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RESPONSE

Response to comments on my article: Levinthal's question revisited and answered. Ben-Naim, A. (2012), Journal of Biomolecular Structure and Dynamics, *30,* 113–124

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General comment

Reading through the articles that were written in response to my article (Ben-Naim, 2012a), I felt a deep sense of satisfaction that the points I raised in my article were largely understood and accepted. There were a few exceptions, however. A few authors misunderstood my main message. I hope that my response will clarify the misunderstanding. A more detailed discussion is also provided in the two monographs: Ben-Naim (2011) and Ben-Naim (2013).

Authors: Aditya Mittal and Chanchal Acharya

I of course agree with these authors that hydrophobicity is not a "force". However, I do not agree with the statement that:

> Hydrophobicity simply appears to be an interaction as a result of exclusion by water, that too given the right physical parameters (e.g. minimal concentrations of solutes, presence of non-aqueous interface, etc.).

This is a too simplistic view of "hydrophobicity", and far from being complete.

The authors state,

> Ben-Naim's voluntary dismissal or simple unawareness of the discovery of the importance of stoichiometry of amino acids in folded proteins

I did not dismiss, nor am I unaware of the stoichiometry of amino acids … I simply do not think this is relevant to the subject of my article. Nothing in my article depends on or is derived from the suggested "stoichiometry" of amino acids. Therefore, I did not mention this in my article, nor do I feel it is necessary to mention it even after reading the extended comments by these authors.

Finally, these authors write:

> Thus, unfortunately, Ben-Naim (2012a) becomes another addition to the vast body of literature that has failed to quantitatively address Levinthal's question in a manner even remotely close to the elegant calculation known as the Levinthal's paradox.

This statement is quite unfortunate. My article had nothing to do with the Levinthal's paradox. This (non) paradox was discussed separately in another article [Ben-Naim (2012b), and in a recent monograph Ben-Naim (2013)]. This is precisely the reason why I titled my article "Levinthal's question" and not "Levinthal's paradox".

The distinction between the two was made clear in my article, which was unfortunately missed by these authors.

Authors: Jihua Wang, Jiafeng Yu, Liling Zhao, Guodong Hu and Zanxia Cao

In this article, the authors start by making several unfounded statements:

> The energy landscape picture replaces the pathway concept of sequential events with the funnel concept of parallel events and emphasizes the ensemble nature of protein conformations, which derives from advances in both experiment and theory.
>
> It has provided a powerful conceptual framework to rationalize protein folding and unfolding.

Unfortunately, the *energy landscape does not* replace pathways, and it does not provide *any*, let alone

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"powerful", framework to rationalize protein folding. The energy landscape is simply a *function*. As I have discussed in my article and in my book [Ben-Naim (2013)], the study of the entire energy landscape, or the more relevant GEL makes a difficult problem immensely more difficult.

Thus, the claim that:

> The concept of free energy landscape has promoted much of the recent progress in understanding the process of protein folding.

is really an empty claim. The free energy landscape did not promote any understanding – in fact, it cannot, in principle, explain anything.

Furthermore, these authors overlooked my criticism of the "dominance of the hydrophobic effects" and simply repeated the "evidence" in favor of the hydrophobic effect in protein folding, which I have refuted one by one in my article (and in more details in Ben-Naim, 2013).

Author: R. Nagaraj

"Is Levinthal's question answered after a revisit?"

I am very glad that the author agrees with me

> that nature did not solve the protein folding positions (PFP). I think it did not even attempt it! In my opinion, evolutionary arguments cannot provide insights into the PFP.

I am also gratified with the statement

> It is gratifying to note that Ben-Naim concludes in section 3.2 that the Gibbs energy landscape (GEL) approach does not help in solving the PFP and only makes the PFP more difficult than necessary.

By the way, this is exactly an opposing view to the one expressed by the previous authors.

Regarding hydrophilic interaction, the author states that "He was not forceful in his argument as in the article commented upon (Ben-Naim, 2012a)".

Indeed, in my article, I did not elaborate on all the evidence in favor of the hydrophilic effects. This is done in both of my recent monographs Ben-Naim (2011, 2013).

