember that Kauzmann's paper was on "*some factors* in the interpretation of protein denaturation" – not on *all factors* involved. No one knew what were *all the factors* especially those that are solvent-induced. The only factor that could have competed with the HϕO effect was the HB, but the HBs were already deemed to be unimportant in aqueous solutions.

Kauzmann's model of inference from transferring of molecules from water to organic liquid, and the fact that most HϕO groups are found in the interior of the protein were so convincing that the mere expression of doubts could not have rattled the dominance of the HϕO dogma. One needs more than doubts. One needs facts! "Lack of evidence" for an idea cannot be used as evidence against that idea.

This was the main motivation for the examination of the entire question of the solvent-induced effects on the protein folding and protein-protein association that I undertook late in the 1980s [14,15,16,17,18,19]. The results of this examination were stunning; initially to me, then slowly diffusing into the literature.

First, came the realization that the HB-inventory argument was fundamentally faulty [17]. Second, Kauzmann's model, appealing as it was, for over 50 years was found irrelevant to the protein folding process [14,15,16,17,18,19]. Finally, a logical pitfall: The fact that HϕO groups are found in the interior of the protein cannot be used as an argument in favor of the role of the HϕO effect in protein folding.

I shall briefly explain here in qualitative terms each of the abovementioned findings. More can be found in references 4 and 5. The HB-inventory argument was based on the stoichiometric equation (1). The first serious challenge to the HB-inventory argument was expressed in 1990 [17]. It was shown that the very writing of the stoichiometric equation in the form (1) is faulty for two reasons:

1. What is lost on the left hand side are not HB *energies* but *solvation Gibbs energies* of the HϕI groups. On the other hand, a genuine HB energy is gained on the right side of (1).

2. Whatever the water molecules do when they are released from the solvation sphere of the two HϕI groups is irrelevant to the driving force. These water molecules "flow" from the solvation sphere into the pool of water at constant chemical potential. Therefore, they cannot contribute anything to the driving force. This argument has demolished the foundations on which the HϕO has risen.

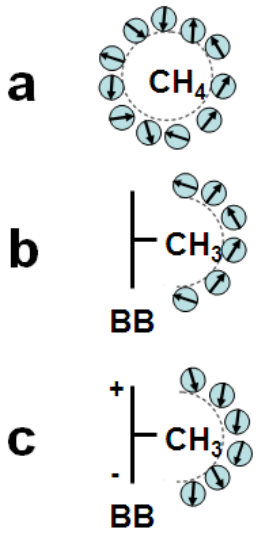

Figure 2. (a) Solvation of a non-polar molecule in water. (b) onditional solvation of a non-polar group attached to non-polar backbone. (c) onditional solvation of a non-polar group attached to polar backbone

Second, the analysis of *all* the solvent-induced factors revealed that Kauzmann's model *does not* feature in the "driving force" for the process of protein folding [15,16,17,18,19]. Instead of the Gibbs energy of *solvation* of a HϕO molecule in water, the *conditional solvation* Gibbs energy of a HϕO group features in the "driving force." These Gibbs energies are very different from the Gibbs energies of solvation in water. The difference could be one or two orders of magnitudes. The main reason is that a HϕO group attached to the backbone (BB) of the protein is surrounded by water molecules which are perturbed by the BB. Figure 2b. On the other hand, the solvation of a HϕO molecule is by unperturbed water molecules, Figure 2a. Thus, at this stage not only the basis on which the HϕO model was built upon was demolished, but the HϕO - model itself was now shown to be inadequate [4,5].

It is unfortunate that despite these findings people still propagate the non-evidential "evidence" in favor of the HϕO effect.

In a recent review, Dill *et al* write: [20]

"There is considerable evidence that hydrophobic interactions must play a major role in protein folding. (a) Proteins have hydrophobic cores, implying nonpolar amino acids are driven to be sequestered from water. (b) Model compound studies show 1-2 kcal/mol for transferring a hydrophobic side chain from water into oil-like media , and there are many of them. (c) Proteins are readily denatured in nonpolar solvents.(d) Sequences that are jumbled and retain only their correct hydrophobic and polar patterning fold to their expected native states…, in the absence of efforts to design packing, charges or hydrogen bonding."

