



Review

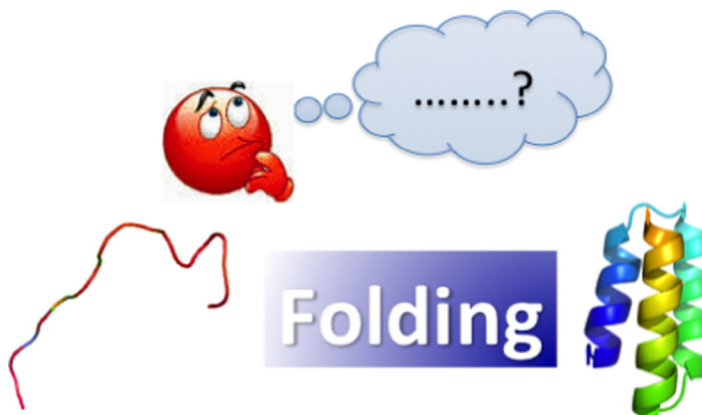
Protein folding: Vexing debates on a fundamental problem

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HIGHLIGHTS

- Several debates are flourishing in the protein folding field.
- Compaction of the denatured state measured by single molecule techniques is challenged by SAXS.
- The presence of nucleation sites probed by Φ analysis is constantly being criticised.
- Long transition path times challenge molecular dynamics simulations.

GRAPHICAL ABSTRACT



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ABSTRACT

The folding of proteins has been at the heart of protein chemistry and biophysics ever since the pioneering experiments by the labs of Fred Richards and Christian Anfinsen. But, despite nearly 60 years of intense research, there are unresolved issues and a lively debate regarding some aspects of this fundamental problem. In this review we give a personal account on some key topics in the field: (i) the nature of the denatured state of a protein, (ii) nucleation sites in the folding reaction, and (iii) the time it takes for individual molecules to traverse the transition state.

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1. Introduction

The amino acid chain of a protein folds into a native structure, which can be globular and well defined, highly disordered, or anything in between. The structure of the protein is dictated by the sequence of the amino acid residues and the environment of the protein. Because the structure of a protein defines its functions, it is clear how the interest in the study of protein folding has been significant over the last six decades, with this scientific field playing a very influential role in biophysics and molecular biology [1]. The protein folding problem can be formulated in a very simple way: how does the unfolded chain find the native conformation? The answer is less simple.

Thanks to the collaborative efforts between experimentalists and theoreticians, some of the general rules of folding have been already drawn and our understanding of the reaction has grown enormously over the last years (discussed for example in these interesting reviews [2–7]). Nevertheless, the employment of innovative techniques, as well as re-analyses of the large amount of data accumulated over the years not only answered questions, but is now igniting new debates in the field, and raising new challenges and questions to address.

Most proteins contain more than 100 amino acid residues and often several independently folding domains. However, due to the complexity of large proteins and limitations in computational power, studies on protein folding have mainly been limited to small (<100 residues) single domain proteins. The most striking experimental observation in protein folding on single domain proteins, is the stark co-operativity of the reaction [8]. In fact, whilst hundreds of interactions form and break upon folding or unfolding, the observed transition from unfolded to folded is often surprisingly as simple as two-state [9]: only the unfolded state and the native state are typically present at equilibrium. Consequently, when a transient intermediate cannot be identified or characterized in the protein folding reaction, the transition state, separating the unfolded and the folded state, is the only source of information regarding the reaction mechanism.

In this review we highlight some emerging controversies in the protein folding field. We will first focus on the unfolded, or perhaps more correctly, the denatured state of the protein, which represents the starting point of the folding reaction. We will then look at the transition state, which contains the key points to address the overall mechanism. Finally, we will discuss the track along which a single molecule diffuses on the free energy landscape, and in particular the transition path time, which is the time it takes to pass the energetic barrier.

2. The denatured state of proteins

What is the denatured state of a protein? This question has spurred many discussions and the lack of clarity regarding the denatured state can be sought in its own definition. In 1957, Klee and Richards [10] and Anfinsen and co-workers [11] observed that the enzymatic activity of RNase A and its spectral properties, reporting on the secondary and tertiary structure, were not necessarily concomitantly perturbed when the enzyme was exposed to different experimental conditions. Therefore, in 1959 White and Anfinsen listed a set of conditions where the activity of RNase A could be abolished along with its spectral properties or not [12]. These findings led to the proposal that the protein contained an “active centre” constituted by a relatively small part of the molecule. The experimental forefathers of protein folding hence put

forward the concept of *denaturation*. Consequently, by following Richards and Anfinsen, the term *denatured* may be referred to a conformation of a protein that is inactive, irrespective of whether or not it contains ‘folded’ regions.

The difference between the terms *denatured* and *unfolded* is therefore not only semantic, it is very important for the understanding of the inherent properties of this heterogeneous state. The denatured state (*D*) represents a functionally inactive conformation that contains a variable degree of native or non-native interactions, and may be populated under conditions that favor folding. On the other hand, the unfolded state (*U*), represents the expanded chain found at equilibrium in the presence of high concentrations of denaturants or at high temperature. While *U* of many proteins resembles a random coil, the structural and dynamic properties of *D* is key to understanding the early events in protein folding, as shown for different protein systems [13–21].

In refolding mixing experiments, it is commonly observed that a rapid dilution of denaturant leads to a variation of fluorescence that precedes the folding reaction [22,23]. This ‘burst phase’ has been associated with a compaction of the unfolded chain, i.e., representing the transition from *U* to *D*. A recurring debate in protein folding pertains to whether such transition is a barrier limited reaction, driven by specific interactions, or a non-specific collapse induced by water exclusion [24–29]. A recently developed methodology, single molecule Förster resonance energy transfer (smFRET), could provide additional insights to this issue [30]. An advantage of smFRET is that it avoids ensemble averaging. Specifically, by performing a statistical analysis of single molecule events, it is possible to reconstruct the properties of a given state, rather than measuring experimental observables belonging to a mixture of states.

By applying smFRET, it has been observed that *D* is characterized by a compact conformation in the absence of denaturants [31–33]. Interestingly, increasing denaturant concentrations led to a gradual increase of the overall radius of gyration of *D*. This observation suggests that in the case of the proteins explored, the transition from *U* to *D* is a second order barrier-less type of transition, characterized by a continuum of states. In fact, if the reaction were of a first order barrier-limited type, smFRET would have detected a discrete change in the relative populations of *U* and *D*. However, the observations by smFRET were recently challenged. In particular, small angle X-ray scattering shows little evidence for *D* state compaction as a function of denaturant concentration [34]. Furthermore, it was shown that double labeled polyethylene glycol (PEG) as measured by smFRET showed a compaction very similar to that of denatured proteins, but neutron scattering experiments demonstrated that unlabeled PEG remains expanded irrespective of the denaturant concentration [35]. These differences question the validity of the smFRET analyses and keep the debate on the nature of the denatured state alive. We interpret these experimental differences as arising, at least in part, from the lower quality and resolution of the SAXS data and encourage additional research on this critical topic in protein folding.

3. The transition state

Like any chemical reaction, the folding of a protein proceeds via a transition state. Because of the co-operativity of the folding reaction, the transition state of folding is often the only experimentally accessible state giving information about the pathway [9], unless intermediates can be identified [36–40]. Consequently, a considerable amount of

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