Authors: Paul S. Agutter

I agree with the statement

> A little surprisingly, he does not exploit his earlier work on the importance of water (as solvent) in the thermodynamics of polymer-polymer binding (Ben-Naim et al., 1990, Ben-Naim, 2002), which is highly relevant to protein structure and to the overall organization of the intracellular milieu; the ideas developed in those earlier papers seem wholly consistent with the proposed dominance of hydrophilic interactions in three-dimensional protein structures

My answer to this comment is the same as my answer to the previous author. I have dealt with the evidence in favor of the hydrophilic effects in my monographs (Ben-Naim 2011, 2013).

Regarding the question of the role of chaperon, I agree with the author's comment. I did not *ignore* the role of chaperons, I simply focused only on those proteins that fold spontaneously *in vitro* without the presence of chaperons.

Author: Rajendra R. Joshi

This author starts by stating that "Today, the landscape and ensemble concept is well accepted to explain the process of protein folding".

As I said before, the "landscape" and "ensemble" concepts are meaningful concepts. They are useful in *formulating* the problem of protein folding, but they do not *explain anything*. In principle, these concepts cannot explain anything, as much as concepts such as "free energy" and "statistical mechanics" by themselves cannot explain anything.

Another apparent misunderstanding of my article is embodied in the statement

> The author also challenges the theory of a funnel-shaped GEL and suggests an 'alternative' process of folding and describes the same page on page 123 (column 1, last para). Reading through the description, it appears that this paragraph essentially describes the well accepted theory of funnel-shaped GEL

No, I have not accepted the "well-accepted theory of funnel-shaped GEL". In fact, I do not think the "funnel theory" has any basis.

I also do not agree with the conclusion of the author that:

> If one has understood the principles of protein folding, one must be able to design a new protein with a new function.

One can fully understand the principles of protein folding – one can also *design* a new protein, but one cannot guarantee that this designed protein will fold to any structure. The arguments are explained in Ben-Naim (2011, 2013).

Author: Robert Bywater

Generally speaking, I agree with the author's statements. There is one point that I wish to clarify. I agree that: "So, hydrogen bonds are the dominant forces in protein and other macromolecular structures". However, I wish to point out that one must make a clear-cut distinction between *direct* intramolecular hydrogen bonding and various hydrophilic interactions. More details on this are in my monographs (2011, 2013).

Authors: Ricardo Graña-Montes and Salvador Ventura

I fully agree with most of the statements made by these authors. I have a comment though regarding a statement made by the authors:

> There is little doubt that the tight packing of the hydrophobic core plays a key role in the stability of proteins by providing ….

Indeed, there is no doubt about the role played by the hydrophobic core. The question I raised is that this hydrophobic core is not necessarily the dominant effect in protein folding. I believe that various hydrophilic interactions are far more important.

Authors: Jayanth R. Banavar and Saraswathi Vishveshwara

I do not agree with most of the statements of these authors. For instance, I do not agree with the statement that:

> The experimental observation that a given protein or sequence of amino acids folds reproducibly and rapidly into a specific native fold is encapsulated in the folding funnel picture.

I also do not understand the statement

> The folding funnel is a pictorial way of describing the landscape of a minimally frustrated system.

All my attempts to understand the "minimally frustrated principle" have failed. I will be glad if these authors will explain to me how "frustration" is defined in protein, how it is quantified, and what the basis of the "minimal frustration principle" is. Without a clear definition of the concept of frustration as applied to proteins, such statements are just empty words.

I also do not agree with the statement "This is the underlying basis for simple, yet powerful, coarse-grained models such as the HP lattice model of Kenneth Dill and his colleagues (Lau & Dill, 1989). The two approaches are alternative ways of describing the same phenomenon".

I do not believe that any lattice model including the HP model can, in principle, help in understanding the protein-folding phenomena. I have dealt on this in my monograph (2013).

Finally, to the question raised by the authors "How is a funnel-like landscape sculpted from the hydrophilic interactions?"

I can simply answer: *All interactions*, both direct-and solvent-induced interactions, determine the form of the GEL, not necessarily the *funnel-like* GEL!