Unfortunately, *none* of these can be used as *evidence* in favor of the role of hydrophobic interaction in protein folding. (a) The fact that hydrophobic groups are found in the interior of the protein does not necessarily mean that the hydrophobic interactions are *responsible* for bringing these groups to the interior of the protein. Such a conclusion is an illusion and cannot be supported by theory. It is similar to the conclusion that the mixing of two ideal gases is the *cause* of the entropy increase upon mixing, or that the "entropy of mixing" is the "driving force" for the process of mixing [21]. Similarly, one cannot say anything about the "driving force" for protein folding merely by watching the hydrophobic groups occupying the interior of the protein. (b) The Gibbs energies of transfer of small model compounds from water to an oil-like media were shown to be irrelevant to the driving force in protein folding [4,5]. (c) The fact that proteins are readily denatured in non-polar solvent means that water is important. It says nothing on the relative importance of hydrophobic vis-à-vis hydrophilic effects. (d) The last "evidence" is anything but evidence in favor of hydrophobic effects. It says nothing even on the role of water, certainly nothing about the relative importance of the hydrophobic vis-à-vis the hydrophilic effects.

It is regrettable that these non-evidence appear in the literature almost twenty years after the strong evidence in favor of the HϕI effects was published!

The evidence in favor of the HϕI effects are overwhelming. First, it was shown that direct HB in aqueous solutions is not insignificant, as believed [17]. Furthermore, the analysis of the solvent-induced effects on protein folding has also opened the door to a host of new solvent-induced effects that were never considered before. These effects involved HϕI rather than HϕO groups. The most important one, and so far the most studied, was the pairwise HϕI interactions between pairs of HϕI groups at a distance between 4-5Å. For this particular HϕI interaction there is overwhelming evidence that it is far stronger than any of the HϕO effects. The evidence comes from theoretical estimates [4,5], simulations [22,23], and experimental data [4,5,24]. This HϕI effect was estimated to contribute about -12kJ/mol, a far larger contribution compared with the corresponding HϕO effect. It should be noted that the number of pairs of HϕI groups in any protein is much larger than the number of HϕO groups.

The qualitative reason for such a strong HϕI interaction at this particular configuration, is shown in Figure 3 where the two HϕI groups *can* be bridged by a water molecule. It should be stressed however that this effect is not due to a *formation* of long-lived HB-bridge, as some have misunderstood. Such a "permanent" bridge could provide two HB *energies*, i.e. about -48 kJ/mol. The real effect is a *mutual solvation* of the two HϕI groups. This effect involves HB energy, but also involves probability of finding a water molecule that can form a HB-bridge between the two HϕI groups. The most direct evidence for the existence of such a HϕI effect is the second peak in the radial distribution function of pure liquid water [4]. Other experimental evidence comes from the relative solubilities of two isomers of the same molecule, having two HϕI groups at two different distances [5]. We have mentioned only one HϕI effect. Other effects involving simultaneous solvation of three or more HϕI groups were found to be even stronger, albeit perhaps less frequent [5].

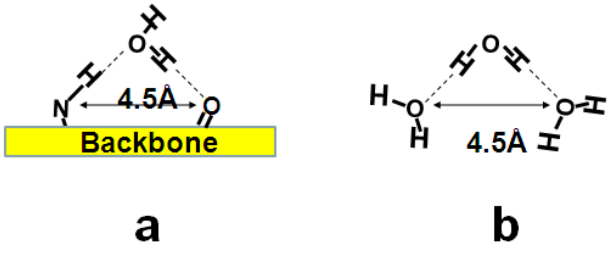

Figure 3. Two HϕI groups at a distance of 25A (a), can be bridged by a water molecule. This is the same distance as the second nearest neighbors in ice (b)

4. The Problem of Protein Folding and Its Answer

There are several problems that are associated with the process of protein folding. Some aspects of the problem such as the existence of a code that translates from a sequence of amino acids into a 3-D structure, or the existence of a global minimum in the Gibbs energy landscape were inspired by Anfinsen's thermodynamic hypothesis [25,26]. These aspects were discussed in reference 5. Here we shall discuss the question that was raised by Levinthal [27,28] and later formulated by the editors of Science [29]. Levinthal made a quick estimate that if the protein were to move at random in its configurational space, it would take eons to reach the native 3-D structure. This question was chosen as one of the 125 "big questions" of Science [29,30,31].

"Can we predict how proteins will fold? Out of near infinitude of possible ways to fold, protein picks one in just tens of microseconds."

There are essentially two questions in this quotation. One, is the question of prediction of the 3D proteins. In my opinion there is no general answer to this question [31]. The second, is the question about the *speed* of the folding process. A hint to the solution to this question was already provided by Levinthal: [27,28]

"We feel that protein folding is speeded and guided by rapid formation of local interactions." Levinthal was almost right in his speculative answer. He did not specify what are the "local interactions" that "speeded and guided" the protein in its folding pathway. The answer to

the question of *how* and *why* protein folds in a relatively short time is simple and straightforward: a protein will fold, if it folds, in a short time because at each stage of the folding process strong *forces* are exerted on the various groups along the protein. The main question is not how and why proteins fold, but rather what the strong forces are that *force* the protein to fold in a narrow range of pathway? This question could not be answered within the HϕO paradigm, simply because the HϕO effect could not deliver strong forces.

It is ironic that in a review article entitled "Dominant Force in Protein Folding" you cannot find a word on real *force*, and certainly nothing about dominant forces [32]! The situation has radically changed once strong HϕI forces - *real forces*, the existence of which was not even suspected - were discovered. Recognizing the existence of strong HϕI forces rendered all the speculations about the shape of the Gibbs energy landscape superfluous. One does not even need to mention the Gibbs energy landscape in connection with the problem of protein folding. The *general* problem of how and why protein folds is now answerable. There remains the question however of the implementation of this general answer to *specific* proteins. In my opinion this should not pose any insurmountable difficulty. At each initial configuration of the unfolded protein, one can calculate the total *forces*- both direct and solvent-induced forces exerted on all the groups of protein. The resultants of these forces should "speed and guide" the protein into its folding pathway.

5. The Problem of Protein-Protein Association and Its Answer

In the editorial of Science in 2005, one finds another "big question" in science: [29,30]

"How do proteins find their partners?" Protein-protein interactions are at the heart of life. To understand how partners come together in precise orientations in seconds, researchers need to know more about the cell's biochemistry and structural organization.

Again, this question could not be answered within the HϕO paradigm. It was therefore deemed to be one of the "big questions" of Science. Indeed, the process of protein-protein association to form a stable and long lived dimer (or higher aggregate) is not less of a mystery than the process of protein folding [5].

In forming a stable dimer, one monomer loses its translational entropy. This is true for any association process. In most cases of dimerization a chemical bond is formed between the monomers. The energy of the bonds compensates for the loss of translational entropy making the dimerization process favorable. In protein association there is no covalent bond between the monomers. Hence the mystery: What is the factor that compensates for the loss of translational entropy? In some textbooks, you might find the answer that the HϕO effect is the dominant factor in this process. However, a careful examination of the process shows that this factor is minor and cannot account for the stability of the dimer (or of any other aggregate for that matter) [5]. On the other hand, the inclusion of the HϕI effect shows how an extremely unlikely process of dimerization turns into an extremely likely process. The details of the HϕI contribution to the process of association may be found in reference 5. Here, we provide only a qualitative description of the basic idea.

Consider two globular proteins at contact. Clearly, the direct interaction between the two monomers is minor. The Gibbs energy for this process will be large and positive. Next, we take into account the solvent-induced effect. We start with completely HϕO monomers, i.e. proteins on the surface of which consists of HϕO group. We find that in this case the Gibbs energy of the process of dimerization remains large and positive. This means an unfavorable dimerization process.