If the authors believe that the landscape has a *funnel-like* form, they should provide the answer to the question of How is a *funnel-like* landscape sculpted from all the interactions?

Author: Seema Mishra

I certainly do not agree with the paragraph:

> Reading Ben-Naim's article was like reading a comprehensive, linguistically-smitten prose and a lawyer's rendition which attempts to find the meaning of a word or sentence, taking care that the word chosen fits perfectly alongside. As an example among the many examples existing throughout the article, 'forces' and 'molecular interactions' are given a separate identity, rather than being used interchangeably as these usually are

Indeed, "force" and "interactions" are used interchangeably. This is unfortunate, since these are very different concepts. This is one point that I have emphasized in my monographs (2011, 2013). I do not think this is merely a question of semantics!

I believe this author understood most of my points. There is, however, one point that was probably misunderstood. The author states:

> Ben-Naim has compared the random process of protein folding pathway to a drunken man's random walk throughout a city to reach a specific location from his original one.

No, I brought up the metaphor only to demonstrate the difference between the target-based and the cause-based theory. I did not claim that the random walk of the drunken man is a model for protein folding.

Authors: Youtao Song, LinanXu, ManliShen and Jianwei He

I fully agree that the phenomenon of misfolding of proteins is very important and needs to be studied further. My article does not deal with this phenomenon. I wish I could contribute to understanding the misfolding of proteins and their relevance to some mental disease.

Authors: Li-Quan Yang, Xing-Lai Ji and Shu-Qun Liu

In the second paragraph of this article, the authors write:

> Although the definition of the variables and the derivation procedure are rigorous and the resulting models are sound, some of his interpretations to the models are limited or even incorrect because, (i) from a global view of the GEL for the protein-solvent system, the native, functionally active protein conformation states should have to reside at the bottom, i.e. the global free energy minimum, or the funnel-shaped GEL; and (ii) although the hydrophilic effect indeed plays a dominant role in driving protein folding during certain stages/processes, the solvent entropic effect (also called hydrophobic effect) dominates the first stage of the protein folding, i.e. the hydrophobic collapse process.

Regarding item (i), I do not agree that the active protein must reside at the bottom – *global free energy minimum*. This is exactly what I have criticized in my article, and perhaps misunderstood by these authors. Regarding item (ii), I certainly do not agree that the hydrophobic collapse dominates the first stage of the protein folding. I also do not agree that the solvent entropic effect is called hydrophobic effect.

The authors also state:

"We do not agree with the opinion of Ben-Naim that there exists no "code" that translates the amino acid sequence into a 3D structure (Ben-Naim, 2012a)".

This is fine. They have the right to believe that such a "code" exists. But then, they explain what they mean by a "code".

"The code here is in fact the thermodynamic question of the protein folding, i.e. what are the factors that drive the protein to fold? And how do they do so?"

If this is the "code", then I have fully answered the question posed in this quotation, namely the hydrophilic forces are the main factors that drive the protein folding. Suppose that one finds the exact answer to the "factors that drive the protein", would that answer consist of a "code" that translates from sequence to structure?

Later on, the author again raises the issue:

"raising the questions of what the factors are which guide and speed the man towards the target …"

This is precisely the question I have answered in the article.

In another place, these authors discuss my view regarding the global minimum:

> We do not agree with Ben-Naim's view that the stable state of the protein is not necessarily in a global minimum in the GEL because (i) he misinterpreted concept of the global free energy minimum and regarded it as a single absolute minimum; (ii) there exist more than one stable comformational states in the global free energy and all these states can be regarded as native conformers.

My impression is that the authors confused the multitude of minima in the GEL at a *specific environment* (T, P, N), and different GELs at *different* environments, having different stable states. This aspect is discussed at great length in my books (2011, 2013).

There are several other statements such as

> the global free energy minimum concept must be understood under the background of the funnel-like GEL …

And similarly

> the shape of the GEL for protein folding is actually funnel-like.

Although such statements are very commonly made in the literature, they are unfounded. This is exactly one point of criticism in my article.

These authors also "complained" that:

> However, Ben-Naim did not answer explicitly the question of what the shape of the GEL for protein folding is in his eyes.

True, I did not answer that question. I simply do not know the shape of the GEL of any protein! In my opinion, no one knows the shape of the GEL, and the funnel-like proposed shape is a result of misinterpretation of Anfinsen's hypothesis.

In the section entitled "Why is the GEL for protein funnel-like?" the authors correctly quote the definition of the GEL and the Gibbs energy functional. Unfortunately, it seems to me that the authors failed to understand the difference between the two concepts. This is further discussed in great details in my book (2013).

I also believe that the authors misunderstood the indirect, solvent-induced forces, which I have discussed in my article. This is clear from their statements:

> Unfortunately, Ben-Naim focused only on the force that is directly induced by individual water molecules, but ignored the forces that are indirectly induced by the solvent.

I have not *ignored*, but *emphasized* the indirect solvent-induced forces!

And

> the hydrophobic force is not a real force but is the consequence of maximizing the solvent entropy.

This is a meaningless sentence on which I cannot comment. In my recent monograph, I have explained that under constant energy, volume, and composition, the Second Law states that the entropy has a maximum. In a system of interacting particles, there is no such "maximum entropy" of only one component (water) of the system. There is also no such "maximum entropy" of the *entire system* under constants T, P, and N.

Finally, these authors repeatedly refer to the "GEL theory". I know what the GEL is. I have no idea what is the "GEL theory", which appears so often in the literature. I will be glad if these authors could teach me what this theory is, what its assumptions are, as well as its results.

Authors: Hridoy R. Bairagya and Bishnu P. Mukhopadhyay

These authors seem to understand the main points in my article. They criticized the metaphor of the drunken man walking in the city. I tend to agree that this metaphor has nothing to do with the protein folding. The only reason I brought it up in the article was to explain the difference between "target-based" and "cause-based" processes. I did not imply that this is a model for protein folding.

Authors: Teodorico C. Ramalho, Lucas A. Santos and Elaine F. F. da Cunha

There is only one thing I wish to point out in this article. The authors state

> In the balance of forces between hydrophobic and hydrophilic effects, it is known from literature that polar forces are stronger (Ben-Naim, 1991). It should be kept in mind, however, that hydrophobic forces can also play an important role in the protein folding process, because if a hypothetical protein only contains polar amino acids residues in its primary sequence, that protein shows stronger interactions with water molecules and the solvation Gibbs free energy is quite negative, decreasing the probability the protein folding occurring.

I am not sure that a protein that contains only polar groups will not fold. In fact, in most proteins, the number of polar groups (both on the backbone and the side chains) are about four to five times larger than the number of hydrophobic groups. And these fold!

Authors: C. A. Taft and C. H. T. P. da Silva

The first paragraph says it all:

> The manuscript "Comments on the paper Levinthal's question, revisited and answered" by Ben-Naim (2012a) [1] is a very good scientific work, well rationalized with good supporting arguments and an excellently written paper regard PFP. Already from the abstract we can divide the author's position in three conclusions, i.e. first, the protein does not need to be at a global minimum of the Gibbs energy, second, adherence to a hydrophilic model instead of an hydrophobic model, third, switching from a target-based to a cause-based approach

A very nice summary of all the points I raised in the article.

Authors: Srinivisarao Karri and Chanchal K. Mitra

I have only one comment to this article. In their final section, they wrote

> but this problem of searching $\sim 20{,}000$ active proteins within a theoretically possible 20100 ($> 10^{130}$) sequences appear to be a far daunting task, far more difficult than the PFP. Unfortunately, he has not provided any resolution of this aspect.

If I understood correctly, the authors imply that evolution had searched (and found) some 20,000 proteins out of possible 10^{100} sequences. This is of course a far daunting task. But no one had undertaken that task, not even evolution!

Surely, I *cannot* provide any resolution to this far daunting task. No one can, nor the millions of years of evolution.

I can only suspect here that there is a fundamental misunderstanding how evolution works. I have elaborated a little more about this in my book (2013), although this aspect is not really relevant to the PFP. I have discussed evolution simply because many authors involved evolution in connection with the PFP.