Next, we gradually increase the mole fraction of HϕI groups on the surface of the protein. Figure 4 shows the dependence of the Gibbs energy of dimerization as a function of the radius of the protein for several mole fractions of the HϕI groups. We see that once the mole fraction of the HϕI group is larger than about 0.4 or 0.6 the curve turns into a negative territory, i.e. making the process of dimerization favorable. The larger the mole fraction x_0 the more negative the Gibbs energy change.

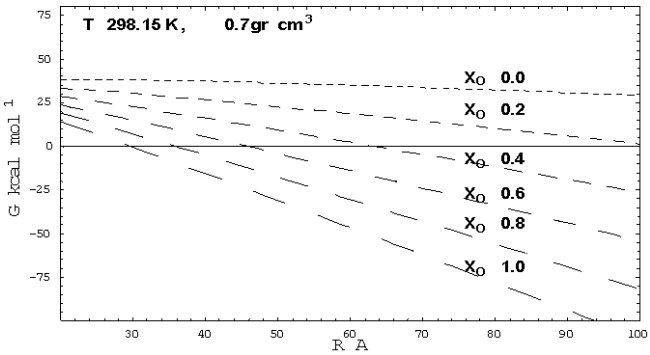

Figure 4. The dependence of the standard Gibbs energy of dimerization of two globular proteins as a function of the radius of the protein, for different values of the mole fraction of the hydrophilic groups

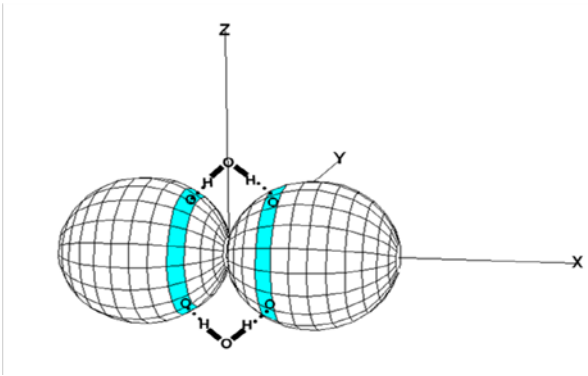

Figure 5. Water-bridges connecting two globular proteins

On the molecular level the reason for the large negative Gibbs energy of dimerization is simple to visualize. When the two proteins are in contact, there exists a strip on the surface of each monomer from which water-bridges HϕI groups may be formed. Figure 5. It was estimated that each water-bridge between two HϕI groups can contribute about -11kJ/mol. This effect is almost an order of magnitude larger than the corresponding HϕO effect. Clearly, as the proteins become larger the number of HϕI groups that can be bridged by water molecules become larger too. As can be seen from Figure 4. For proteins of radius of about 50Å the Gibbs energy of dimerization

becomes large and negative. As can be seen from Figure 4 the Gibbs energy changes become more and more negative for larger proteins. This finding solves the mystery associated with the question of the stability of an assembly of proteins.

6. The Problem of the High Solubility of Globular Proteins

The high solubility of globular proteins is another mystery that did not get sufficient attention in the literature. The reason the solubility of protein was not included in the list of "big questions" of science is simple. Everyone who has given a thought about the question regarding the reasons for the large solubility of globular proteins, correctly concluded that the hydrophilic groups on the surface of the proteins are responsible for its solubility. What was not known is that it is not the mere *number* of hydrophilic groups exposed to the solvent that determine its high solubility, but the specific distribution of the hydrophilic groups on the surface of the protein [4,33]. It was recently shown that the possibility of formation of water-bridges between two or more hydrophilic groups contributes significantly, if not decisively to the large solubility of the protein [4,33].

7. Conclusion

For over 50 years the HϕO -paradigm reigned supreme in the biochemical literature. The HϕO -paradigm has thrived not on evidence but on faith on one hand, and lack of alternatives, on the other. This situation has radically changed when a host of new HϕI effects were discovered and shown to be much stronger than the corresponding HϕO effects. The resulting change in paradigm from the HϕO to HϕI has not only demystified many biochemical processes, but in fact provided answers to long sought problems such as the process of protein folding and self-assembly of proteins [5,31].

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