Author: Kaushik, A. and Gupta, D

I fully agree with most of this article. It seems to me that these authors fully understood all the points raised in my article. In their last paragraph, they discussed the role of chaperons in assisting the folding of proteins. This is an important aspect on which I did not elaborate. Instead, I focused on spontaneous folding of proteins as described in Anfinsen's experiments.

Author: Evgenii L. Kovrigin

This article nicely summarizes all the points raised in my article. I can recommend it to anyone who misunderstood my article. I applaud the author for a clear and sharp presentation of my article.

Author: Alexei V. Finkelstein and Sergiy O. Garbuzynskiy

To the title of this article is "Levinthal's question answered … again?" My answer is No, not again! To the best of my knowledge, the answer I have given to Levinthal's question was *never* given before. The authors found "nothing non-trivial …" in my article. Unfortunately, what they have written shows clearly that they misunderstood all my "trivial" points. To give only one example (out of many meaningless statements made in this article), the authors conclude:

> Summarizing, I have to say that I cannot find in the paper by Ben-Naim anything more than a complicated reincarnation of the old good funnel model of protein folding.

My paper *criticizes* the funnel model, as an *unfounded* model and is not a "*reincarnation* of the old good funnel model". It is strange that the author read in my article exactly the opposite of what I have *written*!

I fail to understand how a famous author would reach such an erroneous conclusion. I can only refer the authors to my new monograph (2013), which I hope will clarify the points raised in the article.

Author: Giuseppe Graziano

The author starts by referring to my book on hydrophobic interactions published in 1980. He then continued by saying (correctly) that:

> The hydrophilic effect consists of two distinct processes: hydrophobic hydration and hydrophobic interaction (Blokzijl & Engberts, 1993). Hydrophobic hydration refers to the transfer of a nonpolar molecule (i.e. methane or neopentane) from a fixed position in the ideal gas phase to a fixed position in water [this is also called Ben-Naim standard (Lee, 1991)].

"Hydrophobic interaction is considered to be a partial reversal of hydrophobic hydration: nonpolar molecules, being poor soluble in water in view of their inability to participate in H-bonds".

However, the author seems to ignore my recent findings that both *solvation* and *hydrophobic interactions* are irrelevant to protein folding. The main reason is that the backbone of the protein is present and that affects both the solvation and the interactions between hydrophobic groups.

In addition, ten year after I published the book on hydrophobic interactions, I discovered that *hydrophilic interactions* are far more important than hydrophobic interactions.

The rest of the article discusses the importance of the cavity work in the process of protein folding. It is true that I did not discuss the cavity work in my article simply because this work is not relevant to the Levinthal question. The role of cavity formation is of course important in all biochemical processes and is discussed in all my books, in particular, in my two recent monographs (2011, 2013).

Authors: Jean-NumaGillet and Indira Gosh

These authors complain that:

> We feel a lack of discussions about the entropy S in the paper presented by Ben-Naim (2012a). However, S is an important quantity for the PFP that has been unfortunately not considered in current molecular dynamics (MD) models.

It is true that entropy is important in any process, and in particular, in protein folding. However, my main concern in my article was not to examine the relative contributions of the entropy and the enthalpy to the Gibbs energy. Instead, I had focused on the Gibbs energy itself and showed that the various hydrophilic effects are far more important than the corresponding hydrophobic effects. In my recent monographs, I did discuss the role of entropy and enthalpy of both the hydrophobic and the hydrophilic effects.

I am glad that these authors say:

> We therefore agree with Ben-Naim (2012a) that considering an excessive importance of the hydrophobic forces to lead the protein folding is not an acceptable paradigm in the PFP.

Author: Montgomery Pettitt

I fully agree with the comment of this author regarding the confusion between energy and Gibbs energy. This issue is more than a mere sloppy usage of words, as some other authors have commented. Not only are the two concepts different in thermodynamics, they also have different *properties* when applied to the PFP. As I have explained in more details in my monograph (Ben-Naim, 2013) some authors use the concept of energy landscape (EL), but when asked what they mean, they admit that they mean GEL. This sounds like a mere sloppy language. Unfortunately, these authors use *properties* of the *energy* landscape to their *Gibbs energy* landscape. The result is a theory, which is based neither on EL nor on the GEL.

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